IMMUNOTHERAPY IN MULTIPLE MYELOMA

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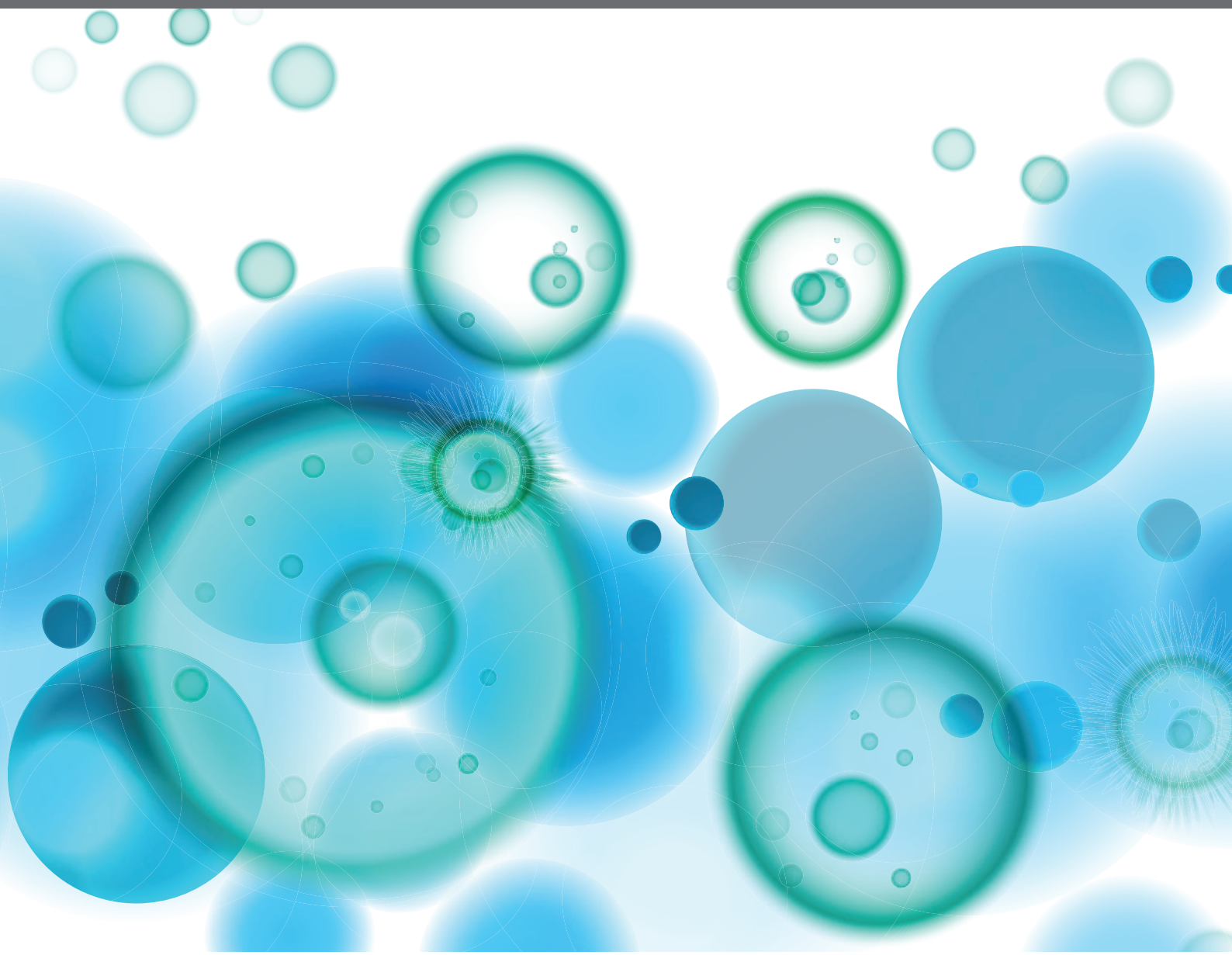

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IMMUNOTHERAPY IN MULTIPLE MYELOMA

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EDITORIAL
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Editorial: Immunotherapy in Multiple Myeloma

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Editorial on the Research Topic

Immunotherapy in Multiple Myeloma

HONORING THE PAST

Multiple myeloma (MM) is a hematological malignancy characterized by a high tendency to relapse and to become drug resistant. In the past, melphalan was considered the standard of the treatment for MM patients (1). Following, the introduction of thalidomide and the proteasome inhibitor bortezomib leaded to a significant improvement of the survival of MM patients: however, none of them reached the cure of the disease. These drugs introduced the concept of the treatment of MM patients targeting not only the malignant clone but also the microenvironment (2). In addition, the introduction of lenalidomide, a thalidomide derivative, expanded this concept by focusing to the immune-microenvironment (2). More recently, the introduction of the monoclonal antibodies (mAbs) seem to change the paradigm of MM treatment, highlighting the possibility to cure MM patients by an immunotherapeutic approach (3).

Immunotherapy is part of a concept that goes back to the beginning of 1900. The "magic bullet" opened the way to the objective of having a tool able to selectively eliminate target cells and at the same to modulate the immune response in a beneficial way. It was necessary to wait for several decades and apparently for unrelated findings coming from different fields before having a picture able to frame the view outside a simple anecdotal hope. The objective was initially made possible by combining results from basic science and the availability of mAbs, a reagent made of a homogeneous population of immunoglobulins (Ig), the main difference from the conventional antisera. mAb is hence able to recognize only a single epitope on the molecular target.

The second key event derived from the identification of a vast number of soluble factors, which share the feature of transmitting signals in the context of similar or different cells (the interleukins). Using this new tools, Reinherz et al. generated a panel of mAbs specific for surface molecules located on the surface of human T lymphocytes (4). At the same time, Smith et al. made available IL-2, a cytokine which made possible to expand clones of normal T lymphocytes (5). Combining these approaches, Reinherz et al. (4) were able to define murine reagents specific for molecules present on all T lymphocytes, while other ones were limited to subsets of the same cells. Another set of mAbs recognized molecules only present in single lymphocytes (defined as idiotypic). These findings modified the simplistic dogma that mAbs were only able to bind the target antigen and to induce cell lysis. This lead to the definition of the concept of agonistic antibodies: the translational inference is that the engagement by a mAb of selected domain of a molecule may surrogate the effects induced by a natural ligand of the same receptor, even when the ligand was not known.

The definition of immune check points molecules lead to the preparation of panels of mAbs able to induce or brake immune responses, according to distinct medical needs. The concept of immune

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modulation was further completed by the identification of activator effector T and B cells, while other cell subsets produce effects going in opposite directions (Treg, Breg, and myeloid-derived suppressor cells are the most important). The considerations derived from the dissection of the main steps of immune cell defense were indirectly confirmed by studies conducted on the different strategies of immune escape of tumors. Indeed, it emerged that some tumors adopt escape or camouflage strategies, which implement genetic programs driving to metabolic reshaping, secretion of immunomodulatory cytokines, or generation of tolerogenic substances, among the others.

STUDYING THE PRESENT

At the moment, available to the medical community there is a panel of therapeutic antibodies, which significantly modified the fate of some diseases, especially neoplasies of hematological origin. There is a general agreement that the therapeutic effects are prevalently obtained by means of antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC), and concurrently by the induction of signals on cell effectors (6). These effects may be enriched by other functions exerted by the target molecule, such as the ability to lead to generation of substrates able to induce immunomodulation. This is the case when a molecule belongs to the family of ectoenzymes, which now encompasses almost 5% of the entire surface molecule express by leukocyte (6). Other mAbs were generated against soluble molecules produced by both MM cells and the bone microenvironment including sclerostin able to block MM-derived bone destruction and in turn MM progression.

In the treatment of MM, the Food and Drug Administration (FDA) approved daratumumab (DARA) and elotuzumab (Elo), two monoclonal IgG-k mAbs, specific for CD38 and SLAMF7 (signaling lymphocytic activation molecule F7), respectively. The approval was for the treatment of relapsed or refractory MM (RRMM) patients, in combination with lenalidomide and dexamethasone (7). CD38 is a transmembrane glycoprotein highly expressed on MM cells that acts as both a receptor and an ectoenzyme. It is also involved in the activation and proliferation of immune cells (Morandi et al.). SLAMF7 is a surface glycoprotein receptor expressed on plasma cells (PCs) and on natural killer (NK) cells that is implicated in adhesion to stromal cells and in the activation of NK cell effector function (8).

Both DARA and Elo share the feature to recruit the immune system to enhance cellular cytotoxicity directed against myeloma cells (9). However, Elo acts only through NK cells and its effects are improved in combination with immunomodulatory drugs (IMiDs) as lenalidomide. On the other hand, DARA and the newer isatuximab (Isa) shows a broad spectrum of activity, including ADCC, ADCP, CDC, and possibly direct induction of apoptosis on MM cells. Further, they exhibit promising results even as a single-agent (9). Beyond mAbs against surface molecules, several agents targeting immune checkpoints (e.g., CTLA-4, LAG3, PD-1/PD-L1, ICOS) expressed on immune cells have also been recently developed as a therapeutic strategy to activate T-cell mediated anti-tumor immunity (10). Specifically, the PD-1/PD-L1 axis has emerged as a central immune checkpoint that controls anti-tumor immune responses and plays a critical role in the metabolic reprogramming of cancer cells within solid tumors. However, its role in MM progression remains to be clarified. Discordant results have been reported on PD-1/PD-L1 expression in MM thus suggesting the need of a more precise definition of PD-1/PD-L1 distribution in the context of cells within the MM tumor microenvironment (Costa et al.). Interestingly, the expression of immunecheckpoint molecules by osteoclast has been recently underlined. However, single-agent studies on PD-1/PD-L1 inhibitors have not demonstrated significant responses in MM patients. On the other hand, other studies have demonstrated the ability of lenalidomide to enhance anti-MM immune activity mediated by PD-1/PD-L1 inhibition despite high grade of toxicity (Jelinek et al.).

The use of mAbs in therapy now led to the observation of antibody resistance, which may appear at different times. Different approaches were designed in order to answer this issue, which is of critical relevance in clinics. Hypothesis or observations explaining the effects may be referred to down modulation of the target molecule by the neoplastic cells. An alternative is represented by a re-distribution of the target molecule on selected surface domains (e.g., polar aggregation or capping). Polar aggregation tends to coalescence the CD38 molecule with ectoenzymes involved in the production of adenosine along with inhibitory complement receptors (CD46, CD55, and CD59) and PD-L1. The availability now of a second antibody with the same the same specificity but recognizing a different epitope may be proposed when the resistance to the first one is observed. This strategy is expected to bypass the resistance mechanisms and to exert new mechanisms of therapeutic action.

TRYING TO DESIGN THE FUTURE

The design of innovative strategies in MM therapy is a difficult challenge, since the disease has been adopted as a model where different immunotherapeutic approaches are under evaluation. For these reasons, we would like to focus to some aspects, sometime not considered to design the future of the immunotherapy in MM. Most details and complete authoritative reviews may be found in the manuscripts of this Special Issue.

1) *New target markers for mAb therapy*. The efforts to identify specific markers exclusively identifying human myeloma cell surface has been quite disappointing. So far, a criteria adopted is quantitative (e.g., for anti-CD38) or based on clear receptorial features (e.g., for CS1 or SLAMF7). B cell maturation antigen (BCMA) was recently adopted in virtue of a quite restricted expression, along with APRIL, one of its ligand. All the potential targets for antibody-mediated therapy are summarized in a recent and complete reviews (11, 12).
2) *Cell-based therapies*. Beside the mAb-mediated approaches alone or as drug carriers or bispecific T cell engager, the cellular approaches using genetically modified T lymphocytes (CAR-T) or NK cells are expanding exponentially and

analyzed (11). Such approaches are reviewed in papers of the Special Issue.

3) *Extension of NK cell life and activity*. Strategies to extend the life and performance of NK cells is one of the hot areas in the field. Paiva group analyzed the gene profile obtained in NK cells exposed to Isa: among the up-modulated appeared CD137, an inducible molecule (also known as Tumor Necrosis Factor Receptor Super Family-9, TNFRSF-9) (13). For this molecule, there are available two different antibodies, used for clinical trials. Their use combined with Isa aimed at increasing the life span of NK cells produced unsatisfactory results, at least in the model adopted (13). However, new observations support the possibility of combinations between therapeutic anti-CD38 and anti-CD137. The disappointing results obtained *in vivo* with anti-CD137 mAbs (urelumab, a human IgG4, and utomilumab, a human IgG2) were likely attributable to negative effects mediated by their interactions with FcRs (14). Now a construct with an arm made of a recombinant trimetric form of the CD137 Ligand (TNFSF-9) associated to the different tumor-associated molecules leads to an *in situ* activation of CD8 cell co-stimulation, with production of IFN-γ (15, 16).

   In order to generate potent antibodies against tumor cells and stimulating anti-tumor cell immunity, recently, trifunctional natural killer (NK) cell engagers (targeting NKp46 and CD16 on NK cells) and a tumor antigen on cancer cells have been developed. This approach produced *in vitro* more potent effects than the therapeutic antibodies used in clinics to target the same tumor antigens (17).

4) *FcR engagement and effects induced by target ligation*. No systematic analysis of this step was conducted on anti-CD38 therapy to date. It is reasonable to expect that the differences in structure between DARA and Isa (one full human, the other one chimeric mAbs) may be reflected on the interactions with the IgG FcRs. Results obtained *in vitro* giving DARA-armed FcR^+ cells instead of soluble mAb is followed by a distinct membrane dynamics. Critical here are the effects induced by antibody ligation on the tumor target molecule. It is reported that this event may be followed by internalization of the target/antibody complex or externalization, followed by a release in the biological fluids in the form of microvesicles.

5) *Combination therapies*. Part of actual therapeutic potential of the different type of antibody approach may be improved by using immune modulators or combination with other mAbs (with similar or different specificities) or recombinant constructs. A limit on the use of reagents targeting one or two different molecules (surface targets or modulators of the immune response) comes from the recent evidence that the MM is characterized by a marked spatial genomic heterogeneity, with an early phase with clonal sweeps followed by a regional evolution in advanced stages of the disease (18).

AUTHOR CONTRIBUTIONS

NG and FM equally contributed to write the manuscript and made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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We would like here to thank and remember Vito Pistoia (1949–2018). Vito was a brilliant witness of a lucky period of the italian renaissance, which blessed the italian medical research in the Seventies. After his M.D. graduation, he started a career in Genova, where a significant number of scientists with an international background were operating. The synergies among the groups of Benvenuto Pernis, Ruggero Ceppellini, Carlo Grossi, Manlio Ferrarini, Soldano Ferrone, and the Moretta group were the ground of his basic education in science. This was completed by a period in Alabama at the school of Max Cooper. B lymphocyte area became the first area of his interests.

Lately he became Director of the Oncology Lab at the Gaslini Institute (Genova, Italy), a further source of synergies with the groups of Lorenzo and Alessandro Moretta. His Lab became reference for studies of cancer microenvironment, seen as potential regulator (positively and negatively) of the immune responses.

From the above interests stemmed his interest on the analysis of the events taking place in the bone marrow and in human multiple myeloma, the focus of intense interest by basic and clinical scientists. His skills, experience in science, and personal enthusiasm was the starting point of the common effort behind this Special Issue.

His scientific personality was also enriched and completed by non-common personal qualities. His positive approach to life, his refined humor, and a smiling face (mirroring internal equilibrium and peace) were his master characteristics.

Unfortunately, Vito passed away before the completion of this Special Issue. For this, it is our honor and a pleasure for us to dedicate the Issue in his name and memory.

REFERENCES

1. Cherry BM, Korde N, Kwok M, Roschewski M, Landgren O. Evolving therapeutic paradigms for multiple myeloma: back to the future. *Leuk Lymphoma*. (2013) 54:451–63. doi: 10.3109/10428194.2012.717277
2. Kawano Y, Moschetta M, Manier S, Glavey S, Gorgun GT, Roccaro AM, et al. Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev*. (2015) 263:160–72. doi: 10.1111/imr.12233
3. Franssen LE, Mutis T, Lokhorst HM, van de Donk NWCJ. Immunotherapy in myeloma: how far have we come? *Ther Adv Hematol*. (2019) 10:2040620718822660. doi: 10.1177/2040620718822660

4. Reinherz EL, Parkman R, Rappeport J, Rosen FS, Schlossman SF. Aberrations of suppressor T cells in human graft-versus-host disease. *N Engl J Med*. (1979) 300:1061–8. doi: 10.1056/NEJM197905103001901
5. Smith KA, Lachman LB, Oppenheim JJ, Favata MF. The functional relationship of the interleukins. *J Exp Med*. (1980) 151:1551–6. doi: 10.1084/jem.151.6.1551
6. Malavasi F, Faini AC. Mechanism of action of a new anti-CD38 antibody: enhancing myeloma immunotherapy. *Clin Cancer Res*. (2019) 25:2946–8. doi: 10.1158/1078-0432.CCR-19-0260
7. Varga C, Maglio M, Ghobrial IM, Richardson PG. Current use of monoclonal antibodies in the treatment of multiple myeloma. *Brit J Haematol*. (2018) 181:447–59. doi: 10.1111/bjh.15121
8. Veillette A, Guo H. CS1, a SLAM family receptor involved in immune regulation, is a therapeutic target in multiple myeloma. *Crit Rev Oncol Hematol*. (2013) 88:168–77. doi: 10.1016/j.critrevonc.2013.04.003
9. Sherbenou DW, Mark TM, Forsberg P. Monoclonal antibodies in multiple myeloma: a new wave of the future. *Clin Lymphoma Myeloma Leuk*. (2017) 17:545–54. doi: 10.1016/j.clml.2017.06.030
10. Ok CY, Young KH. Checkpoint inhibitors in hematological malignancies. *J Hematol Oncol*. (2017) 10:103. doi: 10.1186/s13045-017-0474-3
11. Szalat R, Munshi NC. Novel agents in multiple myeloma. *Cancer J*. (2019) 25:45–53. doi: 10.1097/PPO.0000000000000355
12. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood*. (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
13. Moreno L, Perez C, Zabaleta A, Manrique I, Alignani D, Ajona D, et al. The mechanism of action of the anti-CD38 monoclonal antibody isatuximab in multiple myeloma. *Clin Cancer Res*. (2019) 25:3176–87. doi: 10.1158/1078-0432.CCR-18-1597
14. Ochoa MC, Perez-Ruiz E, Minute L, Onate C, Perez G, Rodriguez I, et al. Daratumumab in combination with urelumab to potentiate anti-myeloma activity in lymphocyte-deficient mice reconstituted with humanNK cells. *Oncoimmunology*. (2019) 8:1599636. doi: 10.1080/2162402X.2019.1599636
15. Claus C, Ferrara C, Xu W, Sam J, Lang S, Uhlenbrock F, et al. Tumor-targeted 4-1BB agonists for combination with T cell bispecific antibodies as off-the-shelf therapy. *Sci Transl Med*. (2019) 11:eaav5989. doi: 10.1126/scitranslmed.aav5989
16. Sanmamed MF, Etxeberria I, Otano I, Melero I. Twists and turns to translating 4-1BB cancer immunotherapy. *Sci Transl Med*. (2019) 11:eaax4738. doi: 10.1126/scitranslmed.aax4738
17. Gauthier L, Morel A, Anceriz N, Rossi B, Blanchard-Alvarez A, Grondin G, et al. Multifunctional natural killer cell engagers targeting NKp46 trigger protective tumor immunity. *Cell*. (2019) 177:1701–13 e16. doi: 10.1016/j.cell.2019.04.041
18. Rasche L, Chavan SS, Stephens OW, Patel PH, Tytarenko R, Ashby C, et al. Spatial genomic heterogeneity in multiple myeloma revealed by multi-region sequencing. *Nat Commun*. (2017) 8:268. doi: 10.1038/s41467-017-00296-y

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MICA-129 Dimorphism and Soluble MICA Are Associated With the Progression of Multiple Myeloma

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Natural killer (NK) cells are immune innate effectors playing a pivotal role in the immunosurveillance of multiple myeloma (MM) since they are able to directly recognize and kill MM cells. In this regard, among activating receptors expressed by NK cells, NKG2D represents an important receptor for the recognition of MM cells, being its ligands expressed by tumor cells, and being able to trigger NK cell cytotoxicity. The MHC class I-related molecule A (MICA) is one of the NKG2D ligands; it is encoded by highly polymorphic genes and exists as membrane-bound and soluble isoforms. Soluble MICA (sMICA) is overexpressed in the serum of MM patients, and its levels correlate with tumor progression. Interestingly, a methionine (Met) to valine (Val) substitution at position 129 of the α2 heavy chain domain classifies the MICA alleles into strong (*MICA-129Met*) and weak (*MICA-129Val*) binders to NKG2D receptor. We addressed whether the genetic polymorphisms in the MICA-129 alleles could affect MICA release during MM progression. The frequencies of *Val/Val*, *Val/Met*, and *Met/Met* MICA-129 genotypes in a cohort of 137 MM patients were 36, 43, and 22%, respectively. Interestingly, patients characterized by a *Val/Val* genotype exhibited the highest levels of sMICA in the sera. In addition, analysis of the frequencies of MICA-129 genotypes among different MM disease states revealed that *Val/Val* patients had a significant higher frequency of relapse. Interestingly, NKG2D was downmodulated in NK cells derived from *MICA-129Met/Met* MM patients. Results obtained by structural modeling analysis suggested that the Met to Val dimorphism could affect the capacity of MICA to form an optimal template for NKG2D recognition. In conclusion, our findings indicate that the *MICA-129Val/Val* variant is associated with significantly higher levels of sMICA and the progression of MM, strongly suggesting that the usage of soluble MICA as prognostic marker has to be definitely combined with the patient MICA genotype.

Keywords: multiple myeloma, natural killer cells, NKG2D receptor, MICA polymorphism, predictive biomarker

INTRODUCTION

Natural killer (NK) cells represent innate immune effectors playing a pivotal role in tumor surveillance. NK cell activation is regulated by a delicate balance between activating and inhibitory signals, with the latter being primarily transduced by receptors for MHC class I molecules (KIRs, CD94/NKG2A). Recognition of abnormal self on tumor cells triggers a number of non-MHC class I-restricted activating receptors, such as NK group 2D (NKG2D), DNAX accessory molecule-1 (CD226), and the natural cytotoxicity receptors (1).

NKG2D is an activating receptor expressed on the surface of NK cells, $CD8^+$ T cells, and subsets of $CD4^+$ T cells, invariant NKT cells (iNKT), and γδ T cells (1). NKG2D recognizes two families of ligands in humans: the MHC class I chain-related protein A/B (MICA/B) and the UL16-binding proteins (ULBP1-6) (1). In general, healthy adult tissues do not express NKG2D ligands on the cell surface, but the expression levels of these molecules can be significantly induced by various physiological and pathological "stress" circumstances, including infection by different pathogens (1), cell division (2), and neoplastic transformation (3). Among all known NKG2D ligands, *MICA* is the most polymorphic non classical class I gene, with 104 alleles identified to date (http://www.ebi.ac.uk/imgt/hla/, release 3.25.0). Some MICA polymorphisms have raised a great interest since they can affect MICA biology. For instance, the MICA*008 allele (rs67841474) contains a guanine (G) insertion that causes a premature stop codon that, in turn, crops 10 amino acids of the transmembrane domain as well as the cytoplasmic tail. In contrast to other MICA alleles that are shed as truncated soluble species after metalloproteinase-mediated cleavage, it is released from cells associated to exosomes (4). In addition, the single-nucleotide polymorphism causing a valine (Val) to methionine (Met) modification at position 129 of the α2 heavy chain domain classifies these MICA alleles into high-affinity (*MICA-129Met*) and low-affinity (*MICA-129Val*) binders to NKG2D receptor (5). It has also been recently reported that *MICA-129Met*, characterized by stronger and faster NKG2D signaling, is able to trigger relatively higher NK cell cytotoxicity and IFNγ release accompanied by rapid downregulation of NKG2D (6). Significant differences in binding affinities of MICA alleles for NKG2D could have different effects on NK cell activation, in particular under conditions of suboptimal MICA expression.

Multiple myeloma (MM) is a clonal B cell malignancy characterized by the expansion of plasma cells (PCs) in the bone marrow (BM) and is still an incurable disease with a median survival of few years. Its prognosis has been improved by the use of autologous hematopoietic stem cell transplantation (7) and new immunochemotherapeutic approaches (8–10). NK cells play a pivotal role in MM immunosurveillance by exerting direct cytotoxic effects through a number of activating receptors, including NKG2D (11, 12). However, several mechanisms have been identified that permit the escape of tumors bearing NKG2D ligands, including their release by tumor cells through proteolytic cleavage (13–17) or exosome secretion (4). Furthermore, it has been demonstrated that MICA is transferred to NK cells upon target conjugation and that this transfer is directly linked to molecular interactions between NKG2D and MICA, following accumulation of the ligand at the immunological synapse (18). Soluble MICA has been identified as an independent prognostic factor for the overall survival and progression-free survival of MM patients (19). In addition, endogenous anti-MICA antibodies and ligand shedding are critical determinants of host immunity during MM progression (20). It is, however, unknown whether functionally relevant polymorphisms of the *MICA* gene may also contribute to disease progression.

The aim of this study was to investigate the association of MICA genetic polymorphisms and MICA sera levels with progression of MM. Interestingly, our findings indicate that the *MICA-129Met/Val* dimorphism is associated with: (i) differential expression of both soluble and cell-surface MICA, (ii) expression levels of NKG2D on *ex vivo* NK cells isolated from the BM and peripheral blood (PBL) of MM patients, and (iii) the disease state.

RESULTS

MICA-129Val Allele Is Associated With Higher Amount of Soluble MICA in the Sera of MM Patients

Soluble MICA has been proposed as a prognostic marker in MM since its levels correlate with tumor progression (19). However, the generation of soluble MICA can be affected by polymorphisms, regulating cell-surface expression, altering the efficacy of cleavage, and favoring MICA recruitment into exosome-like vesicles (4, 16, 21, 22). At first, we investigated whether soluble NKG2D ligands other than MICA in the sera derived from a cohort of MM patients at different disease states, namely MGUS (monoclonal gammopathy of undetermined significance), smoldering, onset, and relapse, were associated with MM progression. As shown in **Figure 1A**, we established that only soluble MICA but not other soluble NKG2D ligands including MICB and ULBP1-3 (data not shown) were associated to MM progression. Since we confirmed the importance of soluble MICA, we further explored whether MICA polymorphism could affect the amount of soluble MICA as well as MM progression. Therefore, MICA genotype was examined by isolating PBMCs DNA from a cohort of 137 MM patients at different disease states (Figure S1A in Supplementary Material). We also identified the sequence of a new MICA allele and the name MICA*085 has been officially assigned by the WHO Nomenclature Committee for factors of the HLA System. MICA alleles were further classified in three subgroups, *MICA-129Val/Val*, *MICA-129Val/Met*, and *MICA-129Met/Met* (**Tables 1** and **2**). Similarly to other studies (6, 23–25), the frequencies of *MICA-129Val/Val*, *MICA-129Val/Met*, and *MICA-129Met/Met* genotypes were 36, 42, and 22%, respectively (Figure S1B in Supplementary Material). Remarkably, the analysis of *MICA-129* genotype frequencies among different MM states revealed that *MICA-129Val/Val* patients displayed a significantly higher percentage of relapse (**Figure 1B**; Figure S1C in Supplementary Material). In contrast, the frequencies of *MICA-129Val/Met*

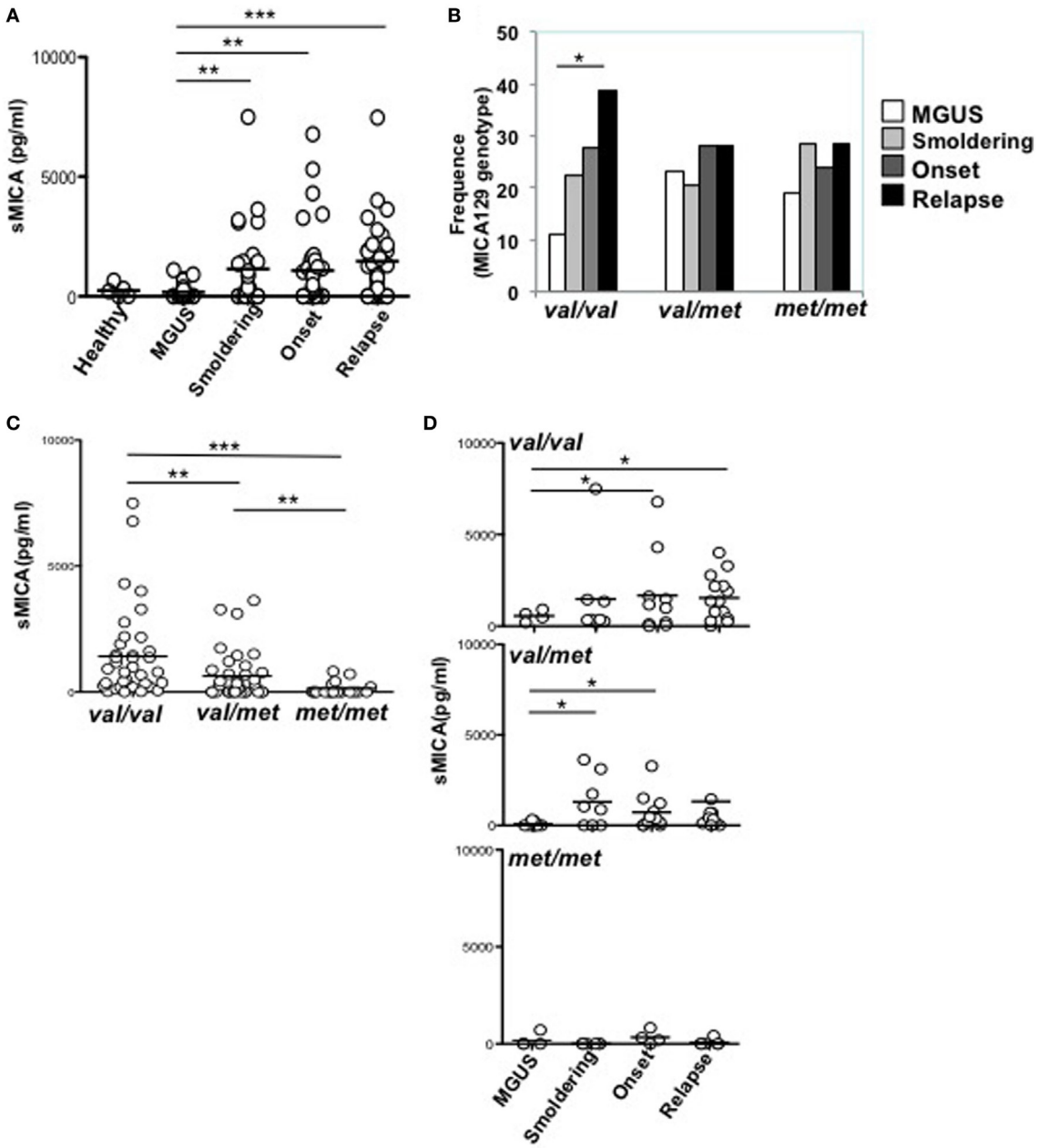

FIGURE 1 | *MICA-129Val* allele is associated with higher amount of sMICA in the sera of multiple myeloma (MM) patients. **(A)** Sera derived from healthy donors, MGUS, and MM patients at different state disease were analyzed for the presence of soluble MICA through a specific enzyme-linked immunosorbent assay. Total number of patients, 97 (healthy, $n = 5$, MGUS, $n = 16$, smoldering, $n = 22$, onset, $n = 26$, relapse, $n = 32$). **(B)** Frequency distribution of MICA-129 genotypes among different MM disease states. χ^2 test with $n - 1$ degrees of freedom was performed. **(C,D)** sMICA is associated with the presence of the *MICA-129* valine (*Val*) allele in MM patients. Total number of patients analyzed, $n = 91$ (Val/Val, $n = 37$; Val/Met, $n = 36$; Met/Met, $n = 18$).

and *MICA-129Met/Met* genotypes were similar throughout all the different disease states (**Figure 1B**). Interestingly, MM patients characterized by the *MICA-129Val/Val* genotype also exhibited the highest levels of soluble MICA in the sera (**Figure 1C**). Consequently, correlation of soluble MICA with MM progression was observed only in the presence of the *MICA-129Val* allele (**Figure 1D**). We further explored the outcome of patients in response to the therapy among different MICA-129 genotypes (**Figures 2A–C**). As shown in **Figure 2A**, a similar response to chemotherapy was observed among the three genotypes. Interestingly, we observed that the highest frequency of relapse was developed by *MICA-129Val/Val* patients also after chemotherapeutic treatment (**Figure 2B**) suggesting that MICA polymorphism impacts on MM relapse.

TABLE 1 | MICA genotype and 129 polymorphism in patients at different disease state.

MGUS patients MICA genotype-129 polymorphism		Smoldering patients MICA genotype-129 polymorphism		Onset patients MICA genotype-129 polymorphism		Relapse patients MICA genotype-129 polymorphism	
002:01/018:01	Met/Met	002:01/018:01	Met/Met	009:01/018:01	Val/Met	085/085	Val/Val
008:01/016	Val/Val	018:01/018:01	Met/Met	002:01/18:01	Met/Met	002:01/008:01	Met/Val
004/012:01	Val/Met	008:01/008:01	Val/Val	004/009:02	Val/Val	002:01/016	Met/Val
016/019	Val/Val	017/019	Met/Val	004/008:01	Val/Val	004/016	Val/Val
008:01/018:01	Val/Met	008:01/011	Val/Met	002:01/010:01	Met/Val	009:01/009:01	Val/Val
002:01/018:01	Met/Met	002:01/018:01	Met/Met	004/008:01	Val/Val	002:01/004	Met/Val
001/018:01	Met/Met	006/008:01	Val/Val	010:01/017	Val/Met	002:01/018:01	Met/Met
006/008:01	Val/Val	009:01/018:01	Val/Met	008:01/018	Val/Met	009:01/009:01	Val/Val
002:01/011	Met/Met	001/018:01	Met/Met	008:01/008:01	Val/Val	016/019	Val/Val
009:01/018:01	Val/Met	002:01/018:01	Met/Met	027/027	Val/Val	008:01/009:01	Val/Val
010:01/018:01	Val/Met	009:01/009:01	Val/Val	008:01/010:01	Val/Val	008:01/009:01	Val/Val
008:01/011	Val/Met	010:01/012:01	Val/Met	004/004	Val/Val	008:01/019	Val/Val
009:01/018:01	Val/Met	004/008:01	Val/Val	004/008:01	Val/Val	009:01/016	Val/Val
004/018:01	Met/Val	008.01/018:01	Val/Met	008:01/018:01	Val/Met	008:01/009:01	Val/Val
002:01/008:01	Val/Met	004/008:01	Val/Val	004/016	Val/Val	002:01/004	Met/Val
004/008:01	Val/Val	002:01/047	Met/Met	002:01/018:01	Met/Met	009:02/018:01	Val/Met
002:01/008:01	Met/Val	009:01/009:02	Val/Val	008:01/018:01	Val/Met	004/009:02	Val/Val
004/011	Val/Met	008:01/009:01	Val/Val	007:01/016	Met/Val	008:01/016	Val/Val
002:01/017	Met/Met	008:01/012:01	Val/Met	004/008:01	Val/Val	001:01/018	Met/Met
002:01/008:01	Met/Val	011/018:01	Met/Met	012:01/018	Met/Met	001/002:01	Met/Met
008:01/008:01	Val/Val	006/008:01	Val/Val	002:01/004	Met/Val	002:01/007:01	Met/Met
008:01/012:01	Val/Met	008:01/010:01	Val/Val	006/009:01	Val/Val	008:01/018:01	Val/Met
002:01/009:02	Met/Val	002:01/004	Met/Val	008:01/018:01	Val/Met	009:01/018:01	Val/Met
011/012:01	Met/Met	002:01/009:01	Met/Val	008:01/018:01	Val/Met	009:01/016	Val/Val
009:01/016	Val/Val	002:01/018:01	Met/Met	002:01/002:01	Met/Met	002:01/018:01	Met/Met
004/016	Val/Val	002:01/008:01	Met/Val	002:01/012:01	Met/Met	004/008:01	Val/Val
002:01/018:01	Met/Met	009:02/018:01	Val/Met	002:01/016	Met/Val	007:01/008:01	Met/Val
002:01/007:01	Met/Met			018:01/027	Met/Val	008:01/010:01	Val/Val
011/047	Met/Met			001/008:01	Met/Val	002:01/007:01	Met/Met
008:01/016	Val/Val			002:01/018:01	Met/Met	009:01/016	Val/Val
004/011	Val/Met			007:01/008:01	Met/Val	001/016	Met/Val
				002:01/008:01	Met/Val	002:01/016	Met/Val
				009:01/009:01	Val/Val	008:01/016	Val/Val
				001/004	Met/Val	002:01/016	Met/Val
				006/018:01	Val/Met	008:01/010:01	Val/Val
				007:01/009:01	Met/Val	009:01/016	Val/Val
				002:01/018:01	Met/Met	008:01/009:01	Val/Val
				004/018:01	Val/Met	002:01/010:01	Met/Val
						018:01/018:01	Met/Met
						002:01/008:01	Met/Val
						008:01/017	Val/Met
						009:01/018:01	Val/Met
						002:01/008:01	Met/Val

Val, valine; Met, methionine.

TABLE 2 | Patient characteristics.

Patients characteristics	Val/Val	Val/Met	Met/Met
Gender			
Male	25	31	13
Female	24	26	18
Age mean (range)	70 (47–83)	65(41–83)	62 (41–87)
% of PCs mean (range)	MGUS 3 (1–10) Smoldering 16 (5–38) Onset 29 (11–45) Relapse 27 (4–90)	MGUS 4 (1–9) Smoldering 18 (4–40) Onset 36 (7–90) Relapse 29 (5–58)	MGUS 3 (1–10) Smoldering 26 (13–58) Onset 29 (2–52) Relapse 33 (4–54)

PCs, plasma cells; Val, valine; Met, methionine.

We asked whether MICA cell-surface expression levels on primary malignant PCs isolated from patients could be related to the MICA genotype. As shown in **Figures 3A–C**, MICA expression on malignant PCs, was significantly higher in *MICA-129Val/Val* MM patients compared to *MICA-129Met/Met* MM patients, thus suggesting that the increased amount of soluble MICA in the sera of *MICA-129Val/Val* patients could be related to an higher expression of this allelic variant. Finally, we further classified MICA alleles into MICA short and long, based on the presence of the truncated MICA*008 allelic variant, but no differences regarding soluble MICA serum levels and the correlation with the disease state were found (data not shown).

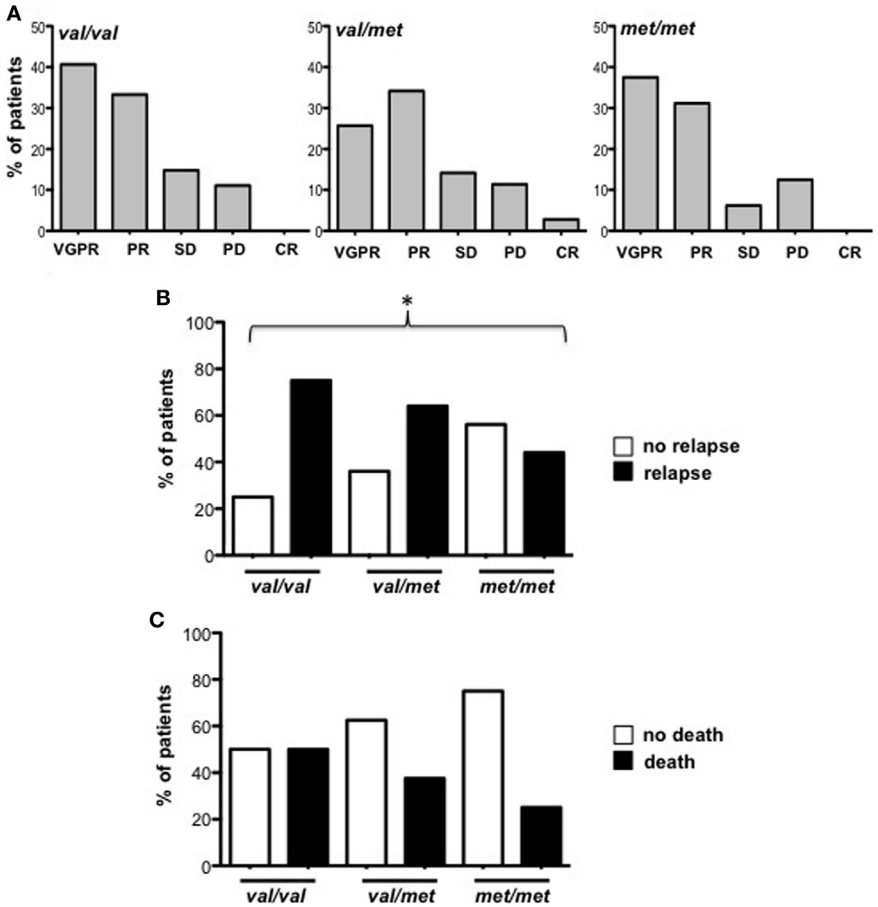

FIGURE 2 | Patient outcome related to MICA-129 genotype. **(A)** Response to therapeutic treatment of multiple myeloma (MM) patients (Onset and Relapse). VGPR, very good partial response; PR, partial response; SD, stable disease; PD, partial disease, CR, complete remission. **(B)** Frequency of relapse development in MM patients after chemotherapy. χ^2 test, $p = 0.0413$. **(C)** Frequency of deceased patients in MM patients after chemotherapy. Total patients, $n = 74$; Val/Val, $n = 26$; Val/Met, $n = 32$; Met/Met, $n = 16$. Abbreviations: Val, valine; Met, methionine.

MICA-129Met Allele Is Associated With an Increased NKG2D Downmodulation on NK Cells Isolated From Patients

We next investigated whether MICA-129 polymorphism was also associated with different levels of NKG2D expression in MM patients. To this aim, NKG2D expression levels on *ex vivo* NK cells isolated from either PBL or BM of MM patients were evaluated. Cells were stained with anti-human CD45, -CD56, -CD3, -CD138 mAbs, along with mAbs specific for NKG2D and DNAM-1 activating receptors. After CD138 (corresponding to PCs) gate exclusion, analysis was performed on $CD45^+CD56^+CD3^-$ total NK cells. Interestingly, our results demonstrate that NKG2D was significantly reduced on NK cells derived from *MICA-129Met/Met* patients compared to both *MICA-129Val/Val* and *MICA-129Val/Met* patients (**Figures 4A,B**). Indeed, the *MICA-129Met* allele that has a higher affinity to NKG2D, is able to induce significantly stronger downmodulation of NKG2D in both NK and CD8 T lymphocytes (6). Importantly, the expression levels of DNAM-1, used as control, were similar among all three different genotypes (**Figures 4A,B**), indicating that NKG2D downmodulation is an event likely associated to *MICA-129* dimorphism. Decreased NKG2D expression on *MICA-129Met/Met* patients was also observed on NK cell subsets expressing low and high levels of CD16 as shown in Figures S2A,B in Supplementary Material. These results suggest that NKG2D downmodulation in MM patients depends essentially on *MICA* genotype and it is not associated with soluble MICA levels. To further support this observation, sera containing different amounts of soluble MICA (derived from patients carrying at least one Val allele) were incubated with PBL derived from healthy donors and NKG2D expression was evaluated after 16 h by immunofluorescence and FACS analysis by gating on $CD56^+CD3^-$ NK cells. As shown in **Figure 5**, we did not observe a significant correlation between the levels of soluble MICA and the extent of NKG2D reduction.

Residue Met129 Is Essential for Appropriate Positioning of the α_{2-1} Helix for NKG2D Recognition

Two crystal structures of MICA have been hitertho determined, one alone (26) and the other in complex with its receptor NKG2D

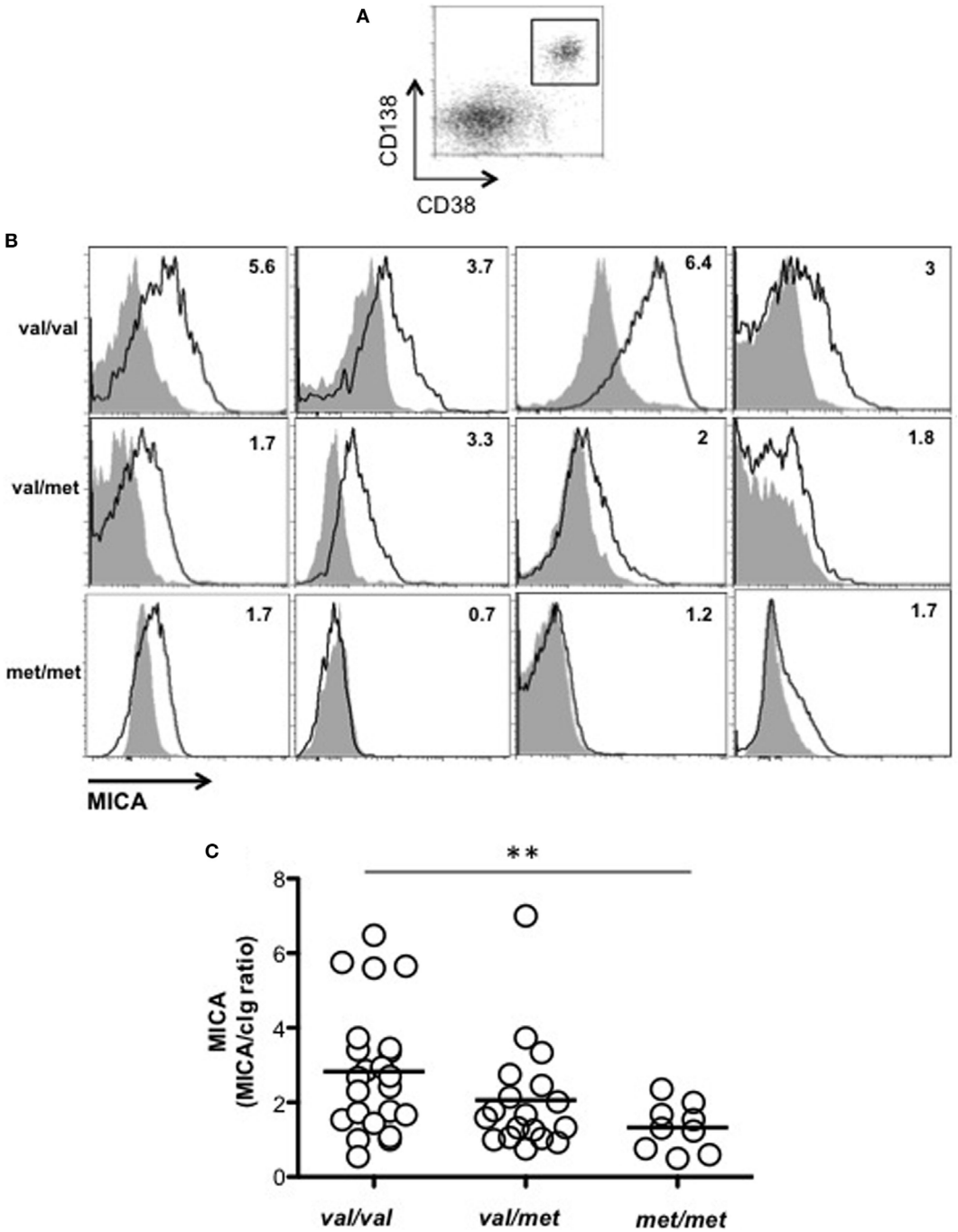

FIGURE 3 | MICA cell-surface expression on malignant plasma cells (PCs) resulted higher in MICA-129Val/Val patients. Total cells derived from the bone marrow (BM) of patients at different disease states were stained with isotypic clg or anti-MICA together with anti-CD38 and anti-CD138 mAbs. **(A)** MICA expression was analyzed by gating $CD38^+/CD138^+$ cells. **(B)** Representative histograms of different patients are reported. Values indicated in each histogram represent the ratio between the MFI value of MICA divided by the MFI value of the isotypic clg. Solid gray histogram: clg; black line: MICA. **(C)** Total number of patients analyzed, $n = 50$ (Val/Val = 23, Val/Met $n = 18$, Met/Met = 9) that were smoldering ($n = 10$), onset ($n = 19$), and relapse ($n = 21$). Abbreviations: Val, valine; Met, methionine.

(27). At first sight, MICA resembles classical MHC class I (MHC-I) molecules with three extracellular domains (α1, α2, and α3), a transmembrane segment that can vary significantly between different MICA alleles, and a carboxy-terminal cytoplasmic tail. However, in contrast to MHC-I, MICA does not bind to the $\beta_2$microglobulin and does not present peptides in the cleft. Comparison of the two structures revealed that the receptor-free form of MICA is disordered within a section of the α_2 region

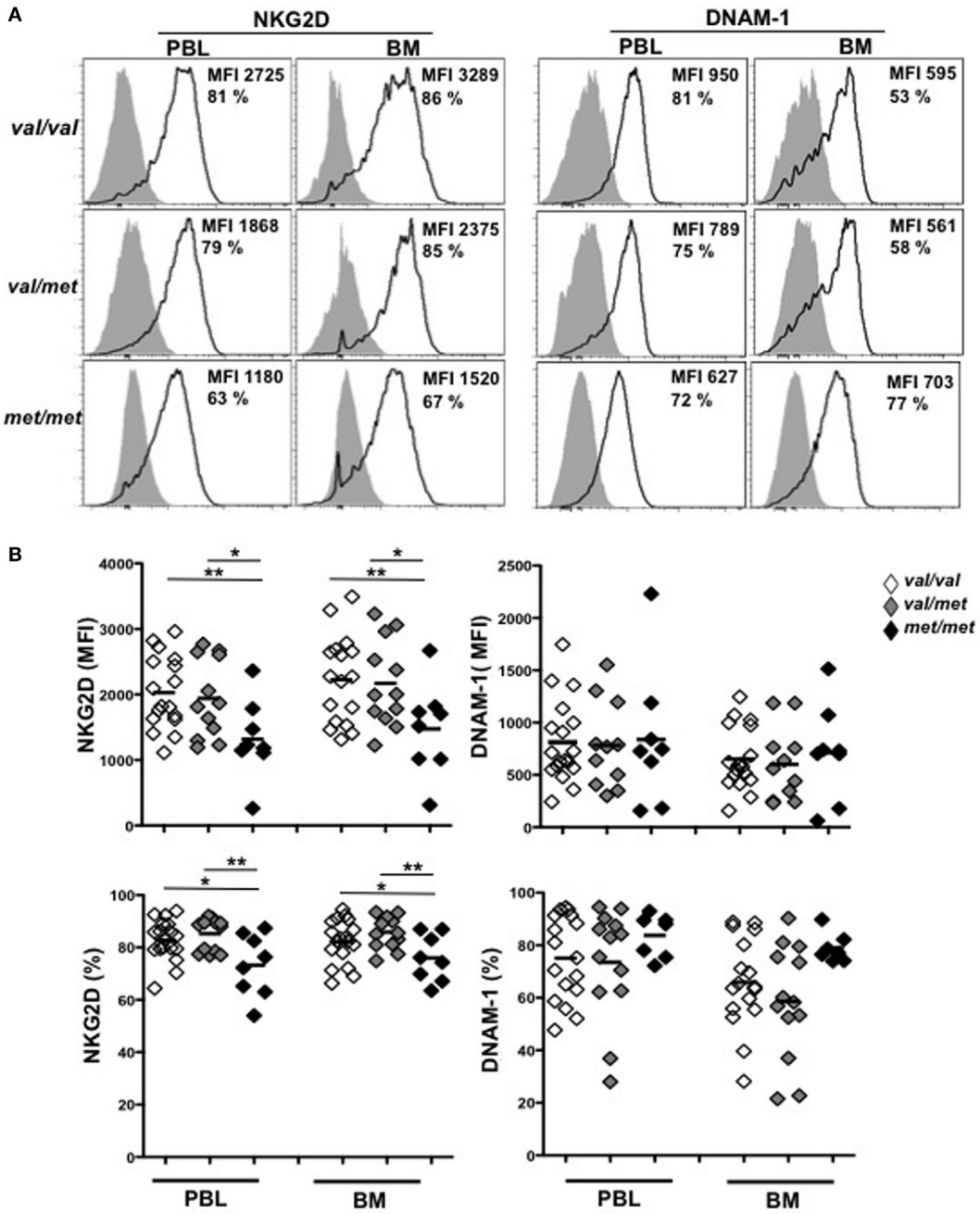

FIGURE 4 | *MICA-129Met* allele is associated with an increased NKG2D downmodulation on natural killer (NK) cells gated from patients. Total cells derived from the bone marrow (BM) or the peripheral blood (PBL) derived from patients at different disease states (including smoldering, onset, and relapse) were stained with antibodies against CD45, CD138, CD56, and CD3. NKG2D or DNAM-1 expression was evaluated by gating on NK cells ($CD45^+CD138^-CD3^-$ $CD56^+$). **(A)** Representative histograms are shown. **(B)** Values reported represent the MFI values of NKG2D or DNAM-1 subtracted from the MFI value of the isotypic clg or the percentage of NKG2D and DNAM-1. Total number of patients analyzed, $n = 40$ (Val/Val = 19, Val/Met $n = 13$, Met/Met = 8). Abbreviations: Val, valine; Met, methionine.

corresponding to residues 152 to 161, essential for NKG2D recognition (27) (**Figure 6A**). Interestingly, the crystal structure of MICA in complex with NKG2D revealed that these nine MICA residues, which link the helices α_{2-1} and α_{2-2}, are ordered upon binding to NKG2D (**Figure 6B**). The crystal structures also revealed that residue Met129 is localized at the end of the small β8-strand, far away from the MICA/NKG2D interface. This residue forms, together with Trp127, Phe110, Phe117, and Leu118, a hydrophobic base on which the helix α_{2-1} docks (**Figure 6C**). The importance of such hydrophobic nucleus for the correct folding and orientation of a helix has been previously demonstrated (28). The hydrophobic residues Leu138, Ala139, Met140, and Val142

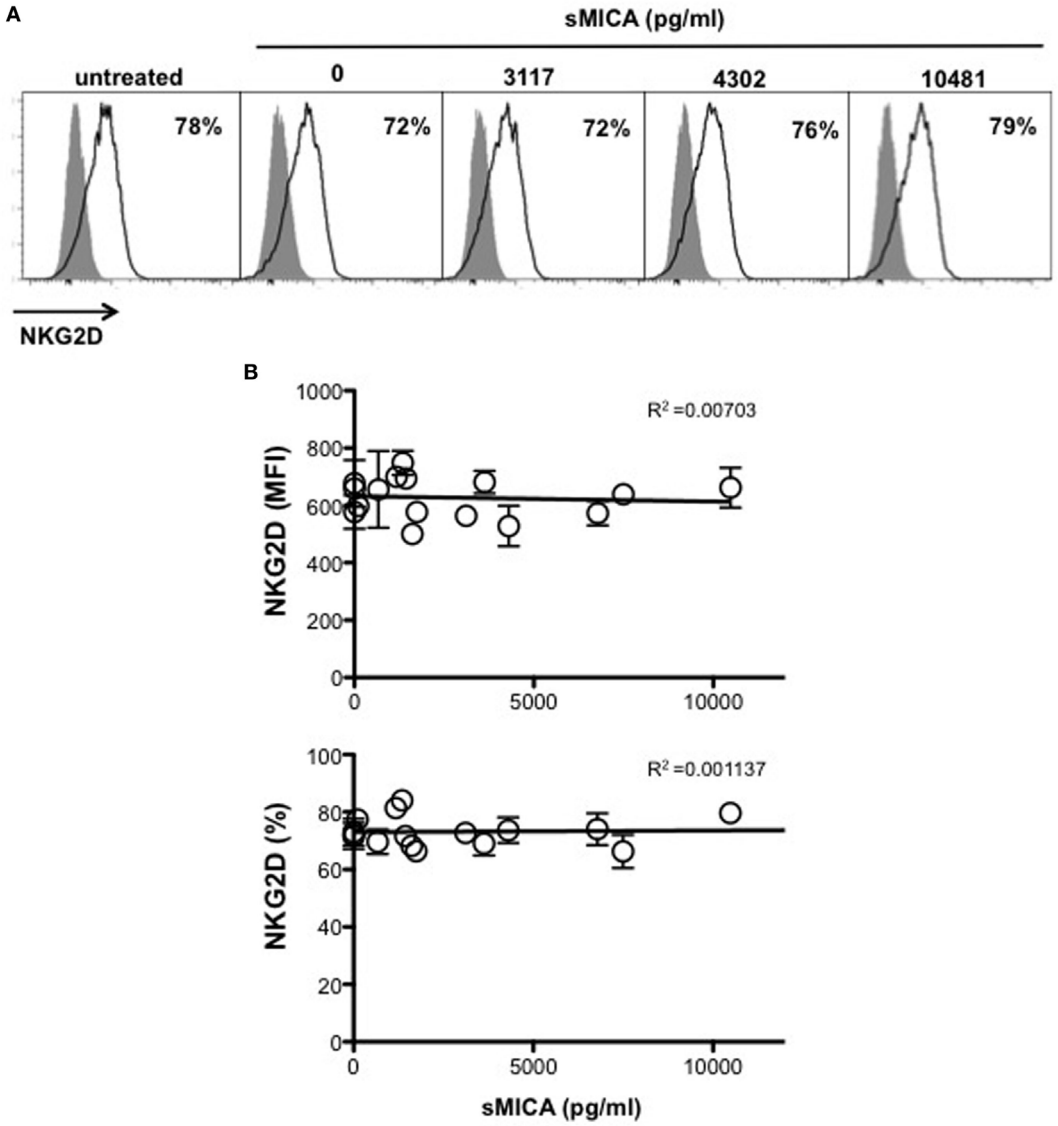

FIGURE 5 | Soluble MICA levels in the serum of multiple myeloma (MM) patients carrying the Val allele do not correlate with change in NKG2D expression. Peripheral blood derived from healthy donors were incubated for 16 h with medium alone or serum derived from MGUS or MM patients at different disease states carrying at least one Val allele and containing variable levels of soluble MICA. Cells were harvested and NKG2D expression was evaluated by gating on $CD3^{-}CD56^{+}$ natural killer (NK) cells. **(A)** A representative experiment is shown. **(B)** Values reported on *y* axis represent the MFI value of NKG2D subtracted from the MFI value of the isotypic clg (high panel) or the percentage of NKG2D positive cells (lower panel) and were correlated with soluble MICA levels of each patient as indicated on *x* axis. Total number of serum patients analyzed, $n = 16$ (3 MGUS, 6 smoldering, 6 onset, 1 relapse).

on the α_{2-1} helix face and interact with the β-sheet docking site described above (**Figure 6C**). The large and hydrophobic residue Met129 is at the heart of this putative nucleating site, forming van der Waals interactions with residues Gln136, Ala139, and Met140 all localized on the α_{2-1} helix (**Figure 6C**).

The clear differences in binding affinity between the two MICA variants and NKG2D have been previously suggested to be due to conformational changes (5). However, this is in our opinion unlike since comparison of the crystal structure of MICB (30) which comprises a Val residue at position 129, with the crystal structure of *MICA-129Met*, reveals the similarity of their conformations (**Figure 6D**). Instead, molecular modeling analysis indicated that replacement of residue Met129 with the much smaller Val residue removes at least three van der Waals interactions between the α_{2-1} helix and the β-sheet on which it docks, and generates a solvent accessible cavity in this hydrophobic core. Furthermore, comparative analysis of the two crystal structures of MICA indicates that the α_{2-1} helix rotates slightly upon NKG2D binding around the contact between Met129 and Ala139, resulting in significant movements at both ends of this helix (**Figure 6E**). Mutation of residue Met129, which we believe acts a lever stop for the helix α_{2-1} to build the conformation optimal for the NKG2D binding, would clearly have a significant impact on the direction and tilting of the helix. Thus, the Met to Val dimorphism could affect

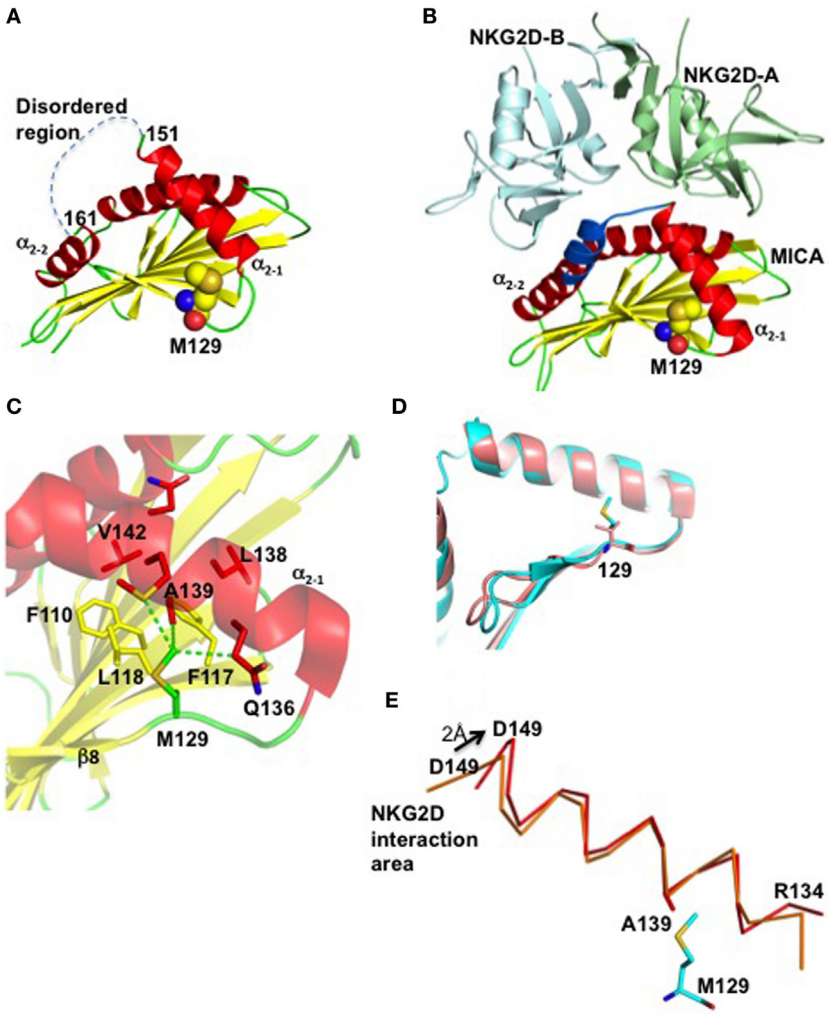

FIGURE 6 | Residue Met129 is essential for appropriate positioning of the α_{2-1} helix for NKG2D recognition. **(A)** The crystal structure of the free form of MICA is colored according to its secondary structure, with helices, β-strands and loops in red, yellow, and green, respectively. Only the α1 and α2 regions of MICA are displayed. The region comprising residues 152–161 and which is not visible in the electron density due to disorder, is indicated by a dashed line. All atoms forming residue Met129 are indicated as balls with oxygen, nitrogen, sulfur, and carbon atoms colored in red, blue, orange, and yellow, respectively. **(B)** The crystal structure of the MICA/NKG2D complex reveals that the flexible 152–161 region (in blue) is stabilized through interactions with NKG2D. The two subunits of NKG2D receptor are displayed in green and cyan. Residue Met129 is localized far away from the MICA/NKG2D interface. **(C)** Residue Met129 plays a key role within a hydrophobic core formed between helix α_{2-1} and several β-sheet residues that surround and interact with Met129. The van der Waals interactions formed between Met129 and residues (in red) on the helix α_{2-1} are indicated by green dashed lines. **(D)** Superposition of the α1α2 domains of MICA and MICB, in pink and cyan, respectively, reveals that Met129 in MICA and Val129 in MICB take the same position and orientation, and demonstrates that MICA-129Met/Val dimorphism does not affect the conformation of the two MIC alleles (29). **(E)** The MICA α_{2-1} helix rotates following complex formation with NKG2D. Both ends of free MICA (red) are deviating from their position in the complex with NKG2D (orange), by 1.5 and 2.0 Å at the N- and C-termini, respectively.

the MICA transition from disorder to form an optimal template for NKG2D recognition, providing a reasonable, although still hypothetical, explanation for the difference in the binding capacity of the two MICA variants to NKG2D.

DISCUSSION

In this study, we investigated the association of MICA genetic polymorphisms and sera levels with the progression of MM. Interestingly, our findings indicate that the *MICA-129Met/Val* dimorphism is associated with: (i) differential expression of both soluble and cell surface MICA, (ii) expression levels of NKG2D on *ex vivo* NK cells isolated from the BM and PBL of MM patients, and (iii) the disease state.

Polymorphisms of MICA have been largely investigated for their role in infections, autoimmune diseases, and cancer (31). Due to its functional consequences, a number of disease association studies with the *MICA-129* dimorphism have been previously performed (32). Interestingly, we found an higher frequency of relapse in MM patients carrying the *MICA-129Val/Val* genotype that was also observed by analyzing the patients outcome in response to the therapy.

Other studies in different cancer models have reported that the *MICA-129Val/Val* genotype can be associated with higher risk for nasopharyngeal (NC) and breast cancer (33, 34). Increasing evidence has pointed out a key role for the NKG2D activating receptor and its ligands in the surveillance of MM. In particular Rebmann and coworkers have shown that soluble MICA levels correlate with tumor progression, and this molecule has been proposed as a prognostic marker in MM (19). Our findings demonstrate that increased levels of soluble MICA can be found in MM patients sera during the progression from MGUS to relapse and clearly show for the first time that the presence of soluble MICA is associated with the *MICA-129Val* allele. As matter of fact, in other pathological conditions, including Ulcerative Colitis and Hepatitis B infection (35, 36), the *MICA-129Val/Val* genotype has been associated with the highest soluble MICA serum levels.

In line with our *in vivo* results showing both a higher expression of cell surface and soluble MICA in *MICA-129Val/Val* patients, Isernhagen and colleagues reported that soluble MICA levels as well as its cell-surface expression were higher in a panel of tumor and melanoma cell lines carrying the *MICA-129Val/Val* genotype (21). It is possible that the *MICA-129Val* allele has a higher transcriptional activity which might explain its effect on soluble and cell-surface MICA levels (37). Another non-excluding possibility is that the higher amount of soluble MICA-*129Val* could be directly due to the 30-times lower affinity of this variant to NKG2D compared to MICA-*129Met* resulting in a reduced transfer to NK cells and its accumulation on the surface of MM target cells.

Previous studies suggested that elevated levels of soluble MICA in the sera of cancer patients correlate with an increased NKG2D downregulation on PBL NK cells and T lymphocytes (22). However, it is still unclear whether soluble MICA has the capability to directly induce NKG2D downregulation and/or if additional soluble factors in the sera of cancer patients contribute to this effect. Infact, Paschen and coworkers demonstrated that elevated levels of soluble NKG2D ligands (i.e., MICA and ULBP2) in the sera of melanoma patients were not associated with a significant downregulation of NKG2D expression on peripheral NK cells (38). Even NK cells from rheumatoid arthritis patients with relatively high soluble MICA levels, did not show diminished NKG2D expression (39). Furthermore in MM, soluble MICA was not significantly associated with NKG2D downregulation and *in vitro* experiments with MM patients' serum and culture supernatants, did not result in changes in NKG2D expression (40). Similarly, by incubating NK cells with sera from patients containing different amounts of soluble MICA, we did not find a significant correlation between the levels of soluble MICA and the extent of NKG2D downmodulation. Interestingly, we observed that the lowest levels of NKG2D on NK cells from MM patients, were associated with the *MICA-129Met/Met* genotype. In line with these findings, it has been shown that the *MICA-129Met* allele, with proven higher affinity to NKG2D, is able to induce significantly stronger downmodulation of NKG2D in both NK and CD8 T lymphocytes, and to better stimulate IFNγ production as compared to the *MICA-129Val* allele (6). It should be considered that receptor endocytosis not only leads to reduced cell-surface receptor abundance but also controls signaling outcome in NK cells as shown by Molfetta and coworkers who reported that ubiquitin-dependent NKG2D/DAP10 endocytosis was required for the activation of extracellular signal-regulated kinase and NK cell functions (41, 42). It is possible that cancer cells carrying the MICA-129*Met* allele could better induce NK cell activation that corresponded to a substantial NKG2D reduction observed in patients. By performing *in vitro* degranulation assays on NKG2D-sensitive targets, "*ex vivo*" unstimulated NK cells derived from patients had very low levels (below 5%) of degranulation, independently of the MICA genotype (data not shown). This result is in accordance with previous evidences indicating that stimulation of NKG2D alone is not sufficient to trigger cytotoxicity and/or cytokine production in resting human NK cells (43). In fact, effector functions mediated by this receptor rely on different factors including the activation status of NK cells, the cooperation with other NK activating receptors or with distinct cytokines (44). Thus, in MM patients, the tumor microenvironment, the cytokine milieu and the expression of other NK cell activating ligands on cancer cells can dictate the final outcome of the NKG2D-mediated NK cell response.

Our results obtained by structural modeling analysis suggested that the Met to Val dimorphism could affect the capacity of MICA to form an optimal template for NKG2D recognition. It is possible that the lowest NKG2D levels in MM patients with a MICA-*129Met/Met* genotype reflects the capacity of the *MICA-129Met* allele to more efficiently engage NKG2D and trigger NK cell activity in a cell–cell contact manner and this event appears to be independent from soluble MICA sera levels that are instead predominant in individuals carrying the *MICA-129Val* allele.

In conclusion, our data indicate that the *MICA-129Val* allele is associated with significantly higher levels of soluble MICA and an higher frequency of relapse and strongly suggest that the MICA genotype could be used as prognostic marker in alternative to soluble MICA if further data with higher numeric dimension will confirm these findings. Altogether, these observations could help to develop more personalized predictive biomarkers in MM.

MATERIALS AND METHODS

Clinical Samples

Sera, PBMCs, and BM samples were obtained from MM patients enrolled at the Division of Hematology (Sapienza University of Rome). Informed and written consent in accordance with the Declaration of Helsinki was obtained from all patients, and approval was obtained from the Ethics Committee of the Sapienza University of Rome (Rif. 3373). Patients were classified according to the disease state. Patients (Onset and Relapse) were treated according to standard therapeutic protocols including the usage of VMP (Bortezomib, Melphalan, Prednisone), VD (Bortezomib, Dexamethasone), and RD (Lenalidomide, Dexamethasone).

MICA Gene Typing

For the genotyping of MICA, genomic DNA derived from patients PBMCs was isolated from 1×10^6 cells using the Genomic DNA purification kit according to the manufacturer's instructions

(Bioline, London, UK). Sequence-based typing of MICA was performed as described before (45). The sequence of a new MICA allele was identified and the name MICA*085 has been officially assigned by the WHO Nomenclature Committee for factors of the HLA System in February 2015 (Genbank accession: KP262025).

Immunofluorescence and FACS Analysis

Analysis of MICA expression on patient-derived PCs was performed by gating the $CD38^+CD138^+$ PC population using the antibodies anti-MICA (clone 159227, R&D Systems, Minneapolis, MN, USA), anti-CD38/APC, and anti-CD138/FITC (both from BD Bioscience, San Jose, CA, USA) as previously reported (16); samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA, USA) and a FACSCalibur (Becton Dickinson). Analysis of NKG2D and DNAM-1 on NK cells from PBMCs or BM aspirates was performed by gating on the $CD45^+CD138^-CD3^-CD56^+$ population using the antibodies anti-CD3/allophycocyanin-H7, anti-CD56/PE, anti-CD138/FITC, anti-CD45/PE-Cy7, anti-NKG2D/APC, or anti-DNAM-1/APC (BD Bioscience). In some experiments, anti-CD16/PerCP mAb was also used (BD Bioscience). In the experiments relative to **Figure 4** and Figure S2 in Supplementary Material, all the patients-derived samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA, USA). In the experiments relative to **Figure 5**, PBMCs derived from healthy donors were incubated with medium alone or serum derived from MGUS or MM patients for 16 h. After harvesting, cells were stained with antibodies from BD Bioscience: anti-CD3/PerCP, anti-CD56/APC, and anti-NKG2D/PE; samples were acquired using a FACSCalibur (Becton Dickinson).

Data analysis was performed using the FlowJo 9.3.2 program (TreeStar, Ashland, OR, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays for soluble MICA, MICB, and ULBP1 were from R&D Systems (Minneapolis, MN, USA), and performed as previously described (46), with modifications (AMO1 anti-MICA capture mAb, 2 μg/ml, BAMOMAB, Germany) (47). Soluble ULBP2 was detected as previously described (15). Absorbance values of triplicate samples were obtained by subtracting readings at 540 nm from readings at 450 nm. Net absorbance was obtained by subtracting the reagent blank absorbance. Before the assay, sera samples were diluted in PBS/0.1% Triton X-100 (vol/vol) and incubated for 30 min at 37 C.

Molecular Modeling of the MICA-Val129 Variant and Structural Analysis

In order to evaluate the structural consequences of the MICA polymorphism, we created a three-dimensional molecular model of the *MICA-Val129* variant, using the crystal structures of the free *MICA-Met129* molecule (PDB code 1B3J) and the *MICA-Met129/NKG2D* complex (PDB code 1HYR) (26, 27) as templates. The creation of the molecular model of the *MICA-Val129* variant, as well as all comparative structural analyses, was performed using the program Coot (48). **Figure 5** was created using the program PyMol (PyMol Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Statistic

In all the experiments, statistic was performed using the unpaired Mann–Whitney test, except for **Figure 1D** in which the unpaired *t*-test with Welch's correction was used. *<0.05; **<0.01; ***<0.001. χ^2 test was used to analyze frequency data.

ETHICS STATEMENT

Informed and written consent in accordance with the Declaration of Helsinki was obtained from all patients, and approval was obtained from the Ethics Committee of the Sapienza University of Rome.

AUTHOR CONTRIBUTIONS

FC, MG, and IN, extracted patients' DNA, collected patients' sera, and performed ELISA experiments. EV and AP performed experiments on bone marrow and peripheral blood of patients. DF, TSaribekyan, and JM performed MICA gene typing. AA and TSandalova performed structural modelling. EM, MP, and MR managed patients and evaluated clinical parameters. CF, ASoriani, MC, and CC, analyzed and discussed data. AZ and ASantoni designed the experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00926/full#supplementary-material.

REFERENCES

1. Lanier LL. NKG2D receptor and its ligands in host defense. *Cancer Immunol Res* (2015) 3:575–82. doi:10.1158/2326-6066.CIR-15-0098
2. Cerboni C, Zingoni A, Cippitelli M, Piccoli M, Frati L, Santoni A. Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis. *Blood* (2007) 110:606–15. doi:10.1182/blood-2006-10-052720
3. Nausch N, Cerwenka A. NKG2D ligands in tumor immunity. *Oncogene* (2008) 27:5944–58. doi:10.1038/onc.2008.272
4. Ashiru O, Boutet P, Fernandez-Messina L, Aguera-Gonzalez S, Skepper JN, Vales-Gomez M, et al. Natural killer cell cytotoxicity is suppressed by exposure

to the human NKG2D ligand MICA*008 that is shed by tumor cells in exosomes. *Cancer Res* (2010) 70:481–9. doi:10.1158/0008-5472.CAN-09-1688
5. Steinle A, Li P, Morris DL, Groh V, Lanier LL, Strong RK, et al. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics* (2001) 53:279–87. doi:10.1007/s002510100325
6. Isernhagen A, Malzahn D, Viktorova E, Elsner L, Monecke S, von Bonin F, et al. The MICA-129 dimorphism affects NKG2D signaling and outcome of hematopoietic stem cell transplantation. *EMBO Mol Med* (2015) 7:1480–502. doi:10.15252/emmm.201505246
7. Vallet S, Pecherstorfer M, Podar K. Adoptive cell therapy in multiple myeloma. *Expert Opin Biol Ther* (2017) 17:1511–22. doi:10.1080/14712598.2017.1375095
8. Fionda C, Soriani A, Zingoni A, Santoni A, Cippitelli M. NKG2D and DNAM-1 ligands: molecular targets for NK cell-mediated immunotherapeutic intervention in multiple myeloma. *Biomed Res Int* (2015) 2015:178698. doi:10.1155/2015/178698
9. Boussi L, Niesvizky R. Advances in immunotherapy in multiple myeloma. *Curr Opin Oncol* (2017) 29:460–6. doi:10.1097/CCO.0000000000000407
10. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, et al. Restoring natural killer cell immunity against multiple myeloma in the era of new drugs. *Front Immunol* (2017) 8:1444. doi:10.3389/fimmu.2017.01444
11. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* (2005) 105:251–8. doi:10.1182/blood-2004-04-1422
12. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* (2009) 113:3503–11. doi:10.1182/blood-2008-08-173914
13. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother* (2006) 55:1584–9. doi:10.1007/s00262-006-0167-1
14. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICA in malignant diseases. *Int J Cancer* (2006) 118:684–7. doi:10.1002/ijc.21382
15. Waldhauer I, Steinle A. Proteolytic release of soluble UL16-binding protein 2 from tumor cells. *Cancer Res* (2006) 66:2520–6. doi:10.1158/0008-5472.CAN-05-2520
16. Zingoni A, Cecere F, Vulpis E, Fionda C, Molfetta R, Soriani A, et al. Genotoxic stress induces senescence-associated ADAM10-dependent release of NKG2D MIC ligands in multiple myeloma cells. *J Immunol* (2015) 195(2):736–48. doi:10.4049/jimmunol.1402643
17. Zingoni A, Vulpis E, Nardone I, Soriani A, Fionda C, Cippitelli M, et al. Targeting NKG2D and NKp30 ligands shedding to improve NK cell-based immunotherapy. *Crit Rev Immunol* (2016) 36:445–60. doi:10.1615/CritRevImmunol.2017020166
18. McCann FE, Eissmann P, Onfelt B, Leung R, Davis DM. The activating NKG2D ligand MHC class I-related chain A transfers from target cells to NK cells in a manner that allows functional consequences. *J Immunol* (2007) 178:3418–26. doi:10.4049/jimmunol.178.6.3418
19. Rebmann V, Schutt P, Brandhorst D, Opalka B, Moritz T, Nowrousian MR, et al. Soluble MICA as an independent prognostic factor for the overall survival and progression-free survival of multiple myeloma patients. *Clin Immunol* (2007) 123:114–20. doi:10.1016/j.clim.2006.11.007
20. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc Natl Acad Sci U S A* (2008) 105:1285–90. doi:10.1073/pnas.0711293105
21. Isernhagen A, Schilling D, Monecke S, Shah P, Elsner L, Walter L, et al. The MICA-129Met/Val dimorphism affects plasma membrane expression and shedding of the NKG2D ligand MICA. *Immunogenetics* (2015) 68:109–23. doi:10.1007/s00251-015-0884-8
22. Chitadze G, Bhat J, Lettau M, Janssen O, Kabelitz D. Generation of soluble NKG2D ligands: proteolytic cleavage, exosome secretion and functional implications. *Scand J Immunol* (2013) 78:120–9. doi:10.1111/sji.12072
23. Fuerst D, Neuchel C, Niederwieser D, Bunjes D, Gramatzki M, Wagner E, et al. Matching for the MICA-129 polymorphism is beneficial in unrelated hematopoietic stem cell transplantation. *Blood* (2016) 128:3169–76. doi:10.1182/blood-2016-05-716357
24. Boukouaci W, Busson M, Peffault de Latour R, Rocha V, Suberbielle C, Bengoufa D, et al. MICA-129 genotype, soluble MICA, and anti-MICA antibodies as biomarkers of chronic graft-versus-host disease. *Blood* (2009) 114:5216–24. doi:10.1182/blood-2009-04-217430
25. Lopez-Hernandez R, Valdes M, Lucas D, Campillo JA, Martinez-Garcia P, Salama H, et al. Association analysis of MICA gene polymorphism and MICA-129 dimorphism with inflammatory bowel disease susceptibility in a Spanish population. *Hum Immunol* (2010) 71:512–4. doi:10.1016/j.humimm.2010.02.003
26. Li P, Willie ST, Bauer S, Morris DL, Spies T, Strong RK. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. *Immunity* (1999) 10:577–84. doi:10.1016/S1074-7613(00)80057-6
27. Li P, Morris DL, Willcox BE, Steinle A, Spies T, Strong RK. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. *Nat Immunol* (2001) 2:443–51. doi:10.1038/87757
28. Wensley BG, Kwa LG, Shammas SL, Rogers JM, Clarke J. Protein folding: adding a nucleus to guide helix docking reduces landscape roughness. *J Mol Biol* (2012) 423:273–83. doi:10.1016/j.jmb.2012.08.003
29. Muller S, Zocher G, Steinle A, Stehle T. Structure of the HCMV UL16-MICB complex elucidates select binding of a viral immunoevasin to diverse NKG2D ligands. *PLoS Pathog* (2010) 6:e1000723. doi:10.1371/journal.ppat.1000723
30. Holmes MA, Li P, Petersdorf EW, Strong RK. Structural studies of allelic diversity of the MHC class I homolog MIC-B, a stress-inducible ligand for the activating immunoreceptor NKG2D. *J Immunol* (2002) 169:1395–400. doi:10.4049/jimmunol.169.3.1395
31. Chen D, Gyllensten U. MICA polymorphism: biology and importance in cancer. *Carcinogenesis* (2014) 35:2633–42. doi:10.1093/carcin/bgu215
32. Isernhagen A, Malzahn D, Bickeboller H, Dressel R. Impact of the MICA-129Met/Val dimorphism on NKG2D-mediated biological functions and disease risks. *Front Immunol* (2016) 7:588. doi:10.3389/fimmu.2016.00588
33. Douik H, Ben Chaaben A, Attia Romdhane N, Romdhane HB, Mamoghli T, Fortier C, et al. Association of MICA-129 polymorphism with nasopharyngeal cancer risk in a Tunisian population. *Hum Immunol* (2009) 70:45–8. doi:10.1016/j.humimm.2008.10.008
34. Ouni N, Ben Chaaben A, Kablouti G, Lajnef M, Ayari F, Abaza H, et al. MICA-129Met/Val polymorphism is associated with early-onset breast cancer risk. *Immunol Invest* (2017) 46(6):603–14. doi:10.1080/08820139.2017.1336175
35. Tong HV, Toan NL, Song LH, Bock CT, Kremsner PG, Velavan TP. Hepatitis B virus-induced hepatocellular carcinoma: functional roles of MICA variants. *J Viral Hepat* (2013) 20:687–98. doi:10.1111/jvh.12089
36. Zhao J, Jiang Y, Lei Y, Zou K, Wang C, Huang S, et al. Functional MICA-129 polymorphism and serum levels of soluble MICA are correlated with ulcerative colitis in Chinese patients. *J Gastroenterol Hepatol* (2011) 26:593–8. doi:10.1111/j.1440-1746.2010.06524.x
37. Lo PH, Urabe Y, Kumar V, Tanikawa C, Koike K, Kato N, et al. Identification of a functional variant in the MICA promoter which regulates MICA expression and increases HCV-related hepatocellular carcinoma risk. *PLoS One* (2013) 8:e61279. doi:10.1371/journal.pone.0061279
38. Paschen A, Sucker A, Hill B, Moll I, Zapatka M, Nguyen XD, et al. Differential clinical significance of individual NKG2D ligands in melanoma: soluble ULBP2 as an indicator of poor prognosis superior to S100B. *Clin Cancer Res* (2009) 15:5208–15. doi:10.1158/1078-0432.CCR-09-0886
39. Groh V, Bruhl A, El-Gabalawy H, Nelson JL, Spies T. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. *Proc Natl Acad Sci U S A* (2003) 100:9452–7. doi:10.1073/pnas.1632807100
40. von Lilienfeld-Toal M, Frank S, Leyendecker C, Feyler S, Jarmin S, Morgan R, et al. Reduced immune effector cell NKG2D expression and increased levels of soluble NKG2D ligands in multiple myeloma may not be causally linked. *Cancer Immunol Immunother* (2010) 59:829–39. doi:10.1007/s00262-009-0807-3

41. Quatrini L, Molfetta R, Zitti B, Peruzzi G, Fionda C, Capuano C, et al. Ubiquitin-dependent endocytosis of NKG2D-DAP10 receptor complexes activates signaling and functions in human NK cells. *Sci Signal* (2015) 8:ra108. doi:10.1126/scisignal.aab2724
42. Molfetta R, Quatrini L, Zitti B, Capuano C, Galandrini R, Santoni A, et al. Regulation of NKG2D expression and signaling by endocytosis. *Trends Immunol* (2016) 37:790–802. doi:10.1016/j.it.2016.08.015
43. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* (2006) 107:159–66. doi:10.1182/blood-2005-04-1351
44. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* (2006) 214:73–91. doi:10.1111/j.1600-065X.2006.00457.x
45. Furst D, Solgi G, Recker K, Mytilineos D, Schrezenmeier H, Mytilineos J. Sequence-based typing of major histocompatibility complex class I chain-related gene A alleles by use of exons 2-5 information. *Tissue Antigens* (2011) 77:201–5. doi:10.1111/j.1399-0039.2010.01601.x
46. Cerboni C, Ardolino M, Santoni A, Zingoni A. Detuning CD8+ T lymphocytes by down-regulation of the activating receptor NKG2D: role of NKG2D ligands released by activated T cells. *Blood* (2009) 113:2955–64. doi:10.1182/blood-2008-06-165944
47. Cox MC, Battella S, La Scaleia R, Pelliccia S, Di Napoli A, Porzia A, et al. Tumor-associated and immunochemotherapy-dependent long-term alterations of the peripheral blood NK cell compartment in DLBCL patients. *Oncoimmunology* (2015) 4:e990773. doi:10.4161/2162402X.2014.990773
48. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* (2010) 66:486–501. doi:10.1107/S0907444910007493

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MINI REVIEW
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Daratumumab for the Treatment of Multiple Myeloma

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This mini-review will summarize the present state of development of the CD38 antibody daratumumab for the treatment of multiple myeloma.

Keywords: daratumumab, myeloma, CD38, immunomodulation, adenosine, complement, trogocytosis, neonatal Fc-receptors

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Now as we are close to the 10-year anniversary of dosing the first patient with daratumumab (March 26, 2008), it seems appropriate to review how far we have come in the development of this CD38 antibody for the treatment of multiple myeloma. Based on preclinical development by scientists at the Danish-Dutch biotech company Genmab in collaboration with scientists at the University Hospital in Utrecht, daratumumab was selected among several hundred CD38 antibodies for clinical development. It was clearly recognized at that time that there was an unmet need for new treatment options because of the poor prognosis of patients who were double refractory to both proteasome inhibitors and IMIDS. At the same time, there was a certain level of anxiety surrounding the clinical use of monoclonal antibodies because of a recent disaster with a CD28 antibody that had been tested in a clinical phase I trial the year before we started testing daratumumab. Also, the fact that the target molecule of daratumumab, CD38, is widely expressed in the human body was a cause of concern. In addition to being expressed by leukocytes, erythrocytes, platelets, and immature cells of the bone marrow, CD38 is also expressed by neuronal cells and glial cells of the central nervous system, peripheral nerves, pancreas islets cells, osteoclasts, skeletal muscle cells, cardiac muscle cells, and bronchial epithelium. Every precaution was taken during the initial phase I/II clinical trial GEN501 to avoid serious damage to occur to the patients because of unwanted reactions with normal tissues. The possibility of testing in animal models for toxicity of daratumumab was limited by the lack of cross reactivity of daratumumab with the CD38 molecule of other species. Six chimpanzees were chosen for preclinical testing of daratumumab, one of them died from a cytokine storm and others developed significant drops of the platelet counts. These adverse events have not been seen in humans.

The reason for moving forward with testing of daratumumab for the treatment of multiple myeloma was the very high level of expression of CD38 by myeloma cells. Preclinical studies showed that daratumumab may kill myeloma cells by complement-mediated cytotoxicity, by antibody-dependent cellular cytotoxicity, and by antibody-dependent cellular phagocytosis (1–3). Due to the anticipated risk of significant side effects, the initial clinical testing of daratumumab took offset from extremely low doses of antibody starting with 0.005 mg/kg with a step-by-step increase of the dose up to a planned maximum of 24 mg/kg (4). Because of the safety precautions during the trial it took 3 1/2 years to enroll the first 23 patients. When the dose of daratumumab had been increased to a level of 2 and 4 mg/kg, we started to see signs of clinical efficacy with a drop in the patient's M-protein. This created a lot of interest and since at the same time we had seen no major side effects to the treatment subsequent enrollment into this and other clinical trials with daratumumab made rapid progress. Pharmacokinetic studies showed that target saturation may be achieved at a dose of 16 mg/kg with a schedule that was defined as eight weekly dosing, followed by eight bi-weekly dosing and then dosing of daratumumab every 4 weeks. A maximum tolerated

dose was not reached at a dose up to 24 mg/kg. The superiority of 16 over 8 mg/kg dosing has been confirmed in a clinical trial (5).

The first clinical trials conducted with single agent daratumumab demonstrated that about 30% of patients with relapsed refractory myeloma may respond to daratumumab (6). Interestingly, about 50% of all the patients participating in the trials had a significant prolongation of survival although they did not fulfill the criteria for a response to daratumumab according to IMWG criteria. A plausible reason for this effect of daratumumab was revealed when studies conducted at the University hospital in Amsterdam in collaboration with Janssen demonstrated an immunomodulatory effect of daratumumab (7). Immunoregulatory cells belonging to the T cell, B cell, and monocyte–macrophage system express CD38 and are eliminated during treatment with daratumumab (**Figure 1**). Since these immunosuppressive cells may inhibit cytotoxic T cells from exerting antitumor control the elimination of the cells causes expansion of cytotoxic T cells in a clonal fashion in myeloma patients treated with daratumumab, a process that is correlated with the clinical response and most likely causally related to the improved survival seen even in patients who do not have a significant reduction in the M-protein. In addition to the immunomodulatory effect of daratumumab exerted *via* elimination of immuneregulatory cells, it was recently shown that antibody-mediated inhibition of the enzymatic activity of CD38 on cytotoxic T cells may directly boost the antitumor activity of these cells (8).

Preclinical studies of daratumumab *in vitro* and *in vivo* models had demonstrated significant additive or synergistic efficacy in combination with other anti-myeloma agents. These findings have now been confirmed in multiple clinical trials testing daratumumab in combination with many different anti-myeloma agents. The ability of daratumumab to combine with other anti-myeloma agents is excellent because there is no overlapping toxicity, and the impressive clinical response rates and duration of responses have now placed daratumumab in a very central position for the treatment of multiple myeloma in second and now also in first line (4–6, 9–14).

Still some patients fail to respond to daratumumab and some patients have progressive disease while being treated with daratumumab. The reason for failure of daratumumab is not understood. Immediately after initiating therapy with daratumumab, the level of CD38 expression by myeloma cells is reduced to much lower levels (15). However, this does not seem to be the cause of failure of the treatment because many patients continue to maintain a response despite the low levels of CD38 expression by myeloma cells. The reasons for low levels of CD38 expression by myeloma cells during treatment with daratumumab may be antibody-mediated "capping" of the daratumumab–CD38 complex on the plasma membrane followed by exocytosis or endocytosis and degradation of the antigen–antibody complex or due to rapid elimination of myeloma cells expressing high levels of CD38, or as recently shown due to "trogocytosis", a process where phagocytes nipple fragments of the plasma membrane carrying antigen–antibody complexes (16).

At the time of failure of daratumumab, there is an increase in the expression of complement regulatory molecules such as CD55 and CD59 (15). These molecules may interfere with complement-mediated cytotoxicity and impair the clinical efficacy of daratumumab. It is also known that myeloma cells and cells in

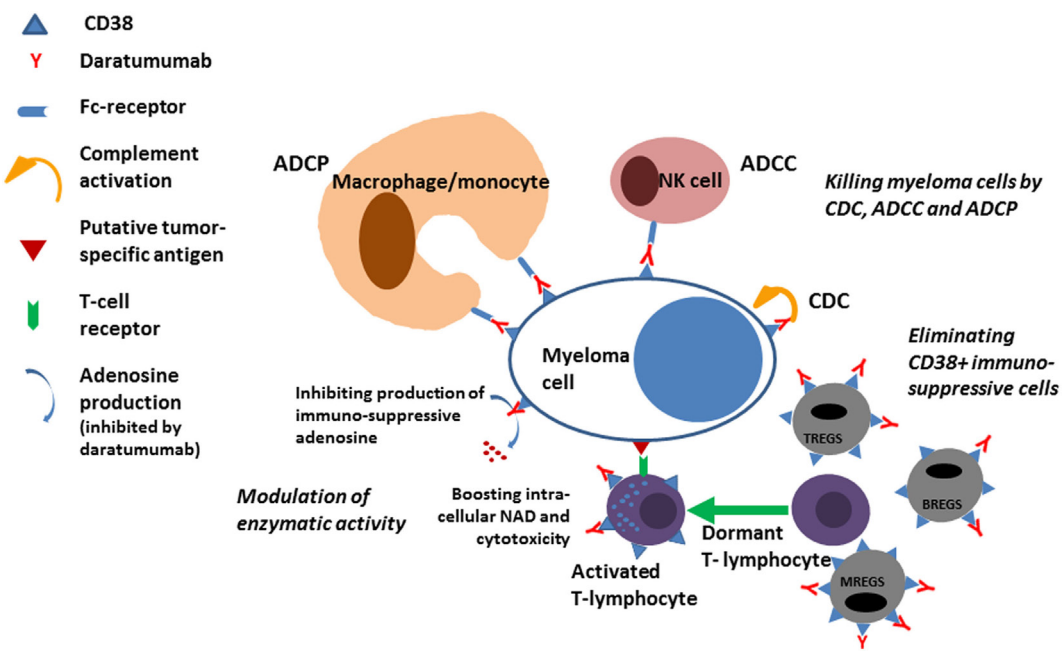

FIGURE 1 | ADCP, antibody-dependent cellular phagocytosis; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-mediated cytotoxicity; TREGS, BREGS, and MREGS: regulatory cells of the T-cell, B-cell, and Myeloid-derived suppressor cells; NAD, nicotinamide adenine dinucleotide.

the microenvironment may express molecules such as PD-L1 that may interfere with the activity of cytotoxic T cells. Much hope has been put into combining daratumumab with checkpoint inhibitor antibodies such as PD-1 or PD-L1 antibodies to boost antitumor cytotoxicity. However, for the time being, the clinical trials in this field have been put to hold by FDA due to an excess mortality in the experimental arm of myeloma patients treated with checkpoint inhibitor antibodies and IMID in phase III trials.

The CD38 molecule is an ectoenzyme that may generate immunosuppressive adenosine and this process may be inhibited by daratumumab (17, 18). Thus, inhibiting the formation of immunosuppressive adenosine daratumumab may boost the T cell immune system and improve disease control. Immunosuppressive adenosine may be generated by CD38 expressed on the surface of myeloma cells, from CD38 expressed by cells in the microenvironment of the myeloma cells or, as recently suggested, by vesicles shed by myeloma cells and carrying CD38 out into the microenvironment surrounding the myeloma cells (19). Hypothetically, such microvesicles could, on top of contributing to generation of adenosine in the microenvironment, also cause off-target binding of daratumumab and contribute to treatment failure.

As it has been hypothesized that the low level of CD38 expressed by myeloma cells immediately after initiating treatment with daratumumab may be a reason for failure to respond to treatment attempts that have been made to increase the level of CD38 expression on myeloma cells with the hope to improve the efficiency of daratumumab (20). A clinical trial is now being conducted with ATRA in combination with daratumumab to increase CD38 expression by myeloma cells and improve responses. Preclinical studies have also shown that panobinostat may increase the expression of CD38 by myeloma cells and improve the response to daratumumab *in vitro* (21). However, our own limited clinical experience outside of a clinical trial testing panobinostat in combination with daratumumab for the treatment of patients progressing on daratumumab has not been successful.

In a model system of non-small cell lung cancer, it has been shown that CD38 is a growth and survival factor for the cancer cells (Gibbons D; ASCO-SICT Clinical Immuno-Oncology Symposium, February 23–25, 2017). Perhaps, the situation in myeloma is similar: high levels of CD38 may be beneficial for myeloma cell survival and conversely the low levels of expression imposed by treatment with daratumumab may render the myeloma cells more vulnerable to other anti-myeloma treatments. Recently, it was shown that myeloma patients refractory to daratumumab and lenalidomide when given separately may respond to the combination of daratumumab and lenalidomide (22). This could be due to daratumumab sensitizing myeloma cells to killing by lenalidomide or to boosting of an exhausted T-cell system in daratumumab refractory patients or both. The hypothesis that daratumumab may sensitize myeloma cells to other anti-myeloma agents fits well with the extraordinary good responses seen when daratumumab is combined with any other anti-myeloma agent not just IMIDs. If further substantiated that an implication may be that daratumumab should be part of any anti-myeloma treatment. Given the pleiotropic effects of daratumumab, it is in fact difficult to imagine how a myeloma patient can become truly refractory to daratumumab.

From a practical point of view, treatment with daratumumab is very easily managed, but it is important to take a few aspects into consideration.

The most important side effect is the infusion-related reaction that may occur during the first infusion in about half of the patients and rarely thereafter. It is a key to success to be prepared for this type of reaction, to look for subtle early signs of the reaction, and to pause the infusion and give extra premedication as soon as the first sign of an infusion-related reaction develops. Prior to the infusion, the patients receive pre-medications with glucocorticoids, antihistamine, montelukast, and paracetamol. If an infusion-related reaction develops the treatment with glucocorticoids and antihistamine can be repeated while the infusion is interrupted for about an hour. When the symptoms have subsided, the infusion can be resumed going back one step regarding the rate of infusion. Often the infusion can then gradually be accelerated and finished with only minor delay. It is recommended to give post-infusion medications with glucocorticoids for 2 days after the infusion, but we tend to admit that after the first two infusions of daratumumab to reduce the glucocorticoid exposure and risk of side effects. Patients with chronic obstructive pulmonary disease may require special attention and more prolonged treatment with glucocorticoids. From the third and subsequent infusions, the infusion rate of daratumumab can be increased so that the IV infusion is finished within 90 min (23). This is of importance in busy outpatient clinics where the number of patients in need of treatment often supersedes the space available. In future, the use of subcutaneous daratumumab that is now being developed in clinical trials may further improve the situation.

Another practical aspect to take into consideration is the expression of CD38 by erythrocytes. Consequently, immediately after starting treatment with daratumumab, this antibody will appear in the serum of patients as an irregular antibody that may cause trouble in the blood bank (24–27). To avoid delays in availability of blood units for transfusion, it is important to inform the staff at the blood bank about the situation, so they can be prepared and manage the difficulties. Daratumumab will cause reactivity in the *antibody screen test*, the *indirect Coombs test*, and the *crossmatch test* used by the blood bank to ensure that blood provided for transfusion will match the recipient. Although the erythrocytes express low levels of CD38, the direct Coombs test is not positive because erythrocytes binding daratumumab rapidly disappear from the circulation. Since only a very small drop of the hemoglobin level is observed after initiation of treatment with daratumumab, erythrocytes that have bound daratumumab may be cleared of the CD38–daratumumab complex on the plasma membrane by a process such as trogocytosis possibly mediated by phagocytes in the spleen and then recirculated.

The level and turnover of IgG in serum are regulated by the so-called "neonatal Fc-receptors" that may protect IgG from degradation. Thus, higher levels of serum IgG will tend to accelerate the turnover of IgG. In a recent study, it was found that despite identical dose levels and schedule the serum level

TABLE 1 | Pivotal clinical trials evaluating daratumumab.

Study name (reference)	Number of patients	Patient population	Response	Adverse events
GEN 501 Phase I/II daratumumab monotherapy (4)	32 patients in dose escalation 72 patients in cohort expansion	RRMM patients mostly refractory to bortezomib or lenalidomide with a median of four prior lines of treatment	16 mg/kg cohort ORR of 36%. 8 mg/kg cohort ORR of 10%. Median PFS in the 16 mg/kg cohort was 5.6 months. 65% of responding patients had not progressed after 12 months of follow-up.	No MTD in phase I (dose escalation up to 24 mg/kg) Daratumumab administration was safe with mostly mild grade 1–2 IRRs predominantly occurring during the first infusion. Most prominent IRRs were rhinitis, cough, or dyspnea.
SIRIUS Phase II daratumumab monotherapy (5)	124 patients 18 patients: 8 mg/kg 106 patients: 16 mg/kg	RRMM patients mostly refractory to bortezomib or lenalidomide with a median of five prior lines of treatment	16 mg/kg cohort ORR of 29% and median PFS was 3.7 months with a 1-year OS of 65%. 8 mg/kg cohort ORR 11%.	Similar to the GEN501 study, the most prominent side effects were IRRs of grade 1 or grade 2.
GEN 503 Phase I/II daratumumab + lenalidomide + dexamethasone (13)	13 patients in dose escalation 32 patients in cohort expansion (16 mg/kg)	RRMM patients with a median of two prior lines of therapy	ORR was 84% in phase I and 81% in phase II with a total of 13 sCR, 3 CR, 13 VGPR, and 8 PR. In phase II, the 18-month PFS was 72% and OS was 90%.	Grade 3–4 adverse events (≥5%) included neutropenia, thrombocytopenia, and anemia. IRRs occurred in 56% of patients after the first infusion.
POLLUX Phase III Lenalidomide + dexamethasone ± daratumumab (9)	569 patients. 286 in the DRd group and 283 in the control group	RRMM patients with a median of one prior line of therapy.	Higher ORR in the DRd group than in the control group (92.9% versus 76.4%). The hazard ratio for disease progression or death in the daratumumab group versus the control group was 0.37. In the DRd group the MRD-negative rate at 10^{-5} was 22.4% versus 4.6% in the Rd group.	Neutropenia grade 3 or 4 in 51.9% of the patients in the DRd group versus 37% in the control group. IRRs of grade 1 or 2 severity in 47.7% of the patients in the DRd group.
CASTOR Phase III bortezomib + dexamethasone ± daratumumab (10)	498 patients. 251 in the DVd group and 247 in the control group	RRMM patients with a median of two prior lines of therapy.	Higher ORR in the DVd group than in the control group (82.9% versus 63.2%). The hazard ratio for disease progression or death in the DVd group versus the control group was 0.39. In the DVd group, the MRD-negative rate at 10^{-5} was 12 versus 2% in the control group.	|Thrombocytopenia grade 3 or 4 in 45.3% of the patients in the DVd group versus 32.9% in the control group. Neutropenia grade 3–4 was 12.8% for DVd versus 4.2% for Vd. |Daratumumab-related IRRs (mostly of grade 1 or 2) occurred in 45.3% of the patients, with the majority occurring during the first infusion.
EQUULEUS Phase Ib daratumumab + pomalidomide and dexamethasone (blood 130, Suppl. 1, 510)	103 patients	RRMM patients with a median of four prior lines of therapy.	ORR was 60%, median PFS 8.8 months and median OS 17.5 months. Out of 17 patients with CR or sCR, 29% were MRD negative by NGS at 10^{-5}.	Neutropenia, anemia, fatigue, diarrhea, and thrombocytopenia were the most common side effects and considered to be mainly caused by pomalidomide. IRRs occurred in 50% of patients and mainly during the first infusion. One case of laryngeal edema occurred during the second infusion.
ALCYONE Phase III melphalan + prednisolon + bortezomib ± daratumumab (11)	706 patients 350 in the D-MPV group and 356 in the control group	Newly diagnosed multiple myeloma patients who are ineligible for stem-cell transplantation.	ORR in the D-MPV group was 90.9 versus 73.9% in the control group. The hazard ratio for disease progression or death in the D-MPV group versus the control group was 0.5. In the D-MPV group, the MRD negative rate at 10^{-5} was 22.3 versus 6.2% in the MPV group.	Infections grade 3 or 4 in 23.1% of the patients in the D-MPV group versus 14.7% in the control group. Daratumumab-related IRRs (mostly of grade 1 or 2) occurred in 27.7% of the patients, with the majority during the first infusion.

RRMM, relapsed/refractory multiple myeloma; ORR, overall response rate; PFS, progression-free survival; OS, overall survival; MTD, maximum tolerated dose; IRR, infusion-related reactions; MRD, minimal residual disease.

of daratumumab is lower in patients with IgG versus patients with IgA myeloma (28). This did, however, not translate into a poorer response to daratumumab in patients with IgG myeloma. For myeloma patients receiving immunoglobulin replacement therapy to prevent infections, it may be advisable to separate as much as possible in time the infusion of daratumumab and normal human IgG, especially if the IgG is given intravenously resulting in high serum peak concentrations, since the amount of normal IgG infused is about 20 times higher than the standard dose of daratumumab.

Regarding assessment of response in myeloma, it is important to know that daratumumab may appear in the serum of patients as an IgG kappa-type M-protein. This may cause trouble when assessing the quality of a response to treatment, and therefore, new assays have been developed to discriminate between daratumumab and the patient's own M-protein (29, 30). Often the level of M-protein represented by daratumumab is low, around 0.5–1 g/L, so when a patient approaches this level of M-protein it may be advisable to request the so-called "daratumumab interference assay" and then perform a bone marrow to confirm CR if it is shown that the residual M-protein is indeed daratumumab.

CD38 is a valuable marker for identification of plasma cells and together with CD138 it is routinely used to quantify the plasma cell compartment. However, during therapy with daratumumab a significant reduction of CD38 expression by MM cells occurs early during treatment (16). Therefore, other markers of plasma cells such as CD269 (BCMA) or CD319 (SLAMF7) may be needed as a substitute for CD38 (31, 32). In addition, daratumumab may affect the accessibility of some of the CD38 epitopes for binding of commercially available CD38 antibodies, so it is important to select an antibody which binds to an epitope on the CD38 molecule that is not occupied by daratumumab (33).

The efficacy and tolerability of daratumumab for the treatment of myeloma have led to rapid implementation of this new drug alone and in combination with standard of care anti-myeloma agents (**Table 1**). It has been approved by FDA, EMA, and many countries across the Globe for the treatment of relapsed-refractory myeloma, and data are now emerging from clinical trials that will likely result in the approval of daratumumab as first-line treatment of myeloma in combination with other drugs.

AUTHOR CONTRIBUTIONS

Both the authors contributed to drafting and writing the manuscript.

REFERENCES

1. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol* (2011) 186(3):1840–8. doi:10.4049/jimmunol.1003032
2. van der Veer MS, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. The therapeutic human CD38 antibody daratumumab improves the anti-myeloma effect of newly emerging multi-drug therapies. *Blood Cancer J* (2011) 1(10):e41. doi:10.1038/bcj.2011.42
3. van der Veer MS, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. Towards effective immunotherapy of myeloma: enhanced elimination of myeloma cells by combination of lenalidomide with the human CD38 monoclonal antibody daratumumab. *Haematologica* (2011) 96(2):284–90. doi:10.3324/haematol.2010.030759
4. Lokhorst HM, Plesner T, Laubach JP, Nahi H, Gimsing P, Hansson M, et al. Targeting CD38 with daratumumab monotherapy in multiple myeloma. *N Engl J Med* (2015) 373(13):1207–19. doi:10.1056/NEJMoa1506348
5. Lonial S, Weiss BM, Usmani SZ, Singhal S, Chari A, Bahlis NJ, et al. Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. *Lancet* (2016) 387(10027):1551–60. doi:10.1016/S0140-6736(15)01120-4
6. Usmani SZ, Weiss BM, Plesner T, Bahlis NJ, Belch A, Lonial S, et al. Clinical efficacy of daratumumab monotherapy in patients with heavily pretreated relapsed or refractory multiple myeloma. *Blood* (2016) 128(1):37–44. doi:10.1182/blood-2016-03-705210
7. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* (2016) 128(3):384–94. doi:10.1182/blood-2015-12-687749
8. Chatterjee S, Daenthanasanmak A, Chakraborty P, Wyatt MW, Dhar P, Selvam SP, et al. CD38-NAD(+)axis regulates immunotherapeutic anti-tumor T cell response. *Cell Metab* (2018) 27(1):85–100.e8. doi:10.1016/j.cmet.2017.10.006
9. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med* (2016) 375(14):1319–31. doi:10.1056/NEJMoa1607751
10. Palumbo A, Chanan-Khan A, Weisel K, Nooka AK, Masszi T, Beksac M, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med* (2016) 375(8):754–66. doi:10.1056/NEJMoa1606038
11. Mateos MV, Dimopoulos MA, Cavo M, Suzuki K, Jakubowiak A, Knop S, et al. Daratumumab plus bortezomib, melphalan, and prednisone for untreated myeloma. *N Engl J Med* (2018) 378(6):518–28. doi:10.1056/NEJMoa1714678
12. Harousseau JL, Attal M. How I treat first relapse of myeloma. *Blood* (2017) 130(8):963–73. doi:10.1182/blood-2017-03-726703
13. Plesner T, Arkenau HT, Gimsing P, Krejcik J, Lemech C, Minnema MC, et al. Phase 1/2 study of daratumumab, lenalidomide, and dexamethasone for relapsed multiple myeloma. *Blood* (2016) 128(14):1821–8. doi:10.1182/blood-2016-07-726729
14. Chari A, Suvannasankha A, Fay JW, Arnulf B, Kaufman JL, Ifthikharuddin JJ, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. *Blood* (2017) 130(8):974–81. doi:10.1182/blood-2017-05-785246
15. Nijhof IS, Casneuf T, van Velzen J, van Kessel B, Axel AE, Syed K, et al. CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma. *Blood* (2016) 128(7):959–70. doi:10.1182/blood-2016-03-703439
16. Krejcik J, Frerichs KA, Nijhof IS, van Kessel B, van Velzen JF, Bloem AC, et al. Monocytes and granulocytes reduce CD38 expression levels on myeloma cells in patients treated with daratumumab. *Clin Cancer Res* (2017) 23(24):7498–511. doi:10.1158/1078-0432.CCR-17-2027
17. Costa F, Toscani D, Chillemi A, Quarona V, Bolzoni M, Marchica V, et al. Expression of CD38 in myeloma bone niche: a rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation. *Oncotarget* (2017) 8(34):56598–611. doi:10.18632/oncotarget.17896
18. Horenstein AL, Chillemi A, Quarona V, Zito A, Roato I, Morandi F, et al. NAD(+)-metabolizing ectoenzymes in remodeling tumor-host interactions: the human myeloma model. *Cells* (2015) 4(3):520–37. doi:10.3390/cells4030520
19. Chillemi A, Quarona V, Antonioli L, Ferrari D, Horenstein AL, Malavasi F. Roles and modalities of ectonucleotidases in remodeling the multiple myeloma niche. *Front Immunol* (2017) 8:305. doi:10.3389/fimmu.2017.00305
20. Nijhof IS, Groen RW, Lokhorst HM, van Kessel B, Bloem AC, van Velzen J, et al. Upregulation of CD38 expression on multiple myeloma cells by all-trans retinoic acid improves the efficacy of daratumumab. *Leukemia* (2015) 29(10):2039–49. doi:10.1038/leu.2015.123
21. Garcia-Guerrero E, Gogishvili T, Danhof S, Schreder M, Pallaud C, Perez-Simon JA, et al. Panobinostat induces CD38 upregulation and augments the antimyeloma efficacy of daratumumab. *Blood* (2017) 129(25):3386–8. doi:10.1182/blood-2017-03-770776

22. Gavriatopoulou M, Kastritis E, Ntanasis-Stathopoulos I, Fotiou D, Roussou M, Migkou M, et al. The addition of IMiDs for patients with daratumumab-refractory multiple myeloma can overcome refractoriness to both agents. *Blood* (2018) 131(4):464–7. doi:10.1182/blood-2017-10-809293
23. Barr H, Dempsey J, Waller A, Huang Y, Williams N, Sharma N, et al. Ninety-minute daratumumab infusion is safe in multiple myeloma. *Leukemia* (2018). doi:10.1038/s41375-018-0120-2
24. Chapuy CI, Nicholson RT, Aguad MD, Chapuy B, Laubach JP, Richardson PG, et al. Resolving the daratumumab interference with blood compatibility testing. *Transfusion* (2015) 55(6 Pt 2):1545–54. doi:10.1111/trf.13069
25. De Vooght KM, Oostendorp M, van Solinge WW. Dealing with anti-CD38 (daratumumab) interference in blood compatibility testing. *Transfusion* (2016) 56(3):778–9. doi:10.1111/trf.13474
26. Oostendorp M, Lammerts van Bueren JJ, Doshi P, Khan I, Ahmadi T, Parren PW, et al. When blood transfusion medicine becomes complicated due to interference by monoclonal antibody therapy. *Transfusion* (2015) 55 (6 Pt 2):1555–62. doi:10.1111/trf.13150
27. Chapuy CI, Aguad MD, Nicholson RT, AuBuchon JP, Cohn CS, Delaney M, et al. International validation of a dithiothreitol (DTT)-based method to resolve the daratumumab interference with blood compatibility testing. *Transfusion* (2016) 56(12):2964–72. doi:10.1111/trf.13789
28. Yan X, Clemens PL, Puchalski T, Lonial S, Lokhorst H, Voorhees PM, et al. Influence of disease and patient characteristics on daratumumab exposure and clinical outcomes in relapsed or refractory multiple myeloma. *Clin Pharmacokinet* (2018) 57(4):529–38. doi:10.1007/s40262-017-0598-1
29. van de Donk NW, Otten HG, El Haddad O, Axel A, Sasser AK, Croockewit S, et al. Interference of daratumumab in monitoring multiple myeloma patients using serum immunofixation electrophoresis can be abrogated using the daratumumab IFE reflex assay (DIRA). *Clin Chem Lab Med* (2016) 54(6):1105–9. doi:10.1515/cclm-2015-0888
30. McCudden C, Axel AE, Slaets D, Dejoie T, Clemens PL, Frans S, et al. Monitoring multiple myeloma patients treated with daratumumab: teasing out monoclonal antibody interference. *Clin Chem Lab Med* (2016) 54(6): 1095–104. doi:10.1515/cclm-2015-1031
31. Flores-Montero J, de Tute R, Paiva B, Perez JJ, Bottcher S, Wind H, et al. Immunophenotype of normal vs. myeloma plasma cells: toward antibody panel specifications for MRD detection in multiple myeloma. *Cytometry B Clin Cytom* (2016) 90(1):61–72. doi:10.1002/cyto.b.21265
32. Frigyesi I, Adolfsson J, Ali M, Christophersen MK, Johnsson E, Turesson I, et al. Robust isolation of malignant plasma cells in multiple myeloma. *Blood* (2014) 123(9):1336–40. doi:10.1182/blood-2013-09-529800
33. van de Donk NW, Moreau P, Plesner T, Palumbo A, Gay F, Laubach JP, et al. Clinical efficacy and management of monoclonal antibodies targeting CD38 and SLAMF7 in multiple myeloma. *Blood* (2016) 127(6):681–95. doi:10.1182/blood-2015-10-646810

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Lenalidomide and Programmed Death-1 Blockade Synergistically Enhances the Effects of Dendritic Cell Vaccination in a Model of Murine Myeloma

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The therapeutic efficacy of dendritic cell (DC)-based immunotherapy may be potentiated in combination with other anticancer therapies that enhance DC function by modulating immune responses and the tumor microenvironment. In this study, we investigated the efficacy of DC vaccination in combination with lenalidomide and programmed death (PD)-1 blockade in a model of murine myeloma. MOPC-315 cell lines were injected subcutaneously to establish myeloma-bearing mice and the following five test groups were established: PBS control, DCs, DCs + lenalidomide, DCs + PD-1 blockade, and DCs + lenalidomide + PD-1 blockade. The combination of DCs plus lenalidomide and PD-1 blockade more potently inhibited tumor growth compared to the other groups. This effect was associated with a reduction in immune suppressor cells (such as myeloid-derived suppressor cells, M2 macrophages, and regulatory T cells) and an increase in immune effector cells [such as $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, and M1 macrophages] in the spleen. Functional activities of cytotoxic T lymphocytes and NK cells were also enhanced by the triple combination. Levels of immunosuppressive cytokines, such as TGF-β and IL-10, were significantly reduced in the tumor microenvironment. These findings suggest that the combination of DCs plus lenalidomide and PD-1 blockade synergistically establishes a robust anti-myeloma immunity through a two-way mechanism, which inhibits immunosuppressive cells while activating effector cells with superior polarization of the Th1/Th2 balance in favor of the tumor immune response. This result should provide an experimental ground for incorporating check point inhibitors to existing immunotherapeutic modalities against multiple myeloma.

Keywords: myeloma, dendritic cells, lenaldiomide, anti-PD-1, cancer immunotherapy

ONE-SENTENCE SUMMARY

A combination of antigen-loaded dendritic cell (DC) vaccination plus lenalidomide and programmed death (PD)-1 blockade synergistically enhanced anticancer immunity in a model of murine multiple myeloma by inhibiting immunosuppressive cells and stimulating effector cells.

INTRODUCTION

Multiple myeloma (MM) is characterized by the infiltration of clonal malignant plasma cells in the bone marrow (BM) (1, 2). Despite advances in treating MM using novel therapies and hematopoietic stem cell transplantation, most patients experience relapses caused by immune evasion among the tumor, immune system, and tumor microenvironment (3). Thus, new therapeutic options with the potential to overcome impaired immune surveillance are needed.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and play a key role in inducing and maintaining antitumor immunity. DCs are able to recognize, process, and present tumor antigens to generate antigen-specific cytotoxic T lymphocytes (CTLs) (4–9). Immune cells in myeloma patients have quantitative and functional deficiencies that contribute to myeloma-associated immune tolerance (10, 11). By contrast, the function of DCs from patients with MM can be recovered and enhanced by *ex vivo* culture (12–14). Lenalidomide is an immunomodulatory agent that targets tumor cells under immunosuppressive microenvironment (15–20). Our previous studies demonstrated that the combination of DC vaccination and lenalidomide synergistically enhanced antitumor immune responses in mouse tumor models (21, 22). Programmed cell death-1 (PD-1, CD279) and its ligands [either PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273)] play a fundamental role in tumor immune escape by inhibiting effector functions (23–26). The PD-1/PD-L1 blockade was recently found to effectively treat cancer by improving durable response rates and the survival profile with minimal toxicity, suggesting that blockade can be used as a cancer therapeutic agent (27–31). However, recent studies reported that PD-1 blockade alone is insufficient to stimulate anti-myeloma immunity in clinical treatment (32, 33). Thus, combination approaches with immune-checkpoint blockade and therapies that stimulate myeloma-reactive T cells can be effective tools to treat myeloma. Such as with immunomodulatory drugs, cellular therapies are currently being applied in clinical trials. Previous studies demonstrated that lenalidomide reduces the expression of PD-1 on natural killer (NK) cells, helper cells, and CTLs, and inhibits PD-L1 expression on tumor cells and myeloid-derived suppressor cells (MDSCs) in patients with MM (20, 34). Moreover, the combination of lenalidomide and PD-1 or PDL-1 blockade increased IFN-γ expression by BM-derived effector cells in myeloma and were associated with increased apoptosis of MM cells (35).

Thus, in this study, we investigated whether the combination of DCs plus lenalidomide and PD-1 blockade has a synergistic effect in a murine myeloma model. The results demonstrate that this combination enhanced antitumor immunity by inhibiting immunosuppressive cells and cytokines as well as activating and recovering effector cells with superior polarization toward Th1 immune response. This study provides a framework for developing a more advanced immunotherapeutic modality employing DCs, lenalidomide, and PD-1 blockade to inhibit tumor cell growth as well as restore immune functionin MM.

MATERIALS AND METHODS

Mice and Tumor Cell Lines

6- to 8-week-old female BALB/c (H-2^d) mice were purchased from Orient Bio (Iksan, Republic of Korea) and maintained under specific pathogen-free conditions. All animal care, experiments, and euthanasia protocols were approved by the Chonnam National University Animal Research Committee. The murine MOPC-315 plasmacytoma cell line and the YAC-1 cell line were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell lines were maintained in Dulbecco's Modified Eagle's Medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1% (w/v) penicillin/streptomycin (PS).

Immunomodulatory Drug (Lenalidomide) and Programmed Death-1 (Anti-PD-1)

Lenalidomide (Revlimid®) was donated by Celgene Corporation (Summit, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL immediately before use. For injection into mice, lenalidomide stock solutions were diluted in sterile 0.9% (v/v) normal saline to a final concentration of 10 mg/mL. The final concentration of DMSO in all experiments was <0.01% (v/v). Anti-PD-1 was purchased from BioXcell (West Lebanon, NH, USA).

Generation of BM-Derived DCs

BALB/c BM-derived immature DCs (imDCs) were generated as described previously (21, 22, 36). Briefly, BM was harvested from the femurs and tibiae of mice and cultured in RPMI-1640 medium (Gibco-BRL) supplemented with 10% (v/v) FBS (Gibco-BRL) and 1% (w/v) PS in the presence of 20 ng/mL recombinant murine (rm) GM-CSF (R&D Systems, Minneapolis, MN, USA) and 10 ng/mL rmIL-4 (R&D Systems). On culture days 2 and 4, half of the medium was removed and replaced with fresh media containing cytokines. On day 6, imDCs were purified *via* positive selection with $CD11c^+$-magnetic beads (Miltenyi Biotec, Auburn, CA, USA). Next, mature DCs were generated by further cultivation for 48 h of $CD11c^+$ DCs with 10 ng/mL rm TNF-α (R&D Systems), 10 ng/mL rmIL-1β (R&D Systems), and 10 ng/mL rmGM-CSF (R&D Systems).

Generation of Dying Myeloma Cell-Loaded DCs

The generation of dying myeloma cell-loaded DCs was performed as described previously (21, 22, 36). Briefly, MOPC-315 tumor cell death was induced by γ-irradiation (100 Gy; Gammacell-1000 Elite, MDS Nordion, Canada), followed by overnight culture in RPMI-1640 without FBS, and the cells were mixed with imDCs 2 h after maturation at a 2:1 ratio (DCs:dying tumor cells).

Animal Vaccination

The following five vaccination groups were established: (1) PBS control, (2) DC vaccination, (3) DC vaccination plus anti-PD-1, (4) DC vaccination plus lenalidomide injection, and (5) DC

vaccination plus anti-PD-1 and lenalidomide injection. On day 0, mice were injected subcutaneously in the right flank with 5×10^5 MOPC-315 cells in a volume of 0.1 mL. After tumor growth, lenalidomide (0.5 mg/kg/day) was administrated orally once a day for 25 days with a 3-day break after the first 11-day dosing period. Each dose of DCs (1×10^6/mouse) was injected subcutaneously into the left flank of BALB/c mice in a volume of 0.1 mL PBS on days 11, 15, 25, and 29; anti-PD-1 (250 μg/mouse) was injected intraperitoneally in a 0.1-mL volume on the same days as DC vaccination. To assess the antitumor status of vaccinated mice, we measured the length, width, and height of each tumor every 3 to 4 days using a Vernier caliper, and we calculated tumor volume using the standard formula for calculating the volume of an ellipsoid: $V = 4/3\pi$(length × width × height/8).

Phenotypic Analysis of Splenocytes From Vaccinated Mice

At the indicated time points, mice were sacrificed and splenocyte phenotypes were characterized by their cell surface markers using fluorescently labeled monoclonal antibodies (mAbs) and analyzed by flow cytometry. Cells were stained with the following mAbs from eBioscience (San Diego, CA, USA): CD11b-FITC (clone: M1/70), CD11b-PE (clone: M1/70), Gr-1-PE (clone: RB6-8C5), CD4-APC (clone: RM4-5), CD4-PE (clone: H129.19), CD8-FITC (clone: 53-6.7), CD49b-PE (clone: Dx5), CD44-PE (clone: IM7), CD62L-FITC (clone: MEL-14), CD69-FITC (clone: H1.2F3), CD25-FITC (clone: CD25-4E3), Foxp3-APC (clone: MF23), F4/80-FITC (clone: BM8), CD274-PE (clone: MH5), and CD206-APC (clone: C068C2). Isotype-matched controls were run in parallel. Cell debris was eliminated by forward- and side-scatter gating. The samples were acquired on a FACSCalibur cell sorter (Becton Dickinson, Mountain View, CA, USA) and data were analyzed using WinMDI ver. 2.9 (Biology Software Net: http://en.bio-soft.net/other/WinMDI.html).

Tumor Antigen-Specific CTL Activity of Vaccinated Mice

Tumor antigen-specific CTL activity was investigated as described previously (21, 22, 36). Briefly, splenocytes (1×10^6) isolated from vaccinated mice 7 days after the final DC vaccination (day 36) were added to 24-well plates and restimulated with irradiated MOPC-315 cells (5×10^5 cells) for 5 days in RPMI-1640 (Gibco-BRL) containing 10% FBS (Gibco-BRL) and 1% PS supplemented with 20 ng/mL rmIL-2 (R&D Systems). After restimulation, we assessed the splenocytes for tumor antigen-specific CTLs using a mouse IFN-γ enzyme-linked immunospot (ELISPOT) assay (BD Bioscience). The MOPC-315 cell line and NK-sensitive YAC-1 cell line were used as target cells.

In Vitro Analysis of Cytokine Production in Vaccinated Mice

We determined cytokine (IFN-γ, IL-10, and TGF-β) production in vaccinated mice using the BD OptEIA™ enzyme-linked immunosorbent assay (ELISA; BD Bioscience). Supernatants from restimulated splenocytes of vaccinated mice and from single tumor cells of all vaccinated mice were assayed to measure the production of Th1- and Th2-polarizing cytokines. Each sample was analyzed in triplicate, and the mean absorbance for each set of standards and samples was calculated.

Intracellular Staining Assay of Tregs and Macrophages Generated in the Spleens of Vaccinated Mice

To evaluate the proportion of Tregs and macrophages, 1×10^6 splenocytes from vaccinated mice were harvested, washed, and stained with surface-staining antibodies of Tregs (CD4-PE and CD25-FITC) and macrophages (CD11b-FITC and F4/80-PE) for 30 min at 4°C. Fc block was added before incubation with surface-staining antibodies. Next, the cells were washed and permeabilized with Fixation/Permeabilization Solution 2 (eBioscience) for 30 min at room temperature. After washing twice, the cells were stained with an intracellular staining antibody, Tregs [Alexa Fluor-conjugated Foxp3 antibody (Miltenyi Biotec)] and macrophages (CD206-APC) for 30 min at room temperature. The samples were acquired on a FACSCalibur cell sorter (Becton Dickinson), and data were analyzed using WinMDI ver. 2.9.

Statistical Analyses

We performed statistical analyses using GraphPad Prism 4 (La Jolla, CA, USA). *t*-Tests, one-way analysis of variance (ANOVA), and two-way ANOVA were used as appropriate. We analyzed the survival of vaccinated mice using SigmaPlot 10.0 (Systat Software, San Jose, CA, USA). $P < 0.05$ was considered significant. Values are expressed as means ± SDs.

RESULTS

DC Vaccination in Combination With Lenalidomide and Anti-PD-1 Treatment Induced a Synergistic Anti-Myeloma Immunity Effect

Our previous study (36) demonstrated that DCs maturated with GM-CSF, TNF-α, and IL-1β expressed higher levels of several molecules related to DC maturation and produced higher levels of IL-12p70 and lower levels of IL-10 compared to imDCs. In this study, we established myeloma-bearing mice to evaluate the antitumor efficacy of DC-based immunotherapies. Before treatment, we observed that high levels of PD-L1 were expressed on MOPC-315 cell lines (Figure S1A in Supplementary Material). The established myeloma-bearing mice were initially treated with lenalidomide (0.25 or 0.5 mg/kg), PD-1 blockade (250 μg/mouse), and dying myeloma cell-loaded DCs as a single therapy (**Figure 1A**). All single treatment groups showed significant inhibition of tumor growth compared to the PBS control group ($P < 0.05$; Figures S1B,C in Supplementary Material). The combination therapy of DCs plus lenalidomide and PD-1 blockade was examined in an effort to more potently inhibit tumor growth in the murine myeloma model (**Figure 1A**). All tumor-bearing mice vaccinated with PBS showed rapid tumor growth that led

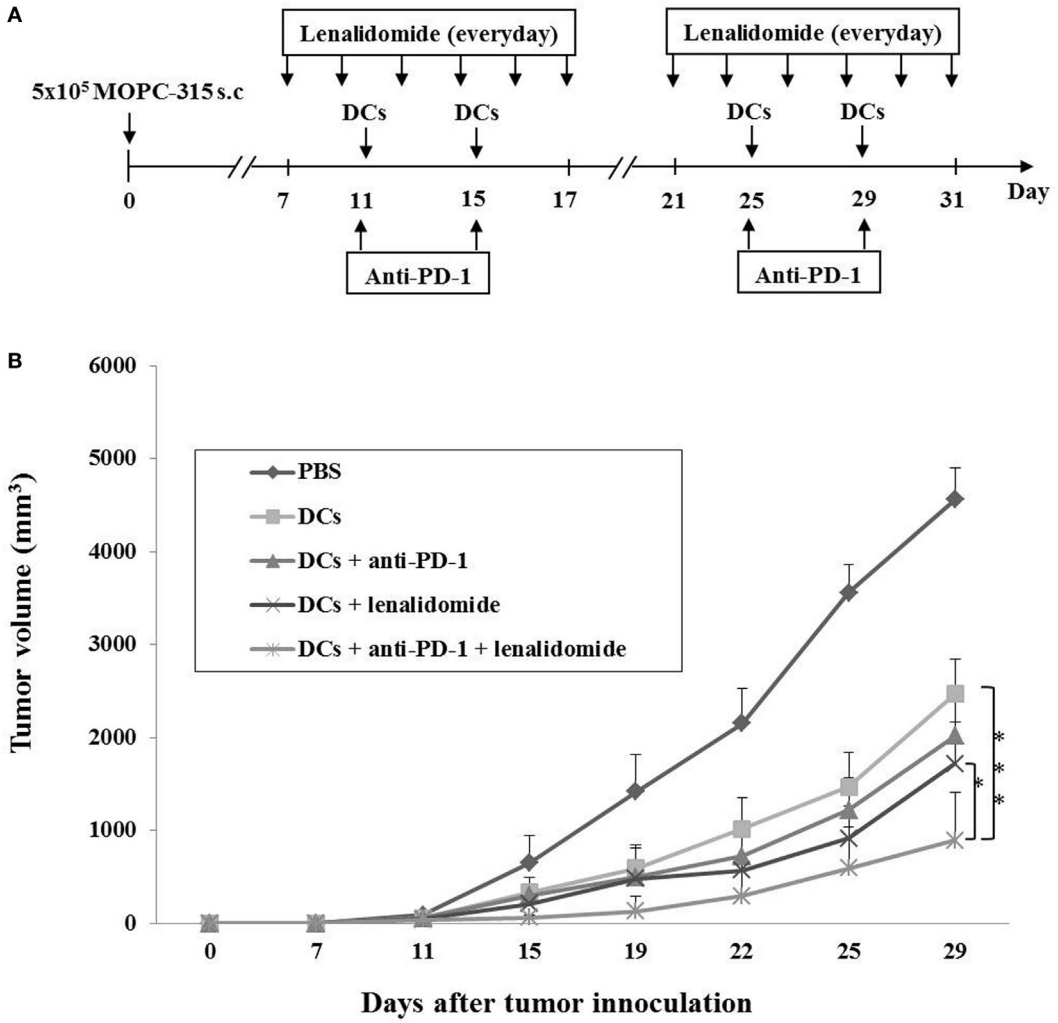

FIGURE 1 | *In vivo* animal vaccination Five vaccination groups were established: (1) PBS control, (2) dying myeloma cell-loaded dendritic cell (DC) vaccination, (3) DC vaccination plus anti-PD-1, (4) DC vaccination plus lenalidomide, and (5) DC vaccination plus lenalidomide and anti-PD-1. On day 0, MOPC-315 cells (5×10^5/mouse) were injected subcutaneously into the right flank of BALB/c mice. **(A)** Schematic representation of the combination of DCs plus lenalidomide and anti-PD-1. After tumor growth, lenalidomide (0.5 mg/kg/day) was administrated orally once a day for 25 days with a 3-day break after the first 11-day dosing period. Each dose of DCs (1×10^6/mouse) was injected subcutaneously into the left flank of BALB/c mice in a volume of 0.1 mL PBS on days 11, 15, 25, and 29. Anti-PD-1 (250 μg/mouse) was injected intraperitoneally on the same days as DC vaccination. **(B)** Data are presented as mean ± SEM and are representative of two independent experiments. The combination of DCs plus lenalidomide and anti-PD-1 significantly inhibited tumor growth (*$P < 0.05$; ***$P < 0.001$ on day 29) and induced a long-term systemic anti-myeloma immune response (29 days).

to sacrifice within 3 weeks. By contrast, tumor-bearing mice vaccinated with DCs showed significantly inhibited tumor growth compared to the PBS control group. Treatment with the combination of DC vaccination plus lenalidomide and PD-1 blockade more strongly inhibited tumor growth ($P < 0.05$) compared to DCs, DCs + lenalidomide, DCs + PD-1 blockade, and lenalidomide + PD-1 blockade (**Figure 1B**; Figures S2A and S3A,B in Supplementary Material). Survival in mice that received the combination of DCs + lenalidomide + PD-1 blockade was significantly prolonged compared to that of mice received DCs, DCs + lenalidomide, DCs + PD-1 blockade, or lenalidomide + PD-1 blockade (Figures S2B and S3C in Supplementary Material). These results indicate that DCs + lenalidomide + PD-1 blockade induce a long-term systemic anti-myeloma immune response in the murine myeloma model.

Activation of CTLs by DC Vaccination Plus Lenalidomide and PD-1 Blockade

To investigate the CTL responses after DC vaccination, we prepared splenocytes and carried out IFN-γ ELISPOT assays. MOPC-315 and YAC-1 cells were used as target cells. Compared to the PBS control, DC vaccination, DCs + lenalidomide, DCs + PD-1 blockade, or DCs + lenalidomide + PD-1 blockade

led to a significant increase in IFN-γ-secreting splenocytes against MOPC-315 and YAC-1 cells ($P < 0.05$). The combination of DCs + lenalidomide + PD-1 blockade showed the highest number of IFN-γ-secreting splenocytes against MOPC-315 cells compared to the PBS control, DCs, DCs + lenalidomide, DCs + PD-1 blockade, and lenalidomide + PD-1 blockade ($P < 0.05$; **Figure 2A**; Figure S4A in Supplementary Material). In addition, cytotoxicity by NK cells, represented by the number of IFN-γ-secreting splenocytes against YAC-1 cells, was similar in all groups that received DCs. These results indicate that the tumor inhibitory effects of DCs + lenalidomide + PD-1 blockade treatment resulted from CTL rather than NK responses. In this study, vaccination with DCs + lenalidomide + PD-1 blockade led to the production of higher levels of IFN-γ compared to the PBS control, DCs, or lenalidomide + PD-1 blockade group (**Figure 2B**; Figure S4B in Supplementary Material). By contrast, TGF-β production in the DCs + lenalidomide + PD-1 blockade group was significantly lower compared to that in the PBS control, DCs, DCs + lenalidomide, or DCs + PD-1 blockade group (**Figure 2C**; Figure S4C in Supplementary Material). These results suggest that the combination of DCs + lenalidomide + PD-1 blockade induced tumor-specific CTL responses enhances through Th1 polarization. Additionally, the DCs + lenalidomide + PD-1 blockade regimen significantly increased percentages of effector $CD4^+$ T cells (**Figure 3A**; Figure S4D in Supplementary Material), effector $CD8^+$ T cells (**Figure 3B**), effector memory T cells (**Figure 3C**; Figure S4E in Supplementary Material), effector NK cells (**Figure 3D**; Figure S4F in Supplementary Material), and M1 macrophages (**Figure 5A**; Figure S6A in Supplementary Material) compared to the other groups.

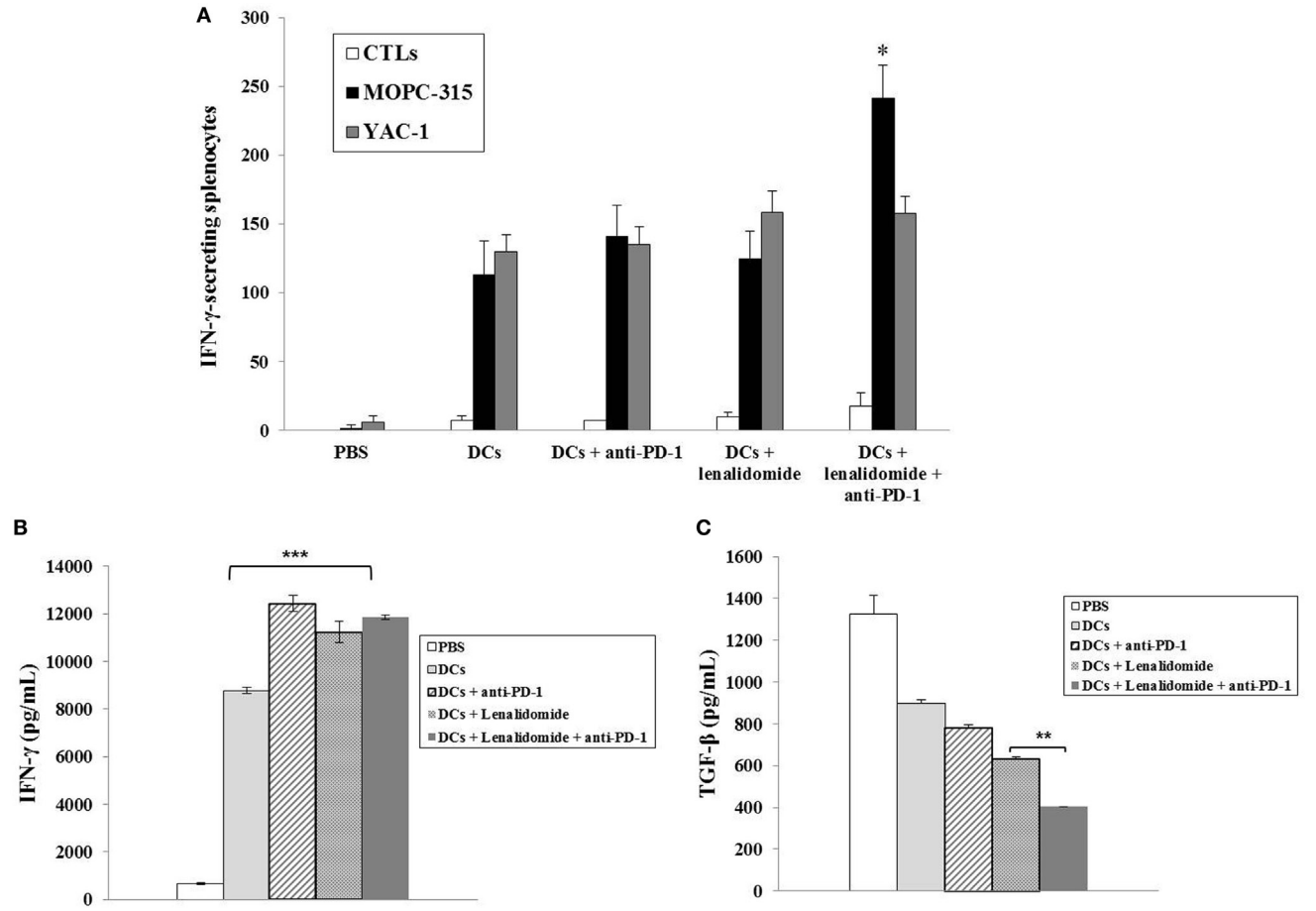

FIGURE 2 | Activation of cytotoxic T lymphocytes (CTLs) and cytokine production induced by treatment with dendritic cells (DCs) plus lenalidomide and anti-PD-1. **(A)** The number of IFN-γ-secreting lymphocytes in the spleens of mice treated with PBS, DCs, DCs plus lenaldiomide, DCs plus anti-PD-1, and DCs plus lenalidomide and anti-PD-1 was counted using IFN-γ enzyme-linked immunospot assay. DC vaccination combined with lenalidomide and anti-PD-1 injection significantly increased the number of IFN-γ-secreting lymphocytes targeting MOPC-315 cells compared to the other groups (*$P < 0.05$). Cytotoxicity by natural killer (NK) cells, represented by the number of IFN-γ-secreting splenocytes against YAC-1 cells, was similar in all DC groups. These results indicate that the tumor inhibitory effects of DCs plus lenalidomide and anti-PD-1 resulted from the CTL-mediated response rather than the NK cell-mediated response. **(B)** IFN-γ and **(C)** TGF-β production in the splenocytes of vaccinated mice was evaluated by enzyme-linked immunosorbent assay. The combination of DCs plus lenalidomide and anti-PD-1 led to the production of higher levels of IFN-γ compared to PBS control and DC vaccination (***$P < 0.001$). By contrast, TGF-β production by DCs plus lenalidomide and anti-PD-1 was lower compared to the other groups (**$P < 0.012$). Data are shown as mean (pg/mL) ± SD of triplicate cultures from three independent experiments.

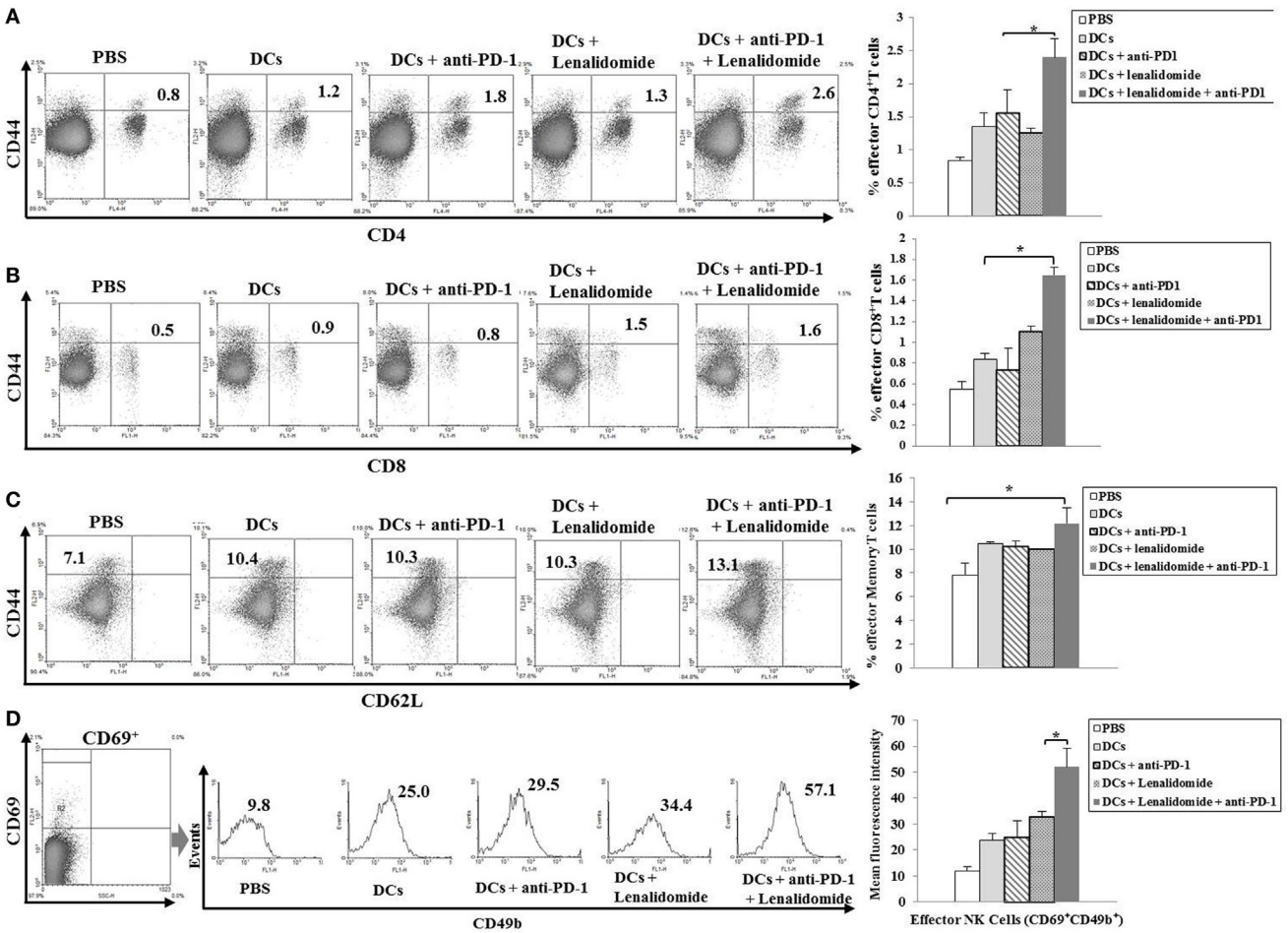

FIGURE 3 | Induction of CD4+ T cells, CD8+ T cells, memory T cells, and natural killer (NK) cells in the spleens of mice treated with a combination of dendritic cells (DCs) plus lenalidomide and anti-PD-1. We measured proportions of **(A)** CD4+ T cells, **(B)** CD8+ T cells, **(C)** memory T cells, and **(D)** NK cells using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The results revealed significant increases in effector cells in the DCs plus lenalidomide and anti-PD-1 combination group compared to the other groups (*$P < 0.05$). Data are representative of three independent experiments.

Suppression of MDSCs, M2 Macrophages, and Regulatory T Cells (Tregs) by the Combination of DC Vaccination Plus Lenalidomide and PD-1 Blockade

To explore the immunological mechanisms underlying the enhanced tumor-specific immune response, we assessed the effects of combination therapy on the proportions of MDSCs ($CD11b^+Gr1^+$), M2 macrophages ($CD11b^+F4/80^+CD206^+$ cells), and Tregs ($CD4^+CD25^+FoxP3^+$ cells) in splenocytes. Percentages of MDSCs (**Figure 4A**; Figure S5A in Supplementary Material) and M2 macrophages (**Figure 5B**; Figure S6B in Supplementary Material) were dramatically reduced in all treatment groups compared to the PBS control group. The DCs + lenalidomide + PD-1 blockade group exhibited the lowest proportion of splenic MDSCs, and M2 macrophages ($P < 0.05$). The proportion of Tregs were significantly higher in the PBS control and DC vaccination groups compared to groups injected with lenalidomide + PD-1 blockade ($P < 0.05$). It is notable that the combination of DCs + lenalidomide + PD-1 blockade resulted in the lowest proportion of splenic Tregs ($P < 0.05$; **Figures 4B–D**; Figures S5B–D in Supplementary Material). These findings suggest that DCs + lenalidomide + PD-1 blockade enhances therapeutic antitumor immunity by also inhibiting immunosuppressive cells in the tumor microenvironment during the vaccination phases.

Efficient Suppression of Inhibitory Cytokine Production by the Combination of DC Vaccination Plus Lenalidomide and PD-1 Blockade in the Tumor Microenvironment of Myeloma-Bearing Mice

To investigate the immunological mechanisms underlying the enhanced tumor-specific immune responses, we evaluated the inhibitory effects of the combination therapy (DC vaccination plus lenalidomide and PD-1 blockade) on the inhibitory cytokine production.

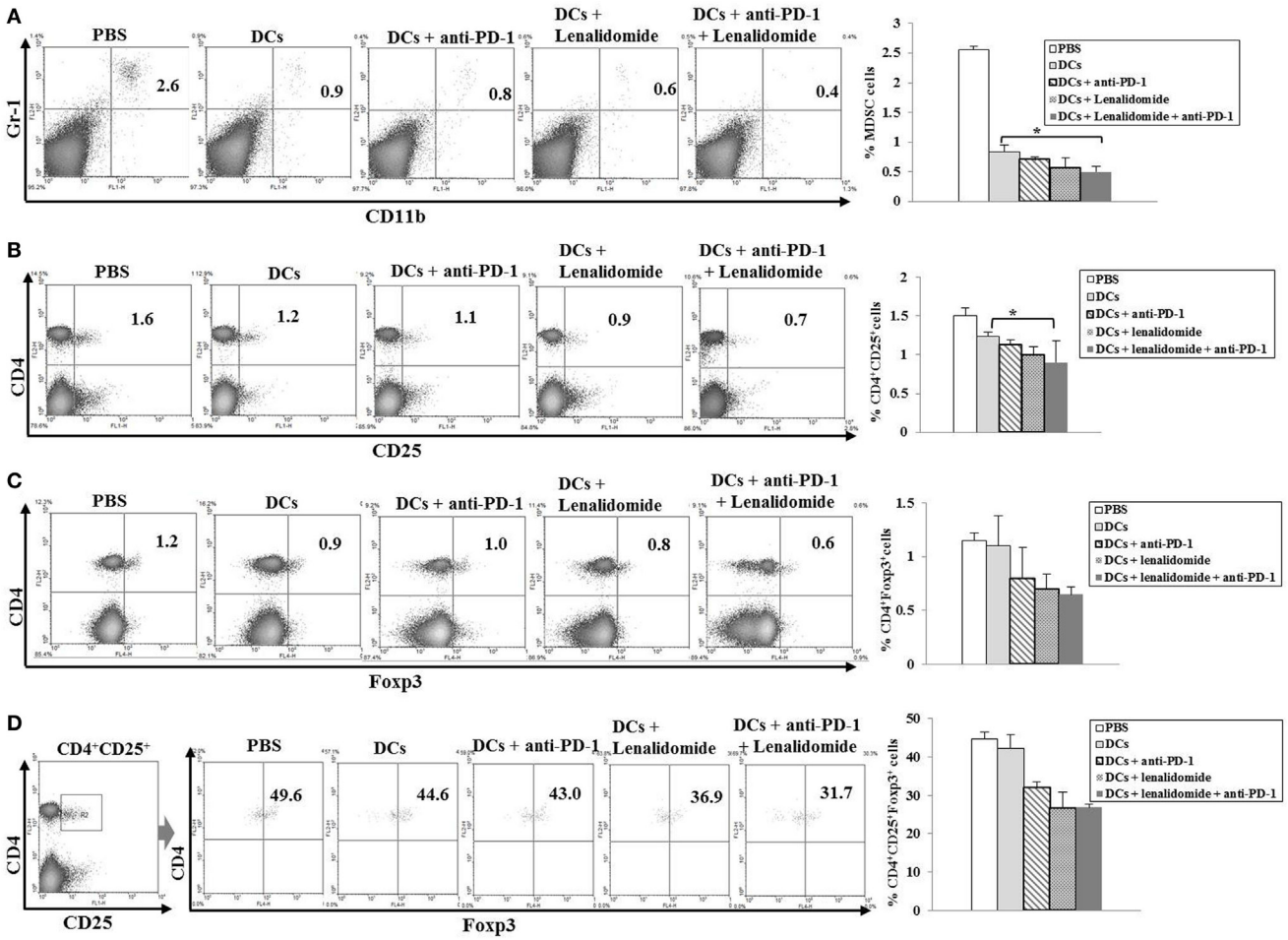

FIGURE 4 | Inhibition of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) in the spleens of mice treated with dendritic cells (DCs) plus lenalidomide and anti-PD-1 We measured proportions of **(A)** MDSCs ($CD11b^+Gr\text{-}1^+$), **(B)** $CD4^+CD25^+$ Tregs, **(C)** $CD4^+Foxp3^+$ Tregs, and **(D)** $CD4^+CD25^+Foxp3^+$ Tregs using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The proportions of MDSCs and Tregs were significantly increased in the PBS control and DC vaccination groups compared to the groups injected with lenalidomide or anti-PD-1 after tumor inoculation. The DC vaccination plus lenalidomide and anti-PD-1 combination group showed significantly decreased proportions of splenic MDSCs and Tregs compared to the other groups (*$P < 0.05$). Data are representative of at least three experiments.

Compared to the treatment groups, the PBS control group was significantly higher in the TGF-β production ($P < 0.001$). However, the production of TGF-β did not significantly differ among the treatment groups (**Figure 5C**; Figure S6C in Supplementary Material). In addition, the combination of DCs + lenalidomide + PD-1 blockade led to the production of the least IL-10 compared to other groups ($P < 0.001$; **Figure 5D**; Figure S6D in Supplementary Material), which suggests that the combination therapy of DC vaccination plus lenalidomide and PD-1 blockade changed the tumor microenvironment toward immunostimulatory by suppressing the production of inhibitory cytokines IL-10 and TGF-β.

DISCUSSION

Dendritic cell-based vaccines serve a promising immunotherapeutic weapon with the potential to prolong the survival of patients with incurable MM (2, 12). Several new tools have been developed and combined to improve clinical outcomes of DC vaccination against MM (17, 18). Recent studies have defined immune checkpoint PD-1/PD-L1 signaling as a key pathway regulating the critical balance between immune activation and tolerance (24, 37–40). The PD-1/PD-L1 pathway plays an important role in shaping the tumor-promoting, immunosuppressive microenvironment of MM. Rosenblatt et al. (41) reported that PD-L1 is highly expressed in plasma cells of MM patients but not in normal plasma cells. Our study confirmed that PD-L1 is also overexpressed on MOPC-315 cell lines (99%). Furthermore, significant PD-1 expression was observed in circulating T cells of advanced MM patients. Inhibition of the PD-1/PD-L1 signaling pathway induces an anti-MM immune response and can be a promising option for anti-myeloma therapy (42, 43).

The tumor microenvironment of MM promotes tumor cell growth and helps them escape from immune surveillance by actively suppressing anti-MM immune effector responses (1, 2).

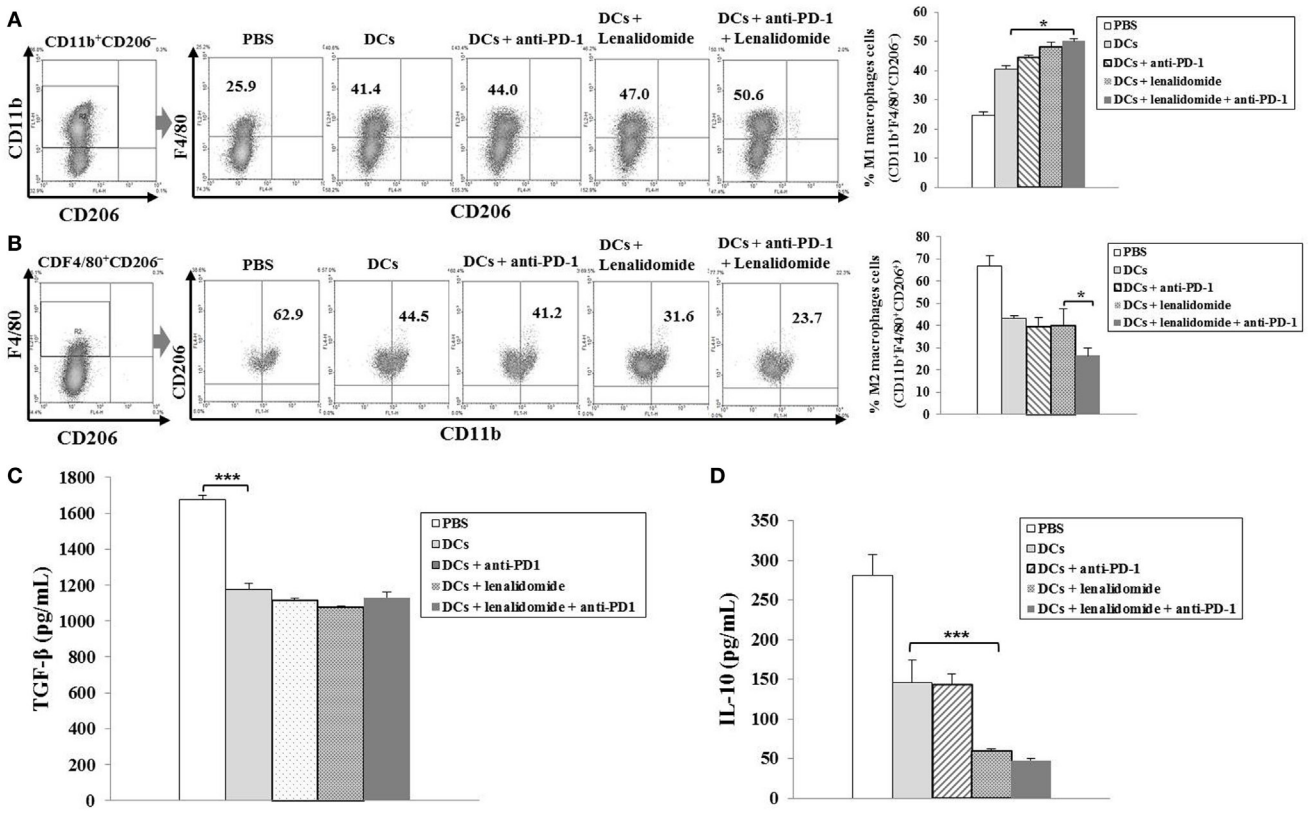

FIGURE 5 | Enhanced M1 and impaired M2 macrophage polarization and reduced inhibitory cytokine production *via* vaccination with dendritic cells (DCs) plus lenalidomide and anti-PD-1 We measured proportions of **(A)** M1 macrophages ($CD11b^+F4/80^+CD206^-$) and **(B)** M2 macrophages ($CD11b^+F4/80^+CD206^+$) in the spleens of vaccinated tumor-bearing mice using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The DCs plus lenalidomide and anti-PD-1 combination group exhibited the highest proportion of M1 macrophages and the lowest proportion of M2 macrophages compared to the other groups (*$P < 0.05$). Data are representative of three independent experiments. The production of **(C)** TGF-β and **(D)** IL-10 inhibitory cytokines in the tumors of tumor-bearing mice was evaluated by enzyme-linked immunosorbent assay. Compared to the treatments, PBS control led to the production of higher levels of TGF-β (***$P < 0.001$). However, the production of TGF-β did not differ significantly among the treatment groups. The production of IL-10 was significantly decreased in the DCs plus lenalidomide and anti-PD-1 combination therapy group compared to the other groups (***$P < 0.001$). Data are representative of at least three experiments.

Lenalidomide, an immunomodulatory drug, inhibits the expression of PD-1 in NK cells, helper T cells, and CTLs of MM patients and downregulates the expression of PD-L1 in myeloma cell lines and primary myeloma cells (20, 34). Lenalidomide was shown to reduce PD-1 expression in all effector cells ($CD4^+$ T cells, $CD8^+$ T cells, NK cells, and NKT cells), and PD-L1 expression in MM cells, MDSC, and monocyte/macrophages in an *in vitro* experiment (35). Additionally, Patients undergoing treatment of lenalidomide demonstrated reduced PD-1 expression in $CD8^+$ T cells (44). Moreover, lenalidomide plus PD-1/PD-L1 checkpoint blockade suppressed MDSCs and stroma-mediated MM growth and enhanced MM-specific cytotoxicity of immune effector cells in BM environments (35). Our previous studies demonstrated that DC-based vaccines were safe and induced the expansion of circulating $CD4^+$ T cells and $CD8^+$ T cells that are specific for tumor antigens, which was synergistically enhanced by the combination of lenalidomide (21, 22, 36). Our expectation was that the therapeutic efficacy of DC vaccination will be far more enhanced if both lenalidomide and PD-1 blockade are combined. Lenalidomide and anti-PD-1 antibody should synergistically improve the MM microenvironment, in which the host immune effector cells induced by the DC vaccination will exert anti-MM effects. This study, as expected, showed that DC vaccination combined to the lenalidomide and PD-1 blockade regiment further inhibited MM tumor growth, consequently prolonging the survival of tumor-bearing mice: the triple combination induced strong anti-myeloma CTL responses and increased the number of effector cells ($CD4^+$ T cells, $CD8^+$ T cells, NK cells, and M1 macrophages), while effectively discouraging suppressor cells (MDSCs, Tregs, and M2 macrophages) in the systemic immune compartment. These findings evidence the induction of systemic immune response potentially being able to eradicate disseminated diseases. DCs combined with lenalidomide and PD-1 blockade also heightened the anti-myeloma cell mediate immunity by inducing the Th1 polarization, as evidenced by the high-level production of IFN-γ, and by suppressing Th2 immune responses, as evidenced by the low-level production of IL-10 and TGF-β. Tregs, MDSCs, and M2 macrophages are major elements molding the potent immunosuppressive environment in tumor tissues. The inhibition of Treg, MDSC, and M2 macrophage

accumulation in the spleen should further contribute to effective anti-myeloma cell mediate immunity in the systemic immune compartment by reciprocally activating DCs or CTLs.

Murine models of myeloma are critical tools to study the mechanisms of disease resistance, pathogenesis, and the development of new therapeutic strategies (45, 46). This study has some limitation to interpret data due to subcutaneous injection of MOPC-315 cells for making plasmacytoma rather than BM involvement model for myeloma.

In conclusion, this study suggests that lenalidomide plus PD-1 blockade treatment synergistically enhances the efficacy of DC vaccination in a murine myeloma model by inhibiting the generation of immunosuppressive cells and the Th2 immune response and enhancing effector cells and the Th1 immune responses. We hereby propose a framework for a more efficacious DC-based vaccination strategy against MM with the combination of immunomodulatory drug lenalidomide and anti-PD-1 antibody.

ETHICS STATEMENT

All animal care, experiments, and euthanasia protocols were approved by the Chonnam National University Animal Research Committee.

AUTHOR CONTRIBUTIONS

M-CV, S-HJ, and J-JL designed the study. M-CV, T-HC, H-JL, TL, and H-SP performed the research and analyzed the data. M-CV, JR, and J-JL wore the article. M-CV, H-JK, and J-JL contributed intellectually to the research.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01370/full#supplementary-material.

FIGURE S1 | Antitumor efficacy of individual therapies in a model of murine myeloma. **(A)** We measured the levels of PD-L1 expressed on MOPC-315 cell lines using flow cytometry. MOPC-315 cell lines showed high-level expression of PD-L1 (99%). Representative histogram shows marker expression (shaded) compared with those of isotype control (black line). **(B)** Representative images of mice vaccinated with lenalidomide (0.25 or 0.5 mg/kg), anti-PD-1 (250 μg/mouse), and dying myeloma cell-loaded dendritic cells (DCs) as single treatments. **(C)** Data are shown as the mean ± SEM and are representative of two independent experiments. All single treatment groups showed significant inhibition of tumor growth compared to the PBS control (*$P < 0.05$). Experiments consisted of five mice per group.

FIGURE S2 | **(A)** Representative images of mice vaccinated with dendritic cells (DCs) plus lenalidomide and anti-PD-1 showed significant inhibition of tumor growth compared to the PBS control, DC vaccination, DCs plus anti-PD-1, and DCs plus lenalidomide groups. **(B)** The survival of the tumor-bearing mice is shown. The combination of DCs plus lenalidomide and anti-PD-1 significantly inhibited tumor growth (*$P < 0.05$; ***$P < 0.001$ on day 29) and induced a long-term systemic anti-myeloma immune response. Experiments consisted of five mice per group.

FIGURE S3 | **(A)** Representative images of mice vaccinated with dendritic cells (DCs) plus lenalidomide and anti-PD-1 showed significant inhibition of tumor growth compared to mice treated with lenalidomide plus anti-PD-1. **(B)** Data are shown as the mean ± SEM and are representative of two independent experiments. **(C)** The survival of the tumor-bearing mice is shown. The combination of DCs plus lenalidomide and anti-PD-1 significantly inhibited tumor growth (*$P < 0.05$ on day 25) and induced a long-term systemic anti-myeloma immune response. Experiments consisted of five mice per group.

FIGURE S4 | Activation of cytotoxic T lymphocytes and natural killer (NK) cells, proportions of $CD4^+$ T cells and memory T cells, NK cells, and cytokine production induced by a combination of dendritic cells (DCs) plus lenalidomide and anti-PD-1 **(A)** We counted the number of IFN-γ-secreting lymphocytes in the spleens of mice treated with lenalidomide plus anti-PD-1 and with DCs plus lenalidomide and anti-PD-1 using the IFN-γ enzyme-linked immunospot assay. The combination of DCs plus lenalidomide and anti-PD-1 significantly increased the number of IFN-γ-secreting lymphocytes targeting MOPC-315 and YAC-1 cells compared to treatment with lenalidomide plus anti-PD-1 (*$P < 0.05$). **(B)** IFN-γ and **(C)** TGF-β production in the splenocytes of vaccinated mice was evaluated by enzyme-linked immunosorbent assay. The combination of DCs plus lenalidomide and anti-PD-1 led to the production of higher levels of IFN-γ compared to treatment with lenalidomide plus anti-PD-1 (***$P < 0.001$). The production of TGF-β did not differ significantly between the two groups. Data are shown as the mean (pg/mL) ± SD of triplicate cultures from three independent experiments. We measured proportions of **(D)** $CD4^+$ T cells, **(E)** memory T cells, and **(F)** NK cells using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The results revealed significant increases in $CD4^+$ T cells and memory T cells in the DCs plus lenalidomide and anti-PD-1 group compared to the lenalidomide plus anti-PD-1 group (*$P < 0.05$; ***$P < 0.001$). Percentages of NK cells did not differ significantly between the two groups. Data are representative of at least three experiments.

FIGURE S5 | Inhibition of myeloid-derived suppressor cells (MDSCs) and Tregs in the spleens of mice treated with a combination of dendritic cells (DCs) plus lenalidomide and anti-PD-1. We measured proportions of **(A)** MDSCs ($CD11b^+Gr\text{-}1^+$), **(B)** $CD4^+CD25^+$ Tregs, **(C)** $CD4^+Foxp3^+$ Tregs, and **(D)** $CD4^+CD25^+Foxp3^+$ Tregs using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The DC vaccination plus lenalidomide and anti-PD-1 combination group showed decreased proportions of splenic MDSCs and Tregs compared to the lenalidomide plus anti-PD-1 group. Data are representative of at least three experiments.

FIGURE S6 | Enhanced M1 and impaired M2 macrophage polarization and reduced inhibitory cytokine production by a combination of dendritic cells (DCs) plus lenalidomide and anti-PD-1. We measured proportions of **(A)** M1 macrophages ($CD11b^+F4/80^+CD206^-$) and **(B)** M2 macrophages ($CD11b^+F4/80^+CD206^+$) in the spleens of vaccinated tumor-bearing mice using flow cytometry (left panel) and compared them using quantitative bar graphs (right panel). The DCs plus lenalidomide and anti-PD-1 combination group showed significantly increased proportions of M1 macrophages compared to the lenalidomide plus anti-PD-1 group (*$P < 0.05$). Percentages of M2 macrophages did not differ significantly between the two groups. Data are representative of at least three experiments. The production of **(C)** TGF-β and **(D)** IL-10 inhibitory cytokines in the tumors of tumor-bearing mice was evaluated by enzyme-linked immunosorbent assay. The production of IL-10 was significantly decreased in the DCs plus lenalidomide and anti-PD-1 combination therapy group compared to the lenalidomide plus anti-PD-1 group (***$P < 0.001$). The production of TGF-β did not differ significantly between the two groups. Data are representative of at least three experiments.

REFERENCES

1. Drach J, Kaufmann H. New developments and treatment in multiple myeloma: new insights on molecular biology. *Ann Oncol* (2002) 4:43–7. doi:10.1093/annonc/mdf637
2. Hoang MD, Jung SH, Lee HJ, Lee YK, Nguyen-Pham TN, Choi NR, et al. Dendritic cell-based cancer immunotherapy against multiple myeloma: from bench to clinic. *Chonnam Med J* (2015) 51(1):1–7. doi:10.4068/cmj.2015.51.1.1
3. Kumar SK, Gertz MA. Risk adapted therapy for multiple myeloma: back to basics. *Leuk Lymphoma* (2014) 55(10):2219–20. doi:10.3109/10428194.2014.905775
4. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998) 392(6673):245–52. doi:10.1038/32588
5. Reid DC. Dendritic cells and immunotherapy for malignant disease. *Br J Haematol* (2001) 112(4):874–87. doi:10.1046/j.1365-2141.2001.02626.x
6. Hong CY, Lee HJ, Choi NR, Jung SH, Vo MC, Hoang MD, et al. Sarcoplasmic reticulum Ca(2+) ATPase 2 (SERCA2) reduces the migratory capacity of CCL21-treated monocyte-derived dendritic cells. *Exp Mol Med* (2016) 48(8):e253. doi:10.1038/emm.2016.69
7. Hoang MD, Lee HJ, Lee HJ, Jung SH, Choi NR, Vo MC, et al. Branched polyethylenimine-superparamagnetic iron oxide nanoparticles (bPEI-SPIONs) improve the immunogenicity of tumor antigens and enhance Th1 polarization of dendritic cells. *J Immunol Res* (2015) 2015:706379. doi:10.1155/2015/706379
8. Choi NR, Lee HJ, Jung SH, Hong CY, Vo MC, Hoang MD, et al. Generation of potent dendritic cells with improved migration ability through p-cofilin and sarco/endoplasmic reticulum Ca(2+) transport ATPase 2 regulation. *Cytotherapy* (2015) 17(10):1421–33. doi:10.1016/j.jcyt.2015.06.002
9. Jung SH, Lee YK, Lee HJ, Choi NR, Vo MC, Hoang MD, et al. Dendritic cells loaded with myeloma cells pretreated with a combination of JSI-124 and bortezomib generate potent myeloma-specific cytotoxic T lymphocytes in vitro. *Exp Hematol* (2014) 42(4):274–81. doi:10.1016/j.exphem.2013.12.008
10. Parrish C, Scott GB, Cook G. Immune dysfunction in multiple myeloma. *Int J Hematol Oncol* (2013) 2:49–59. doi:10.2217/ijh.12.33
11. Ratta M, Faqnoni F, Curti A, Vescovini R, Sansoni P, Oliviero B, et al. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* (2002) 100(1):230–7. doi:10.1182/blood.V100.1.230
12. Jung SH, Lee HJ, Vo MC, Kim HJ, Lee JJ. Immunotherapy for the treatment of multiple myeloma. *Crit Rev Oncol Hematol* (2017) 111:87–93. doi:10.1016/j.critrevonc.2017.01.011
13. Vo MC, Anh-Nguyen Thi T, Lee HJ, Nguyen-Pham TN, Jaya Lakshmi T, Jung SH, et al. Lenalidomide enhances the function of dendritic cells generated from patients with multiple myeloma. *Exp Hematol* (2017) 46:48–55. doi:10.1016/j.exphem.2016.11.004
14. Vo MC, Nguyen-Pham TN, Lee HJ, Jung SH, Choi NR, Hoang MD, et al. Chaetocin enhances dendritic cell function via the induction of heat shock protein and cancer testis antigens in myeloma cells. *Oncotarget* (2017) 8(28):46047–56. doi:10.18632/oncotarget.17517
15. Galustian C, Meyer B, Labarthe MC, Dredge K, Klaschka D, Henry J, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. *Cancer Immunol Immunother* (2009) 58(7):1033–45. doi:10.1007/s00262-008-0620-4
16. Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai YT, et al. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* (2000) 96(9):2943–50.
17. Lagrue K, Carisev A, Morgan Chopra R, Davis DM. Lenalidomide augments actin remodelling and lowers NK cell activation thresholds. *Blood* (2015) 126(1):50–60. doi:10.1182/blood-2015-01-625004
18. List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med* (2006) 355(14):1456–65. doi:10.1056/NEJMoa061292
19. Luptakova K, Glotzbecker B, Mills H, Stroopinsky D, Vasir B, Rosenblatt J, et al. Lenalidomide decrease PD-1 expression, depletes regulatory T-cells and improves cellular response to a multiple myeloma/dendritic cell fusion vaccine in vitro. *Blood* (2010) 116:492.
20. Luptakova K, Rosenblatt J, Glotzbecker B, Mills H, Stroopinsky D, Kufe T, et al. Lenalidomide enhances anti-myeloma cellular immunity. *Cancer Immunol Immunother* (2013) 62(1):39–49. doi:10.1007/s00262-012-1308-3
21. Nguyen-Pham TN, Jung SH, Vo MC, Thanh-Tran HT, Lee YK, Lee HJ, et al. Lenalidomide synergistically enhances the effect of dendritic cell vaccination in a model of murine multiple myeloma. *J Immunother* (2015) 38(8):330–9. doi:10.1097/CJI.0000000000000097
22. Vo MC, Nguyen-Pham TN, Lee HJ, Jaya Lakshmi T, Yang S, Jung SH, et al. Combination therapy with dendritic cells and lenalidomide is an effective approach to enhance antitumor immunity in a mouse colon cancer model. *Oncotarget* (2017) 8(16):27252–62. doi:10.18632/oncotarget.15917
23. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* (2002) 8(8):793–800. doi:10.1038/nm730
24. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* (2000) 192(7):1027–34. doi:10.1084/jem.192.7.1027
25. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* (2011) 364(26):2517–26. doi:10.1056/NEJMoa1104621
26. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* (2012) 24(2):207–12. doi:10.1016/j.coi.2011.12.009
27. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* (2013) 369(2):134–44. doi:10.1056/NEJMoa1305133
28. Rajan A, Gulley JL. Nivolumab (anti-PD-1, BMS-936558, ONO-4538) in patients with advanced non-small cell lung cancer. *Transl Lung Cancer Res* (2014) 3(6):403–5. doi:10.3978/j.issn.2218-6751.2014.09.02
29. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* (2012) 366(26):2443–54. doi:10.1056/NEJMoa1200690
30. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol* (2014) 32(10):1020–30. doi:10.1200/JCO.2013.53.0105
31. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* (2013) 369(2):122–33. doi:10.1056/NEJMoa1302369
32. Suen H, Brown R, Yang S, Weatherburn C, Ho PJ, Woodland N, et al. Multiple myeloma causes clonal T-cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* (2016) 30(8):1716–24. doi:10.1038/leu.2016.84
33. Suen H, Brown R, Yang S, Ho PJ, Gibson J, Joshua D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* (2015) 29(7):1621–2. doi:10.1038/leu.2015.104
34. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116(13):2286–94. doi:10.1182/blood-2010-02-271874
35. Görgün G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res* (2015) 21(20):4607–18. doi:10.1158/1078-0432.CCR-15-0200
36. Vo MC, Lee HJ, Kim JS, Hoang MD, Choi NR, Rhee JH, et al. Dendritic cell vaccination with a toll-like receptor agonist derived from mycobacteria enhances anti-tumor immunity. *Oncotarget* (2015) 6(32):33781–90. doi:10.18632/oncotarget.5281
37. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* (1999) 5(12):1365–9. doi:10.1038/70932
38. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* (1992) 11(11):3887–95.
39. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* (2008) 26:677–704. doi:10.1146/annurev.immunol.26.021607.090331
40. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* (1975) 53(1):27–42. doi:10.1038/icb.1975.3
41. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother* (2011) 34(5):409–18. doi:10.1097/CJI.0b013e31821ca6ce

42. Guillerey C, Nakamura K, Vuckovic S, Hill GR, Smyth MJ. Immune responses in multiple myeloma: role of the natural immune surveillance and potential of immunotherapies. *Cell Mol Life Sci* (2016) 73(8):1569–89. doi:10.1007/s00018-016-2135-z
43. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. *Leukemia* (2013) 27(2):464–72. doi:10.1038/leu.2012.213
44. Danhof S, Schreder M, Knop S, Rasche L, Strifler S, Löffler C, et al. Expression of programmed death-1 on lymphocytes in myeloma patients is lowered during lenalidomide maintenance. *Haematologica* (2018) 103(3):e126–9. doi:10.3324/haematol.2017.178947
45. Lwin ST, Edwards CM, Silbermann R. Preclinical animal models of multiple myeloma. *Bonekey Rep* (2016) 5:772. doi:10.1038/bonekey.2015.142
46. Richard AC, James RB. Animal models of multiple myeloma and their utility in drug discovery. *Curr Protoc Pharmacol* (2008) 40:14.9.1–14.9.22. doi:10.1002/0471141755.ph1409s40

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REVIEW
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Targeting B Cell Maturation Antigen (BCMA) in Multiple Myeloma: Potential Uses of BCMA-Based Immunotherapy

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The approval of the first two monoclonal antibodies targeting CD38 (daratumumab) and SLAMF7 (elotuzumab) in late 2015 for treating relapsed and refractory multiple myeloma (RRMM) was a critical advance for immunotherapies for multiple myeloma (MM). Importantly, the outcome of patients continues to improve with the incorporation of this new class of agents with current MM therapies. However, both antigens are also expressed on other normal tissues including hematopoietic lineages and immune effector cells, which may limit their long-term clinical use. B cell maturation antigen (BCMA), a transmembrane glycoprotein in the tumor necrosis factor receptor superfamily 17 (TNFRSF17), is expressed at significantly higher levels in all patient MM cells but not on other normal tissues except normal plasma cells. Importantly, it is an antigen targeted by chimeric antigen receptor (CAR) T-cells, which have already shown significant clinical activities in patients with RRMM who have undergone at least three prior treatments, including a proteasome inhibitor and an immunomodulatory agent. Moreover, the first anti-BCMA antibody–drug conjugate also has achieved significant clinical responses in patients who failed at least three prior lines of therapy, including an anti-CD38 antibody, a proteasome inhibitor, and an immunomodulatory agent. Both BCMA targeting immunotherapies were granted breakthrough status for patients with RRMM by FDA in Nov 2017. Other promising BCMA-based immunotherapeutic macromolecules including bispecific T-cell engagers, bispecific molecules, bispecific or trispecific antibodies, as well as improved forms of next generation CAR T cells, also demonstrate high anti-MM activity in preclinical and even early clinical studies. Here, we focus on the biology of this promising MM target antigen and then highlight preclinical and clinical data of current BCMA-targeted immunotherapies with various mechanisms of action. These crucial studies will enhance selective anti-MM response, transform the treatment paradigm, and extend disease-free survival in MM.

Keywords: multiple myeloma, B-cell maturation antigen, targeted immunotherapy, monoclonal antibody, chimeric antigen receptor T cell, monoclonal antibody drug conjugate, bi-specific antibody

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INTRODUCTION

Multiple myeloma (MM), the second most common hematologic malignancy in the United States, accounts for 1% of malignancies and 10% of hematologic cancers (1). This tumor is characterized by the expansion of malignant plasma cells (PCs) in the bone marrow (BM), associated with excessive production of monoclonal immunoglobulins in blood and urine in patients. In addition, MM patients develop significant osteolytic bone lesions and have immunodeficiency that compromises both longevity and quality of life (2, 3). For the past two decades, the clinical outcome of MM patients has shown remarkable improvements primarily due to the incorporation of novel therapeutic agents into conventional treatments. Specifically, the addition of proteasome inhibitors (PI) and immumomodulatory drugs (IMiDs) has significantly increased response rate, progression-free, and overall survival in both relapsed and newly diagnosed MM patients, compared with conventional therapies (4–7). The addition of monoclonal antibodies (MoAbs) elotuzumab and daratumumab as immunotherapies in MM has further improved patient outcome. The use of autologous stem cell transplantation also results in better outcome. However, MM remains incurable for most patients, since drug-resistant clones constantly emerge and evolve (8). Persistence of minimal residual disease (MRD) is often seen and patients with MRD-negativity also relapse. Particularly, the overall survival of patients with relapsed disease after PIs IMiDs, and MoAbs treatment is extremely low. Thus, more efficacious therapies and novel strategies are urgently needed if we are to develop curative therapies.

Multiple myeloma develops from a premalignant precursor condition monoclonal gammopathy of undetermined significance, progressing to smoldering MM, then active MM, majorities of which ultimately advancing to end-stage PC leukemia. Genetic and epigenetic processes are present initially and underlie this progression, including hyperdiploidy of chromosomes, translocation of immunoglobulin heavy chain, deregulation of cell cycle genes, alteration of NFκB pathways, and abnormal DNA methylation patterns (9–11). Besides complex molecular aberrations, MM cells are heavily dependent on their BM microenvironment to support their growth, survival, and the development of drug resistance. Tumor cells closely interact with BM accessory cells in bidirectional fashions *via* cell–cell contact and/or production of a variety of factors, which ultimately promotes MM cell expansion, while impairing immune surveillance and effector function against MM cells. These MM-supporting cells include BM stromal cells (BMSCs) (12, 13), osteoclasts (14), endothelial cells (15), macrophages (16), T regulatory cells (17–19), dendritic cells (20), plasmacytoid DCs (pDCs) (21), myeloid-derived suppressor cells (22), and mesenchymal cells (13, 23). These accessory cells secrete various cytokines including interleukin-6 (IL-6) (24), tumor growth factor β (TGFβ) (25, 26), macrophage inflammatory protein-1α (MIP-1α) (27), insulin-like growth factor (28), vascular endothelial growth factor (29), hepatocyte growth factor (30), B cell activating factor (BAFF) (31, 32), and a proliferation-inducing ligand (APRIL) (31, 33), which further maintain an MM-supporting or immunosuppressive BM microenvironment (34). For example, the key myeloma growth factor IL-6 and the critical immune inhibitory factor TGFβ are detected at high levels in the BM of MM patients. The interplay of these two cytokines may affect generation of Th17 cells both directly or *via* other pro-inflammatory cytokines, and thereby downregulate antitumor immune responses (35). Increased Th17 cells and decreased regulatory T cells (Tregs) with less immune suppression is noted in MM patients with long-term survival (36). Since Tregs can inhibit function of antigen-presenting cells and effector T cells (37), increased Treg number allows MM cells to escape from immune surveillance. In fact, immune-suppressive Treg markers Foxp3 and CTLA-4 are significantly upregulated in the BM aspirates of MM patients compared with normal donor controls (17), and increased Tregs are correlated with worse outcomes in MM (36, 38, 39). These studies indicate that molecular and cellular components suppress immune BM milieu, further enhancing MM progression.

Successful targeted anti-MM immunotherapies should both target MM cells and simultaneously restore antitumor activity of immune effector cells (40). Ideally, targets for effective immunotherapies should be selectively and strongly expressed on the surface of MM cells relative to normal cells. Compared with CD38 and SLAMF7, B cell maturation antigen (BCMA) demonstrates highly restricted expression on PCs but no other tissues, is, therefore, an excellent target for immunotherapy in MM (41, 42).

BCMA IS AN IMPORTANT SURFACE PROTEIN SUPPORTING THE SURVIVAL OF MM CELLS

B cell maturation antigen, also termed tumor necrosis factor receptor superfamily member 17 (TNFRS17), is a type III transmembrane protein without a signal-peptide and containing cysteine-rich extracellular domains (43–45). Alignment of the human (44, 45) and murine BCMA protein sequences (43) revealed a conserved motif of six cysteines in the N-terminal part, which strongly suggests that the BCMA protein belongs to the tumor necrosis factor receptor (TNFR) superfamily. BCMA, along with two related TNFR superfamily B-cell activation factor receptor (BAFF-R) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), critically regulate B cell proliferation and survival, as well as maturation and differentiation into PCs. These three functionally related receptors support long-term survival of B cells at different stages of development by binding to BAFF and/or APRIL (46–49), their cognate ligands. Specifically, BCMA is only induced in late memory B cells committed to the PC differentiation and is present on all PCs (46, 50, 51). Expression of BCMA is induced, while BAFF-R is decreased, during PC differentiation from B cells. Studies from BCMA-knockdown mice further indicate that BCMA is most important for long-lived PC survival but is dispensable for overall B cell homeostasis (50, 52). A recent study showed that an enzyme, γ-secretase can cleave membrane BCMA, leading to decreased in membrane form BCMA and formation of soluble form BCMA (sBCMA) (53) (**Figure 1**).

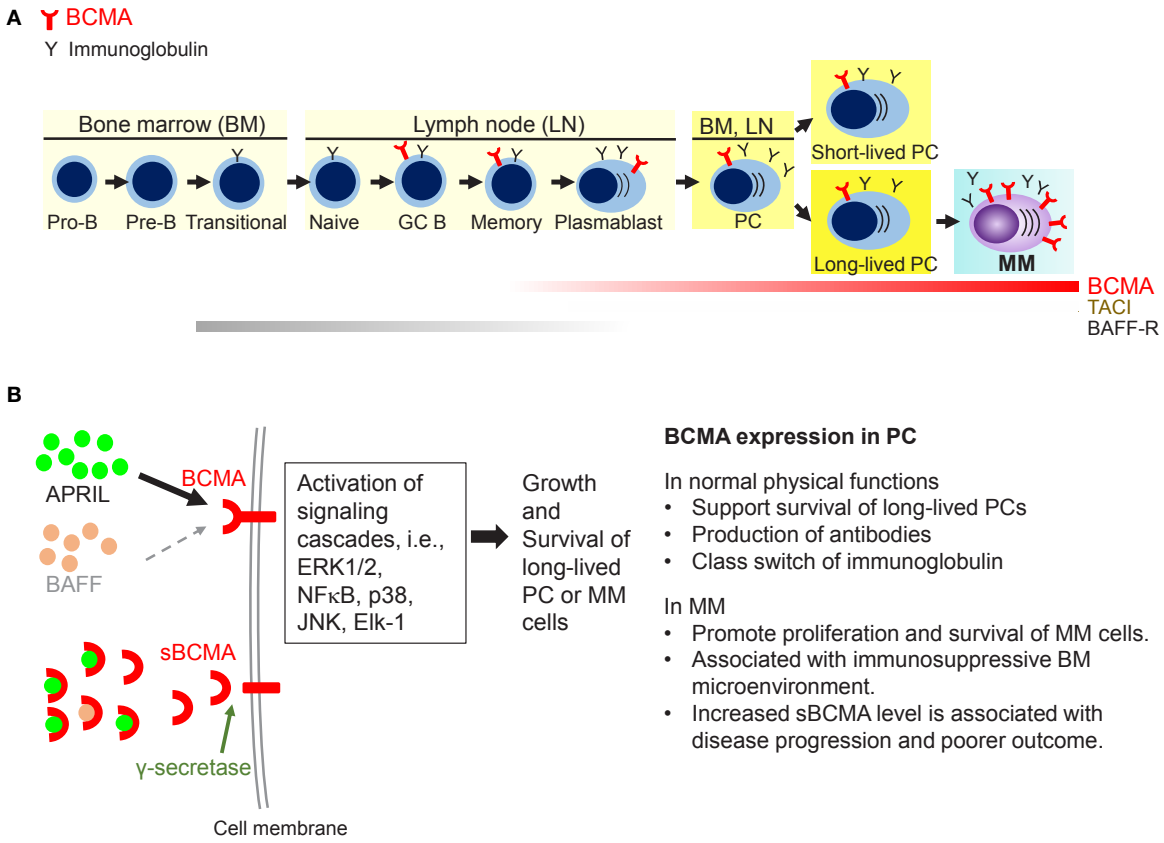

FIGURE 1 | Biological significance of B cell maturation antigen (BCMA) in plasma cells (PCs). **(A)** BCMA is selectively induced during PC differentiation, associated with loss of BAFF-R. It is expressed on late-stage B-cells, short-lived proliferating plasmablasts, and long-lived PCs. BCMA does not maintain normal B-cell homeostasis but is required for the survival of long-lived PCs. In multiple myeloma (MM), expression of BCMA is significantly increased on malignant vs normal PCs. **(B)** A proliferation-inducing ligand (APRIL) and BAFF are two natural ligands for BCMA. Specifically, APRIL binds to BCMA with a significantly higher affinity than BAFF. Activation of BCMA supports growth and survival of PCs *via* activating MEK/ERK, AKT, NFκB, JNK, p38 kinase, and Elk-1. In MM cells, overexpression of BCMA or binding of APRIL to BCMA activates AKT, ERK1/2, and NFκB pathways and upregulate antiapoptotic proteins, i.e., Mcl-1, Bcl-2, Bcl-xL to protect MM cells from dexamethasone- and interleukin-6 deprivation induced apoptosis. Furthermore, BCMA upregulates genes associated with activation of osteoclast, adhesion, and angiogenesis/metastasis. Moreover, overexpressed BCMA can induce the expression immunosuppressive molecules such as PD-L1 in MM cells. Membrane BCMA can be cleaved by γ-secretase, resulting in reduced number of membrane-bound BCMA molecules and increased soluble BCMA. Soluble BCMA can bind to APRIL and BAFF, which may interfere downstream BCMA signaling cascades. TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor; GC, germinal center.

Earlier studies show that overexpression of BCMA in 293 cells activates the mitogen-activated protein kinase pathway, especially JNK and p38 kinase, the nuclear factors NFκB and Elk-1, without stimulation of BAFF or APRIL (54). BCMA expression is positively regulated by B-lymphocyte-induced maturation protein 1 (Blimp-1), a gene controlling proliferation of PCs (55). In KMS12 MM cell line, BCMA co-immunoprecipitates with interferon regulatory factor-4, a master transcription factor mediating survival of MM cells (56). Importantly, BCMA overexpression or APRIL binding to BCMA in MM cells significantly promotes MM cell growth and survival *in vivo* (33, 57). Conversely, BCMA knockdown blocks MM cell proliferation and viability *via* downregulation of cell cycle progression and antiapoptosis molecules. APRIL and BAFF, *via* binding to BCMA and TACI, further activate NFκB pathways and upregulate antiapoptotic proteins (Mcl-1, Bcl-2, Bcl-xL) to protect MM cells against dexamethasone- and serum deprivation-induced cell death (31, 58, 59) (**Figure 1**). These studies establish a pathophysiological role of BCMA and APRIL in MM.

RATIONALE TO TARGETING BCMA IN MM

B cell maturation antigen is exclusively expressed on the surface of plasmablasts and differentiated PCs, but not on memory B, naive B cells, CD34+ hematopoietic stem cells, and other normal tissue cells (41, 50, 51, 60–64). BCMA mRNA and protein are more highly expressed on malignant than normal PCs, as validated by multiple gene expression profiling (41, 42, 65, 66) and immunohistochemistry (IHC) studies (41). In the study by Carpenter et al. (41), cDNA copies of BCMA were detected by qPCR in several hematologic tissues including white blood cells, BM, lymph node, spleen, and tonsil. In normal tissues, low levels of BCMA cDNA copies were detected in the samples of testis, trachea and samples from gastrointestinal organs like

duodenum, rectum, and stomach. When the expression was evaluated by IHC, BCMA protein expression was only detected on MM cells, lymphoid cells, or PCs from normal human organs such as duodenum, rectum, and stomach. However, BCMA protein expression was not detected on the other cell types in these organs (41). Another study examined BCMA expression on various blood cells and Hodgkin lymphoma cells using flow cytometry (63). BCMA expression was negative on naive and memory B cells, weak on founder B cells from germinal center (GC) and Reed–Sternberg cells, positive on GC B cells, but highly positive on plasmacytoid B cells. Based on these findings, BCMA protein is highly and specifically expressed on PCs, low levels of BCMA RNA detected in these normal organs would be due to existence of PCs.

Thus far, majorities of studies indicate that BCMA transcript, protein, and the serum BCMA level are significantly higher in MM cell lines and patient MM cells, when compared with normal donors. One recent study reported that median BCMA expression on patient MM cells was not to be higher compared to normal BMPC as shown in large patient cohorts (66). At protein levels, shedding of BCMA by γ-secretase controls PCs in the BM and may represent a potential biomarker for B-cell involvement in human autoimmune diseases (53). Significantly, sBCMA levels are increased in MM patients vs healthy individuals (67–69). Its level in patient serum is further correlated with disease status and prognosis. Furthermore, anti-BCMA antibodies are detected in MM patients in remission after donor lymphocyte infusion with graft-vs-tumor response, suggesting that antibody responses to cell-surface BCMA may directly contribute to tumor elimination (70). Moreover, low BCMA is detected in pDCs (42), which support survival and drug resistance of MM cells (21). In fact, pDCs are the only cell type other than PCs with detectable BCMA at significantly lower levels (>1-log lower) compared to matched PCs (42). These data confirm BCMA as a very promising MM antigen for targeted immunotherapy.

Both BCMA ligands APRIL and BAFF, to a lesser extent, are critical BM factors supporting growth and survival of malignant PCs in MM (31, 62). The levels of both ligands are significantly increased in serum samples of MM patients vs normal controls (31, 71). APRIL, which does not bind to BAFF-R, preferably binds to BCMA with much higher affinity than BAFF (nM vs μM), whereas BAFF has an approximate 100-fold selectivity for binding to BAFF receptor (BAFF-R) over BCMA (72, 73). Coupled with the fact that APRIL also binds to TACI on PCs *via* interaction with CD138/syndecan-1, APRIL is more specific to PCs than BAFF (57, 74–76). Importantly, APRIL directly promotes MM cell growth and survival *in vivo*, since APRIL knockout mice injected with human MM cell lines live longer than wild-type mice under similar conditions (77). These results strongly supporting targeting BCMA for novel MM treatments.

As described below, GSK2857916, the first therapeutic anti-BCMA antibody–drug conjugates (ADCs) with multiple mechanisms of action against MM cells, used alone and with MM-protecting BM components, rapidly eliminates MM cells in two murine models and significantly prolongs survival of mice (42). These promising data further support clinical development of BCMA-targeted immunotherapies in MM.

BCMA-BASED IMMUNOTHERAPIES

The development of novel agents targeting BCMA is ongoing rapidly, especially following impressive clinical responses in relapsed MM patients using the first chimeric antigen receptor (CAR) T cell therapy (78). Currently, there are multiple BCMA-based treatment modalities including: ADC, bispecific T-cell engager (BiTE), CAR T cell (CAR T), bispecific molecule, and bi/trispecific Abs (**Figure 2**) (summarized in **Table 1**), as well as cancer vaccines.

ANTIBODY–DRUG CONJUGATES

Antibody–drug conjugate, one of the fastest growing class of cancer therapeutics, is composed of recombinant mAbs covalently bound to cytotoxic chemicals (payload) *via* synthetic chemical linkers (107). The mAbs first identify and bind to the antigen on the surface of tumor cells, and then is absorbed or internalized, together with the payload. After the ADC is internalized, the cytotoxic chemicals are released in the lysosomes and transported to cytosol to kill the tumor cells.

GSK2857916

GSK285791 is a humanized and IgG1 mAb with high affinity to BCMA (Kd of ~0.5 nM) (42), which uses non-cleavable linker, maleimidocaproyl (mc), and a new class of antimitotic agents, monomethyl auristatin F, as payload. This structure is characterized by high stability and high antitumor potency, with low by-stander toxicity.

GSK2857916 binds to all CD138+ and BCMA+ MM cell lines and patient MM cells. MM cell proliferation is inhibited *via* G2/M arrest in a dose-dependent manner, and apoptosis is induced by activation of caspase 3/7 and 8. There are minimal effects on surrounding BCMA-negative normal cells. GSK2857916 also triggers

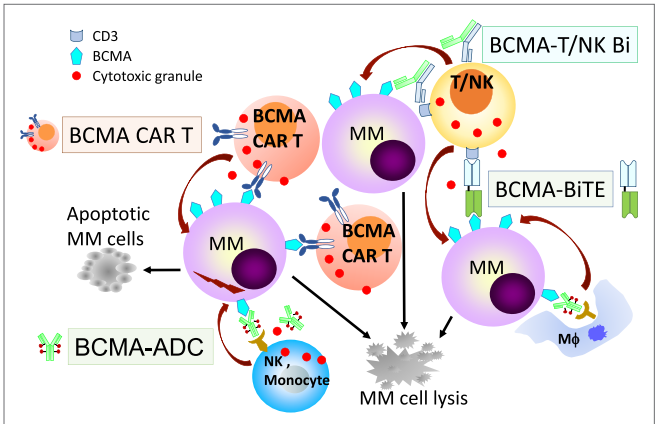

FIGURE 2 | B cell maturation antigen (BCMA)-based immunotherapies with multiple mechanisms of action against MM cells. Various BCMA-based treatment modalities are under clinical development are listed in **Table 1** and shown here. BCMA-NK Bi or Tri Ab, not shown here, can also specifically induce effector cell-mediated lysis of MM cells. ADC, antibody drug conjugate; Bi, bispecific full-length immunoglobulin; BiTE, bispecific T-cell engager; CAR T, chimeric antigen receptor T cell; MM, multiple myeloma cell; NK, natural killer cell; Mϕ, macrophage.

TABLE 1 | List of Anti-B cell maturation antigen (BCMA) formats.

Therapeutic format	Compound (or name)	Company/ sponsor	Characteristics	Clinical development	Reference
Antibody–drug conjugates	GSK2857916	GlaxoSmithKline	1. Humanized and afucosylated IgG1 mAb 2. BCMA binding affinity: Kd of ~0.5 nM 3. Anticancer drug: monomethyl auristatin F 4. Linker: Maleimidocaproyl (non-cleavable)	Phase 1	(42, 79, 80)
	HDP-101	Heidelberg Pharma	1. Antigen-targeted amanitin-conjugates 2. Humanized mAb 3. Anticancer agent: Amanitin 4. Linker: Maleimide (non-cleavable)	Preclinical	(81, 82)
	MEDI2228	MedImmune	1. Fully humanized antibody 2. Anticancer drug: Pyrrolobenzodiazepine 3. Linker: Protease-cleavable linker	Preclinical	(83)
Bispecific T-cell engager	BI 836909 (Amg420)/ Amg701	Boehringer Ingelheim/Amgen	1. Bispecific single-chain variable fragment with hexahistidine tag 2. Targeting CD3 and BCMA	Preclinical	(84, 85)
CAR T	Anti-BCMA chimeric antigen receptor (CAR)	National Cancer Institute	1. Transfection: γ-retroviral vector 2. Extracellular domain: murine scFv 3. Co-stimulation domain: CD28	Phase 1	(41, 78, 86)
	bb2121	Bluebird Bio Celgene	1. Transfection: Lentivirus vector 2. Extracellular domain: Murine scFv 3. Co-stimulation domain: 4-1BB	Phase 1	(87)
	LCAR-B38M	Nanjing Legend Biotech	1. Transfection: lentivirus vector 2. Extracellular domain: Bispecific variable fragments of llama heavy-chain antibodies 3. Co-stimulation domain: 4-1BB	Phase 1	(88, 89)
	CART-BCMA	Novartis	1. Transfection: Lentivirus vector 2. Extracellular domain: fully human scFv 3. Co-stimulation domain: 4-1BB	Phase 1	(90, 91)
	KITE-585	Kite Pharma	1. Transfection: lentivirus vector 2. Extracellular domain: fully human scFv 3. Co-stimulation domain: CD28	Preclinical	(92)
	BCMA CAR	Pfizer Cellectis SA	1. Transfection: lentivirus vector 2. Extracellular domain: fully human scFv 3. Co-stimulation domain: 4-1BB 4. Inactivation of the T cell receptor alpha chain 5. Contained an intra-CAR rituximab-recognition domain to deplete CAR T cells	Preclinical	(93)
	P-BCMA-101	Poseida Therapeutics	1. *In vitro* transcribed mRNA and plasmid DNA, no viral transfection 2. Extracellular domain: human fibronectin type III domain 3. Contain a safety switch	Preclinical	(94–96)
	FHVH74-CD828Z FHVH32-CD828Z FHVH33-CD828Z FHVH93-CD828Z	Tenebrio	1. Antigen-recognition domains composed of single fully human FHVH without light chain variable region domain or linker 2. Co-stimulation domain: 4-1BB or CD28	Preclinical	(97)
	Descartes-08	Cartesian Therapeutics	1. CD8+ anti-BCMA CAR T-cells modified transiently by mRNA transfection	Preclinical	(98)
	P-BCMA-ALLO1	Poseida Therapeutics	1. NextGEN™ (NG) CRISPR gene editing system to disrupt both TCR and MHCI expression 2. Non-viral piggyBac™ (PB) DNA transposition technology to produce CAR-T cells with highly desirable stem cell memory T cell subset	Preclinical	(99)
	EGFRt/BCMA-41BBz	Juno	1. Transfection: lentivirus 2. Extracellular domain: fully human scFv 3. Co-stimulation domain: 4-1BB 4. Suicidal gene: EGFRt	Phase 1 (recruiting)	(89)

(*Continued*)

TABLE 1 | Continued

Therapeutic format	Compound (or name)	Company/ sponsor	Characteristics	Clinical development	Reference
Bispecific molecule	BCMA/CD3 bispecific	Pfizer Alexo Therapeutics Kodiak Sciences	1. Fully-human IgG CD3 bispecific molecule with IgG2A backbone 2. BCMA binding affinity: Kd 20 pM 3. CD3 binding affinity: Kd ~40 nM	Preclinical	(100)
Bispecific antibody	EM801	EngMab AG Celgene	1. Two-arm IgG1-based human antibody 2. One CD3 and two BCMA binding sites 3. BCMA-binding affinity: Kd of 10 nM 4. CD3-binding affinity: Kd of 70 nM	Preclinical	(66)
	[a]BCMA-TCB2/EM901	Celgene	1. Two-arm IgG1-based human antibody 2. One CD3 and two BCMA-binding sites	Preclinical[a]	(101)
	Ab-957	Janssen	1. BCMAxCD3 bispecific antibody 2. Ec50: a. BCMA + cell: 0.06–0.45 nM b. T-cell activation: 0.1–0.28 nM	Preclinical	(102)
	AFM26	Affimed	1. Targeting CD16A (NK cells) and BCMA 2. NK-cell binding affinity: Kd of 1.2 nM	Preclinical	(103, 104)
	TNB383B/TNB-384B	TeneoBio	1. Targeting BCMA and CD3 2. Very low or absence of cytokine release after TNB-383B treatment	Preclinical	(105)
Trispecific antibody	Anti-CD16A/BCMA/ CD200 antibody	Affimed	1. Trispecific antibody format: CD16A/BCMA/CD200 2. Bivalent binding to CD16A 3. Monovalent binding to both BCMA and CD200	Preclinical	(106)

Every effort has been made to obtain reliable data from multiple sources including http://clinicaltrials.gov/, companies, and other web sites, but accuracy cannot be guaranteed.
[a]Most recently, the BCMAxCD3 TCB CC-93269 (EM901) has entered clinical phase I testing (NCT03486067).

ADCC and antibody-dependent cellular-mediated phagocytosis against patient MM cells. The cytotoxicity against MM cells is further enhanced when GSK2857916 is combined with lenalidomide *via* effector-dependent and -independent manners. Most importantly, in both disseminated and subcutaneous human MM xenograft models in mice, GSK2857916 rapidly eliminates MM cells and generated little toxicity in mice treated with continuous dosing for nine times at 4 mg/kg, with tumor-free survival up to 3.5 months in mice (42).

GSK2857916 was evaluated in a phase 1 study of patients with relapsed and refractory multiple myeloma (RRMM), including dose-escalating and expansion parts (79, 80). GSK2857916 monotherapy has demonstrated a 60% response rate and a median progression-free survival of 7.9 months in a group of hard to treat and heavily pretreated RRMM (80). It has recently been awarded Breakthrough Therapy designation from FDA and received PRIME designation from the European Medicines Agency (EMA).

HDP-101

HDP-1, an antibody-targeted amanitin conjugate, is an anti-BCMA ADC with a novel payload amanitin, which binds to the RNA polymerase II in eukaryotic cells and inhibits cellular transcription at very low concentrations (108). HDP-1 was synthesized with the conjugation of maleimide-amanitin compounds and engineered cysteine residues in the heavy chain of the humanized anti-BCMA Thiomab (109, 110).

HDP-101 demonstrated potent *in vitro* cytotoxicity against BCMA-expressing MM cell lines at picomolar range, without effects on BCMA-negative cells. Significant tumor regression including complete remission was observed in the mouse xenograft model in a dose-dependent manner. The tolerability and therapeutic index were good after a series of HDP-101 administrations at different concentrations in Cynomolgus monkeys. Mild-to-moderate elevation of liver enzymes and lactic dehydrogenase were noted, but these abnormalities were transient. HDP-101 has a long half-life in serum (about 12 days) (81, 82).

MEDI2228

The structure of MEDI2228 includes a fully human antibody site-specifically conjugated to a pyrrolobenzodiazepine dimer *via* a protease-cleavable linker. This ADC is rapidly internalized into MM cells and trafficked to lysosomes.

MEDI2228 was highly active in 8 of 10 MM cell lines (IC50 range 6 to 210 ng/mL) including cell lines regardless of BCMA levels (83). MEDI2228 was also active in the presence of BMSCs. A single injection of MEDI2228 induced human MM xenograft regression in mice at very low doses (0.1 mg/kg). MEDI2228 was characterized by weak binding capacity to recombinant monomeric human BCMA, but strong binding to membrane-bound BCMA. It kills an average of 95% of tumor cells in the presence of sBCMA at levels up to 720 ng/mL, without impact on IC50. Clinical trials of this new anti-BCMA will be starting in mid-2018.

BISPECIFIC T-CELL ENGAGER

Bispecific T-cell engager is a single-chain variable fragment (scFv), composed of two linked mAbs (bispecific antibodies) targeting

mainly CD3 on the surface of T-cells and tumor-associated antigens. This unique structure allows BiTE to engage T-cells with tumor cells (111). After the binding, antitumor cytotoxicity and cytokine production of T cells are activated, and the formation of cytolytic immunological synapses are induced (112, 113). BiTE is also characterized by its small size (55 kDa), which makes it a highly potent and efficacious molecule to against cancer (114). However, the small size of BiTE is unstable due to short serum half-life, thus continuous infusion is required.

BI 836909

BI 836909 is the first bispecific scFv with two linked scFvs in MM (84). The scFv targeting BCMA is positioned in N-terminal, and the scFv targeting CD3ε is in C-terminal, followed by a hexahistidine (His6-tag). BI 836909 simultaneously bind to CD3+ T cell and BCMA-expressing MM cells. This makes a cross-link between both cells to induce formation of cytolytic synapse, ultimately leading to activation of T cells and lysis of BCMA+ MM cells. These cytotoxic activities were not observed in BCMA-negative cells. When cocultured with BM stromal cells, BI 836909 retains potent anti-MM activity. Additionally, soluble APRIL and BCMA have only a mild effect on the anti-MM activity of BI 836909.

In mouse xenograft studies, BI 836909 led to tumor shrinkage in a subcutaneous NCI-H929 xenograft model and prolonged survival in an orthotopic L-363 xenograft model. In a cynomolgus monkey study, administration of BI 836909 resulted in significant depletion of BCMA + PCs in the BM of monkeys (84).

A half-life extended anti-BCMA BiTE base on BI 836909 was recently reported to be effective *in vitro* and *in vivo* and is suitable for once-weekly dosing in MM patients (85).

CAR T CELL THERAPY

Adoptive transfer of T cells genetically modified to recognize tumor-associated antigens is a promising cancer treatment (115). By using techniques of genetic modification, T cells can express CAR, which are fusion proteins that have an antigen recognition region, usually scFv derived from antibody on the surface, and a costimulation domain in the cell. Unlike T cell receptor modified T cells, CAR T cells are not restricted by major histocompatibility complex (40).

In MM, several anti-BCMA CAR T cell therapies have shown impressive clinical activities (some reaching 90–100%) with more are developed and under preclinical and/or clinical investigations (see **Table 1**).

OTHER TRIALS OF ANTI-BCMA CAR-T THERAPY

The combined infusion of CD19 and BCMA-specific CAR T Cells for RRMM was investigated in an early phase study (NCT 03196414) (116). The cells contained respective anti-BCMA or anti-CD19 scFv transduced by lentivirus, OX40 and CD28 costimulatory moiety, and CD3z T-cell activation domain. Clinical efficacy was evaluated in five patients monitored for more than 4 weeks, and showed that ORR was 100%, including 1 sCR, 1 VGRP, 2 PR, and 1 SD (116).

ANTI-BCMA CD3 BI- OR TRISPECIFIC MOLECULES

A Fully Human IgG CD3 Bispecific Molecule Targeting BCMA

This fully-human IgG bispecific molecule is characterized by its long half-life (about 3 days in mice) (100). The molecule utilizes hinge mutation technology to pair anti-BCMA and anti-CD3 targeting arms and places them in an IgG2A backbone. The anti-MM cytotoxicity was observed in MM patient samples at very low concentration (EC50 = 0.093 ± 0.1 nM), lower than ADC. This molecule also effectively depleted low BCMA-expressing normal plasma B cells. The evolution of toxicity in cynomologus monkeys model showed favorable safety profile.

EM801

EM801 is asymmetric two-arm IgG1-based human antibody with two binding sites for BCMA and 1 binding site for CD3 (66). EM801 promotes activation of CD4+ and CD8+ T-cells accompanied with release of IFN-γ, granzyme B, and perforin, and CD3+ T cell-dependent killing of MM cell lines. EM801 also induced significant cell death in malignant PCs by autologous T cells in BM samples of previously untreated and RRMM patients at very low concentrations (from 10 pM to 30 nM).

BCMA-TCB2

B cell maturation antigen-TCB2 is a bispecific antibody, which shares similar structure of EM801, but with higher affinity to BCMA (101). BCMA-TCB2 induces lysis of MM cells, activation of T cells, and natural emergence of the checkpoint inhibitor PD-1 on T cells at very low concentration. Combination of BCMA-TCB2 with lenalidomide or daratumumab significantly enhanced antimyeloma efficacy. NK cells were also activated after BCMA-TCB2 treatment.

Ab-957

Ab-957 is bispecific IgG-like Ab generated by Genmab DuoBody® technology to target CD3 on T cells and BCMA on MM cells (102). Preclinical studies also show that Ab-957 potently induces specific cytotoxicity of BCMA + MM cells *in vitro* and *in vivo*, with a concomitant activation of T cells at very low concentration.

AFM26

AFM26 is a bispecific antibody, which targets BCMA on MM cells and CD16A on NK cells (103, 104). AFM26 induces potent NK-cell-medicated cytotoxicity in BCMA+ MM, even when BCMA expression of BCMA was low. AFM26 does not induce NK-cell depletion. It shows similar anti-MM activity, but less inflammatory cytokine secretion, than BiTEs.

TNB383B and TNB-384B

TNB383B and TNB-384B are bispecific antibodies targeting BCMA on MM cells and CD3 on T cells, which are generated

based on the basis of *in silico* analysis of heavy chain only/fixed light chain antibody sequences (105). Both Abs showed significant anti-MM cytotoxicity at very low concentration (nano- or pico-molar) and eradicated MM cell growth in mice. In addition, markedly reduced or absence of cytokine release is observed after TNB-383B treatment.

Anti-CD16A/BCMA/CD200 Antibody

This trispecific antibody is characterized by bivalent binding to CD16A on NK cells and monovalent binding to BCMA and CD200 on MM cells (106). This dual-targeting structure may increase selectivity of MM cells coexpressing both antigens and improve safety.

THERAPEUTIC AGENTS TARGETING APRIL

Therapeutic agents blocking APRIL/BCMA are under investigated as well. A novel mouse anti-human APRIL antibody hAPRIL01A (01A) inhibits the binding of APRIL to BCMA and TACI (117). Importantly, 01A inhibited APRIL- and osteoclast-induced proliferation of MM cells and further induced apoptosis of MM cells in cocultures (33). 01A also enhances the cytotoxicity mediated by IMiDs and PI in the cocultures of MM cells with BCMA-negative BM accessory cells and effector cells. Furthermore, APRIL induces expression of genes involved in immunosuppression, such as PD-L1, TGF-β, and IL-10, are decreased in MM cells following 01A treatment (33). The early phase clinical trial of BION-1301, a fully humanized 01A mAb, is ongoing (118).

PERSPECTIVES AND CONCLUSION

Since its discovery in 1992, accumulating evidence has demonstrated that BCMA is a promising target for immunotherapy in MM (**Table 2**). CAR T therapy first demonstrated promising clinical efficacy in several phase 1 clinical trials in which high response rates are seen in heavily pretreated RRMM patients. GSK2857916, the first therapeutic BCMA-ADC, also shows impressive clinical efficacy and acceptable safety profile in RRMM resistant to multiple lines of current anti-MM treatments (**Table 3**). Similar efficacy in clinical trials can be anticipated for other anti-BCMA formats demonstrating highly selective anti-MM activity in preclinical studies.

Ongoing efforts are attempting to make BCMA CAR T therapy more potent, safe, and affordable for patients. To improve clinical efficacy, novel CAR T therapies are being developed to overcome relapse due to reduced tumor antigen, including modification of T cells with two distinct CAR molecules with two different binding domains, or one CAR molecule with two different binding domains in tandem (122–124). To reduce toxicities of conditioning chemotherapy, possible approaches include usage of less toxic conditioning chemotherapy, treating earlier in the disease course with less tumor burden, and improved supportive care (125). For prediction of severe cytokine releasing syndrome (CRS), several inflammation cytokines (especially IL-6) have been evaluated, and models have been established (125, 126). For the treatment of CRS, cytokine-directed therapy with anti-IL6 receptor inhibitor tocilizumab can abrogate toxicities (127, 128). Other strategies to reduce side effects include modification of CAR structure, such as incorporation of suicide genes into the engineered T cells (129–131); adding an inhibitory CAR on engineered T cells to reduce off-target immune response (132); or usage of a small molecule system to control CARs (133, 134). More

TABLE 2 | Important milestone of anti-B cell maturation antigen (BCMA) immunotherapy for MM.

Years	Major findings	Reference
1992	BCMA gene was first found, which was located on chromosome band 16p13.1 in a human malignant T-cell lymphoma	(45)
1994	The structure of BCMA was investigated. BCMA is expressed in mature B cells	(44)
1998	BCMA gene was identified as a new member of the tumor necrosis factor receptor superfamily	(43)
2000	BCMA is the receptors of BAFF and a proliferation-inducing ligand	(47–49)
	BCMA is expressed both on the surface and in an intracellular perinuclear structure of myeloma cell	(54)
	Overexpressed BCMA can activate the MAPK pathway and the nuclear factors NF-κB and Elk-1	
2001	In mouse model studies, knock out of BCMA had no significant impact on the life span of B cell. The humoral responses and memory responses remained intact	(52)
2002	Gene array study identified expression of BAFF, TACI, and BCMA in myeloma cells	(65)
2004	BCMA is necessary for the survival of long-lived bone marrow plasma cells (PCs)	(50)
	BCMA is highly expressed in malignant PCs	(62)
2007	Anti-BCMA MoAb and antibody–drug conjugate (ADC) were synthesized	(119)
	Preclinical study showed antimyeloma activity in myeloma cell lines	
2013	The first anti-BCMA chimeric antigen receptor (CAR) T was synthesized (by NCI)	(41)
	This study confirmed BCMA to be exclusively expressed on malignant PCs	
2014	Anti-BCMA ADC (GSK2857916) showed antimyeloma activity by induction of apoptosis and ADCC	(42)
2016	First phase 1 clinical trial of anti-BCMA CAR T therapy reported	(78)
	First phase 1 clinical trial of anti-BCMA ADC reported (GSK2857916)	(79)
	Promising results of several phase 1 clinical trials	(78, 79, 90)
2017	High complete response rates to anti-BCMA CAR T therapy in relapsed and refractory multiple myeloma patients	(87, 88)

TABLE 3 | Summary of phase 1 clinical trials of anti-B cell maturation antigen (BCMA) agents.

	Name	Enrollment criteria	No.	Prior treatment	Protocol	Results and efficacy	Adverse event (AE)
Antibody–drug conjugate	GSK2857916 (79, 80)	RR MM or other hematologic malignancies expressing BCMA	Dose-escalating part 24 (multiple myeloma)	83%, ≥4 prior lines (alkylators, PIs, IMiDs, ±stem cell transplantation)	IV infusion for 1 h ever 3 weeks 8 dose levels 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.92, 3.4 mg/kg	1. 1 MR at 0.24 mg/kg 2. 1 VGPR, 3 PR, and 1 MR at doses ≥0.96 mg/kg 3. Clinical benefit rate: 25%	Overall: 23/24 (96%), nausea (42%), fatigue (38%), anemia (29%), chills (29%), pyrexia (29%), thrombocytopenia (29%), dry eye (21%), hypercalcemia (21%) Gr 3/4 SE (>10%): thrombocytopenia, anemia, and neutropenia Severe AEs: 8 (in 6 patients), including 1 unresolved limbal stem cell dysfunction Dose reduction: 4 patients IRR: 7/24 (29%) DLT (–)
			Expansion part 35	50%, ≥5 prior lines (range 1–10) All received PI and IMIDs 97% refractory to PI 91% refractory to IMiDs 40% received DARA (37% refractory) 89% refractory to PI and IMiDs	IV infusion for 1 h ever 3 weeks	1. 1 sCR, 2 CR, 15 VGPR, and 3 PR 2. PFS: 7.9 months	1. All patients had at least one AE 2. Corneal events (63%), thrombocytopenia/platelet count decreased (57%), anemia (29%), AST increased (29%), and cough (26%) 3. Gr 3/4 AEs (≥10%): thrombocytopenia (34%) and anemia (14%) 4. Serious AEs were reported in 40% (14/35) of pts 5. IRRs: 8 (2 Gr 1, 3 Gr 2, 3 Gr 3)
Chimeric antigen receptor (CAR) T	Anti-BCMA CAR (78)	RRMM BCMA expression by either IHC or FCM	12	Median of 7 prior lines (range 3–13)	1. Cy (300 mg/m^2) 3 doses and Flu (30 mg/m^2) 3 doses 2. Followed by dose escalation of CAR T from (0.3, 1, 3, 9) × 10^6 cells/kg	1 sCR, 2 VGPR, 1 PR, 8SD	Gr 3/4 AE: lymphopenia (100%), leukopenia (100%), neutropenia (100%), anemia (50%), thrombocytopenia (50%)
	bb2121 (120)	RRMM 50% BCMA expression on plasma cells	21 (18 evaluable for response)	Median of 7 prior lines (range 3–14) All received auto-HSCT 71% exposed to Bort/Len/Car/Pom/Dara 29% with penta-refractory	1. Lymphodepletion: flu (30 mg/m^2)/Cy (300 mg/m^2) daily for 3 days 2. Followed by 1 infusion of bb2121 3. 3 + 3 design with planned dose levels of 50, 150, 450, 800, and 1,200 × 10^6 CAR T cells	Median follow-up after Bb2121 infusion: 15.4 weeks 1. ORR:89% (16/18) 2. ORR:100% (15/15, with 150 × 10^6 or more CAR T cells) 4CR, 7 VGPR, 4PR (4 MRD-) 3. MTD: 80 × 10^7 CAR + T-cells	1. CRS: 15/21 (71%), grade3 (n = 2) 2. Gr 3/4 AE: lymphodepletion, hyponatremia (n = 4), CRS (n = 2), URI (n = 2), and syncope (n = 2) 3. No DLT 4. 1 death (cardiopulmonary arrest) more than 4 months after bb2121 infusion in a patient with an extensive cardiac history (disease status: sCR)
	LCAR-B38M (88, 121)	RRMM	19 (evaluable)	≥3 prior regimens	1. Median infusion cells: 4.7 (0.6–7.0) × 10^6/kg, 3 infusions in 6 days	1. ORR:100%, with 14 sCR, 4 VRPR, 1 PR	1. CRS:14 (74%), Gr 3/4 (n = 2) 2. No neurologic AEs

(*Continued*)

TABLE 3 | Continued

Name	Enrollment criteria	No.	Prior treatment	Protocol	Results and efficacy	Adverse event (AE)
	RRMM, with extramedullary involvement	5 (2 with EMD)	All relapsed after classical chemotherapy, IMiDs, and PIs 3 with prior auto-HSCT	1. Pre-CAR-T treatment: fludarabine (25 mg/m^2) and cyclophosphamide (250 mg/m^2) daily for 3 days (d-5–d-3) 2. 0.62 × 10^6/kg (median) CAR-T cells for 3 days (d0, d2, and d6)	1. 1 CR, 1VGPR, 3 PR	1. Most common AEs: CRS 2. DLT (–) TRM (–)
CART-BCMA (90)	RRMM	33 consented 28 eligible 21 infused	Median 7 prior lines of therapy (range 3–11) 100% PI and IMIDs refractory 67% Dara refractory 95% had high-risk cytogenetics 67% del17p or TP53 mutation 29% extramedullary disease	1. 3 split-dose infusions of CAR T cells (10, 30, 60%) 2. 3 cohorts a. 1–5 × 10^8 CART cells (n = 9) b. Cy 1,500 m g/m^2 + 1–5 × 10^7 CART cells (n = 5) c. Cy 1,500 mg/m^2 + 1–5 × 10^8 CART cells (n = 7)	18 (86%) received full planned dose, and 3 received 40% of dose Efficacy Cohort 1:1 sCR, 2 VGPR, 1 PR, 2 MR Cohort 2:1 PR, 1 MR Cohort 3:1 CR, 3 PR, 1 MR CAR T cell expansion By qPCR: 100% By FCM:90%	1. Cohort 1 data Grade 3/4 SE: hypophosphatemia (n = 3), hypocalcemia (n = 2), anemia, neutropenia, lymphopenia, thrombocytopenia, hypofibrinogenemia, fatigue, pneumonia, UTI, elevated ALP and AST, hypokalemia, hypertension, and pleural effusion 2. CRS Cohort 1:8 (3 grade 3/4, with 4 receiving tocilizumab) Cohorts 2/3:9 (3 grade 3, none requiring tocilizumab) 3. Neurotoxicity Cohort 1; 2 (grade 4 encephalopathy) Cohorts 2/3:1 (grade 2 confusion/aphasia) 4. DLT (–)

ALP, alkaline phosphatase; AST, aspartate aminotransferase; auto-HSCT, autologous hematopoietic stem cell transplantation; Bort, bortezomib; Car, carfilzomib; CRS, cytokine releasing syndrome; Cy, cyclophosphamide; Dara, daratumumab; DOR, duration of response; DLT, dose-limiting toxicity; EMD, extramedullary disease; Flu, fludarabine; FCM, flow cytometry; Gr, grade, IHC, immunohistochemistry; IMiD, immunomodulatory drug; IRR, infusion-related reaction; Len, lenalidomide; MoAb, monoclonal antibody; MR, minimal response; MRD, minimal residual disease; MTD, maximal tolerated dose; ORR, overall response rate; PCR, polymerase chain reaction; PD, progressive disease; PI, proteasome inhibitor; Pom, pomalidomide; PR, partial response; PRES, posterior reversible encephalopathy syndrome; RRMM, relapsed and refractory multiple myeloma; sCR, stringent complete response; SD, stable disease; URI, upper airway infection; UTI, urinary tract infection; VGPR, very good partial response.

cost-effective, time-saving, and more accessible CAR T cell therapies are being developed, including allogeneic CAR T cells or CAR T cells utilizing novel manufacturing processes (93, 94).

For BCMA ADC, the first clinical trial has demonstrated efficacy and safety. ADC delivering highly toxic chemicals into the tumor cells is a highly selective therapy, which is critical since, the conjugated toxic chemicals are extremely deadly. Currently, several novel promising payloads are under development, including α-amanitin, tubulysins, hizoxin, or spliceostatins (135–137). To improve penetration, novel ADC formats such as non-IgG scaffolds or non-internalizing mAb scaffolds, may be applied to anti-BCMA ADC (138). Besides modification of ADC structure, combinations of ADC with other antitumor agents with different mechanisms of action are also under further investigation. Given that immune checkpoint inhibitors have clinical efficacy in several cancers, studies evaluating the clinical efficacy of combining immune checkpoint inhibitors with BCMA ADC are also warranted in MM (139).

Bispecific T-cell engagers are currently evaluated in preclinical studies. These anti-BCMA agents with excellent anti-MM effect will soon be investigated in clinical trials. Unlike CAR T cell therapy, BiTEs have a relatively short serum half-life and may not stimulate persistent immunity against cancer cells (140). Because it is difficult to maintain serum levels with bolus or intermittent infusion, continuous intravenous infusion may be needed (141). Importantly, long half-life molecules of BCMA BiTEs have been generated (85) and are currently being tested in a clinical trial. As CRS and neurotoxicity are also observed after BiTE treatment, close monitoring and adequate management for these side effects is very important (142). BCMA BiTEs mainly mediate their anti-MM effect by recruiting nearby cytotoxic T-cells to MM cells. However, the function of T cells is severely impaired in heavily pretreated MM patients (143, 144). To optimize BiTE anti-MM activity, studies are evaluating combination therapy with other anti-MM agents or immune checkpoint blockers.

Besides MM, anti-BCMA therapies may have therapeutic potential in other BCMA-expressing malignancies. For example, BAFF-R, BCMA, and TACI are all expressed on primary cells from patients with precursor B-cell acute lymphoblastic leukemia. Moreover, survival of leukemia cells is promoted by binding of BAFF and APRIL to their receptors, suggesting the therapeutic potential of targeting this signaling pathway (145). Other malignancies, such as Waldenstrom macroglobulinemia and glioblastoma/astrocytomas, also express BCMA on their cell surface (146, 147) and may benefit from these BCMA targeted therapies.

As BCMA is exclusively expressed on PCs, anti-BCMA treatment will reduce the number of long-lived PCs. Since long-lived PCs play a critical role in maintaining humoral immunity, the impact of anti-BCMA therapy on immune function needs to be carefully and serially evaluated. To address this issue, more clinical observation and correlative studies are warranted. Another potential complicating factor in anti-BCMA immunotherapy is high serum level of sBCMA, cleaved from BCMA by γ-secretase. In MM patients, high levels of sBCMA have been detected, especially in the setting of progressive disease (68). In preclinical studies, sBCMA slightly influenced the potency (shift in EC50 values) but not the maximal lysis mediated by BI 836909 (84). GSK2857916 still induced significant MM1S cell lysis in the presence of MM1S culture supernatant (42). On the other hand, sBCMA level is markedly decreased in patients after successful CAR T cell therapy (78). More clinical studies are needed to determine whether the level of sBCMA can potentially interfere with efficacy of anti-BCMA treatment. Inhibition of γ-secretase to reduce the formation of sBCMA and enhance the expression of BCMA on MM cells is another novel treatment approach.

In conclusion, BCMA-based immunotherapy is a promising in MM. It is anticipated that most of these anti-BCMA approaches, alone and in combinations with immune checkpoint inhibitors, and as well as cancer vaccines, will be evaluated in clinical studies and offer the promise of more selective, better tolerated, anti-MM therapy.

AUTHOR CONTRIBUTIONS

Y-TT and S-FC reviewed literature and designed and wrote this paper. Y-TT and KA critically reviewed and edited the paper.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* (2018) 68(1):7–30. doi:10.3322/caac.21442
2. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc* (2003) 78(1):21–33. doi:10.4065/78.1.21
3. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med* (2011) 364(11):1046–60. doi:10.1056/NEJMra1011442
4. Richardson PG, Sonneveld P, Schuster MW, Irwin D, Stadtmauer EA, Facon T, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* (2005) 352(24):2487–98. doi:10.1056/NEJMoa043445
5. Kumar SK, Dispenzieri A, Lacy MQ, Gertz MA, Buadi FK, Pandey S, et al. Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. *Leukemia* (2014) 28(5):1122–8. doi:10.1038/leu.2013.313
6. San Miguel JF, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, Kropff M, et al. Bortezomib plus melphalan and prednisone for initial treatment

of multiple myeloma. *N Engl J Med* (2008) 359(9):906–17. doi:10.1056/NEJMoa0801479
7. Rosinol L, Oriol A, Teruel AI, Hernandez D, Lopez-Jimenez J, de la Rubia J, et al. Superiority of bortezomib, thalidomide, and dexamethasone (VTD) as induction pretransplantation therapy in multiple myeloma: a randomized phase 3 PETHEMA/GEM study. *Blood* (2012) 120(8):1589–96. doi:10.1182/blood-2012-02-408922
8. Laubach JP, Voorhees PM, Hassoun H, Jakubowiak A, Lonial S, Richardson PG. Current strategies for treatment of relapsed/refractory multiple myeloma. *Expert Rev Hematol* (2014) 7(1):97–111. doi:10.1586/17474086.2014.882764
9. Kuehl WM, Bergsagel PL. Molecular pathogenesis of multiple myeloma and its premalignant precursor. *J Clin Invest* (2012) 122(10):3456–63. doi:10.1172/JCI61188
10. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer* (2012) 12(5):335–48. doi:10.1038/nrc3257
11. Manier S, Salem KZ, Park J, Landau DA, Getz G, Ghobrial IM. Genomic complexity of multiple myeloma and its clinical implications. *Nat Rev Clin Oncol* (2017) 14(2):100–13. doi:10.1038/nrclinonc.2016.122
12. Fowler JA, Mundy GR, Lwin ST, Edwards CM. Bone marrow stromal cells create a permissive microenvironment for myeloma development: a new stromal role for Wnt inhibitor Dkk1. *Cancer Res* (2012) 72(9):2183–9. doi:10.1158/0008-5472.CAN-11-2067
13. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer* (2007) 7(8):585–98. doi:10.1038/nrc2189
14. An G, Acharya C, Feng X, Wen K, Zhong M, Zhang L, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. *Blood* (2016) 128(12):1590–603. doi:10.1182/blood-2016-03-707547
15. Vacca A, Ria R, Semeraro F, Merchionne F, Coluccia M, Boccarelli A, et al. Endothelial cells in the bone marrow of patients with multiple myeloma. *Blood* (2003) 102(9):3340–8. doi:10.1182/blood-2003-04-1338
16. Zheng Y, Cai Z, Wang S, Zhang X, Qian J, Hong S, et al. Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. *Blood* (2009) 114(17):3625–8. doi:10.1182/blood-2009-05-220285
17. Braga WM, da Silva BR, de Carvalho AC, Maekawa YH, Bortoluzzo AB, Rizzatti EG, et al. FOXP3 and CTLA4 overexpression in multiple myeloma bone marrow as a sign of accumulation of CD4(+) T regulatory cells. *Cancer Immunol Immunother* (2014) 63(11):1189–97. doi:10.1007/s00262-014-1589-9
18. Feng X, Zhang L, Acharya C, An G, Wen K, Qiu L, et al. Targeting CD38 suppresses induction and function of T regulatory cells to mitigate immunosuppression in multiple myeloma. *Clin Cancer Res* (2017) 23(15):4290–300. doi:10.1158/1078-0432.CCR-16-3192
19. Kawano Y, Zavidij O, Park J, Moschetta M, Kokubun K, Mouhieddine TH, et al. Blocking IFNAR1 inhibits multiple myeloma-driven Treg expansion and immunosuppression. *J Clin Invest* (2018) 128(6):2487–99. doi:10.1172/JCI88169
20. Leone P, Berardi S, Frassanito MA, Ria R, De Re V, Cicco S, et al. Dendritic cells accumulate in the bone marrow of myeloma patients where they protect tumor plasma cells from CD8+ T-cell killing. *Blood* (2015) 126(12):1443–51. doi:10.1182/blood-2015-01-623975
21. Chauhan D, Singh AV, Brahmandam M, Carrasco R, Bandi M, Hideshima T, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell* (2009) 16(4):309–23. doi:10.1016/j.ccr.2009.08.019
22. Gorgun GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood* (2013) 121(15):2975–87. doi:10.1182/blood-2012-08-448548
23. Corre J, Mahtouk K, Attal M, Gadelorge M, Huynh A, Fleury-Cappellesso S, et al. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. *Leukemia* (2007) 21(5):1079–88. doi:10.1038/sj.leu.2404621
24. Urashima M, Ogata A, Chauhan D, Vidriales MB, Teoh G, Hoshi Y, et al. Interleukin-6 promotes multiple myeloma cell growth via phosphorylation of retinoblastoma protein. *Blood* (1996) 88(6):2219–27.
25. Urashima M, Ogata A, Chauhan D, Hatziyanni M, Vidriales MB, Dedera DA, et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood* (1996) 87(5):1928–38.
26. Hayashi T, Hideshima T, Nguyen AN, Munoz O, Podar K, Hamasaki M, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* (2004) 10(22):7540–6. doi:10.1158/1078-0432.CCR-04-0632
27. Abe M, Hiura K, Wilde J, Moriyama K, Hashimoto T, Ozaki S, et al. Role for macrophage inflammatory protein (MIP)-1alpha and MIP-1beta in the development of osteolytic lesions in multiple myeloma. *Blood* (2002) 100(6):2195–202.
28. Sprynski AC, Hose D, Caillot L, Reme T, Shaughnessy JD Jr, Barlogie B, et al. The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. *Blood* (2009) 113(19):4614–26. doi:10.1182/blood-2008-07-170464
29. Le Gouill S, Podar K, Amiot M, Hideshima T, Chauhan D, Ishitsuka K, et al. VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. *Blood* (2004) 104(9):2886–92. doi:10.1182/blood-2004-05-1760
30. Derksen PW, de Gorter DJ, Meijer HP, Bende RJ, van Dijk M, Lokhorst HM, et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. *Leukemia* (2003) 17(4):764–74. doi:10.1038/sj.leu.2402875
31. Moreaux J, Legouffe E, Jourdan E, Quittet P, Reme T, Lugagne C, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood* (2004) 103(8):3148–57. doi:10.1182/blood-2003-06-1984
32. Tai YT, Li XF, Breitkreutz I, Song W, Neri P, Catley L, et al. Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* (2006) 66(13):6675–82. doi:10.1158/0008-5472.CAN-06-0190
33. Tai YT, Acharya C, An G, Moschetta M, Zhong MY, Feng X, et al. APRIL and BCMA promote human multiple myeloma growth and immunosuppression in the bone marrow microenvironment. *Blood* (2016) 127(25):3225–36. doi:10.1182/blood-2016-01-691162
34. Kawano Y, Moschetta M, Manier S, Glavey S, Gorgun GT, Roccaro AM, et al. Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev* (2015) 263(1):160–72. doi:10.1111/imr.12233
35. Braga WM, Atanackovic D, Colleoni GW. The role of regulatory T cells and TH17 cells in multiple myeloma. *Clin Dev Immunol* (2012) 2012:293479. doi:10.1155/2012/293479
36. Bryant C, Suen H, Brown R, Yang S, Favaloro J, Aklilu E, et al. Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance. *Blood Cancer J* (2013) 3:e148. doi:10.1038/bcj.2013.34
37. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* (2003) 4(4):330–6. doi:10.1038/ni904
38. Joshua D, Suen H, Brown R, Bryant C, Ho PJ, Hart D, et al. The T cell in myeloma. *Clin Lymphoma Myeloma Leuk* (2016) 16(10):537–42. doi:10.1016/j.clml.2016.08.003
39. Muthu Raja KR, Rihova L, Zahradova L, Klincova M, Penka M, Hajek R. Increased T regulatory cells are associated with adverse clinical features and predict progression in multiple myeloma. *PLoS One* (2012) 7(10):e47077. doi:10.1371/journal.pone.0047077
40. Hoyos V, Borrello I. The immunotherapy era of myeloma: monoclonal antibodies, vaccines, and adoptive T-cell therapies. *Blood* (2016) 128(13):1679–87. doi:10.1182/blood-2016-05-636357
41. Carpenter RO, Evbuomwan MO, Pittaluga S, Rose JJ, Raffeld M, Yang S, et al. B-cell maturation antigen is a promising target for adoptive T-cell therapy of multiple myeloma. *Clin Cancer Res* (2013) 19(8):2048–60. doi:10.1158/1078-0432.CCR-12-2422
42. Tai YT, Mayes PA, Acharya C, Zhong MY, Cea M, Cagnetta A, et al. Novel anti-B-cell maturation antigen antibody-drug conjugate (GSK2857916) selectively induces killing of multiple myeloma. *Blood* (2014) 123(20):3128–38. doi:10.1182/blood-2013-10-535088

43. Madry C, Laabi Y, Callebaut I, Roussel J, Hatzoglou A, Le Coniat M, et al. The characterization of murine BCMA gene defines it as a new member of the tumor necrosis factor receptor superfamily. *Int Immunol* (1998) 10(11):1693–702. doi:10.1093/intimm/10.11.1693
44. Laabi Y, Gras MP, Brouet JC, Berger R, Larsen CJ, Tsapis A. The BCMA gene, preferentially expressed during B lymphoid maturation, is bidirectionally transcribed. *Nucleic Acids Res* (1994) 22(7):1147–54. doi:10.1093/nar/22.7.1147
45. Laabi Y, Gras MP, Carbonnel F, Brouet JC, Berger R, Larsen CJ, et al. A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma. *EMBO J* (1992) 11(11):3897–904.
46. Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* (2003) 21:231–64. doi:10.1146/annurev.immunol.21.120601.141152
47. Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A. Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI. *Curr Biol* (2000) 10(13):785–8. doi:10.1016/S0960-9822(00)00566-2
48. Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, et al. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* (2000) 404(6781):995–9. doi:10.1038/35010115
49. Thompson JS, Schneider P, Kalled SL, Wang L, Lefevre EA, Cachero TG, et al. BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. *J Exp Med* (2000) 192(1):129–35. doi:10.1084/jem.192.1.129
50. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med* (2004) 199(1):91–8. doi:10.1084/jem.20031330
51. Benson MJ, Dillon SR, Castigli E, Geha RS, Xu S, Lam KP, et al. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. *J Immunol* (2008) 180(6):3655–9. doi:10.4049/jimmunol.180.6.3655
52. Xu S, Lam KP. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol Cell Biol* (2001) 21(12):4067–74. doi:10.1128/MCB.21.12.4067-4074.2001
53. Laurent SA, Hoffmann FS, Kuhn PH, Cheng Q, Chu Y, Schmidt-Supprian M, et al. gamma-Secretase directly sheds the survival receptor BCMA from plasma cells. *Nat Commun* (2015) 6:7333. doi:10.1038/ncomms8333
54. Hatzoglou A, Roussel J, Bourgeade MF, Rogier E, Madry C, Inoue J, et al. TNF receptor family member BCMA (B cell maturation) associates with TNF receptor-associated factor (TRAF) 1, TRAF2, and TRAF3 and activates NF-kappa B, elk-1, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase. *J Immunol* (2000) 165(3):1322–30. doi:10.4049/jimmunol.165.3.1322
55. Deng S, Yuan T, Cheng X, Jian R, Jiang J. B-lymphocyte-induced maturation protein1 up-regulates the expression of B-cell maturation antigen in mouse plasma cells. *Mol Biol Rep* (2010) 37(8):3747–55. doi:10.1007/s11033-010-0028-z
56. Shaffer AL, Emre NC, Lamy L, Ngo VN, Wright G, Xiao W, et al. IRF4 addiction in multiple myeloma. *Nature* (2008) 454(7201):226–31. doi:10.1038/nature07064
57. Matthes T, Dunand-Sauthier I, Santiago-Raber ML, Krause KH, Donze O, Passweg J, et al. Production of the plasma-cell survival factor a proliferation-inducing ligand (APRIL) peaks in myeloid precursor cells from human bone marrow. *Blood* (2011) 118(7):1838–44. doi:10.1182/blood-2011-01-332940
58. Neri P, Kumar S, Fulciniti MT, Vallet S, Chhetri S, Mukherjee S, et al. Neutralizing B-cell activating factor antibody improves survival and inhibits osteoclastogenesis in a severe combined immunodeficient human multiple myeloma model. *Clin Cancer Res* (2007) 13(19):5903–9. doi:10.1158/1078-0432.CCR-07-0753
59. Shen X, Guo Y, Qi J, Shi W, Wu X, Ju S. Binding of B-cell maturation antigen to B-cell activating factor induces survival of multiple myeloma cells by activating Akt and JNK signaling pathways. *Cell Biochem Funct* (2016) 34(2):104–10. doi:10.1002/cbf.3169
60. Yang M, Hase H, Legarda-Addison D, Varughese L, Seed B, Ting AT. B cell maturation antigen, the receptor for a proliferation-inducing ligand and B cell-activating factor of the TNF family, induces antigen presentation in B cells. *J Immunol* (2005) 175(5):2814–24. doi:10.4049/jimmunol.175.5.2814
61. Avery DT, Kalled SL, Ellyard JI, Ambrose C, Bixler SA, Thien M, et al. BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J Clin Invest* (2003) 112(2):286–97. doi:10.1172/JCI18025
62. Novak AJ, Darce JR, Arendt BK, Harder B, Henderson K, Kindsvogel W, et al. Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. *Blood* (2004) 103(2):689–94. doi:10.1182/blood-2003-06-2043
63. Chiu A, Xu W, He B, Dillon SR, Gross JA, Sievers E, et al. Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL. *Blood* (2007) 109(2):729–39. doi:10.1182/blood-2006-04-015958
64. Lee L, Draper B, Chaplin N, Philip B, Chin M, Galas-Filipowicz D, et al. An APRIL based chimeric antigen receptor for dual targeting of BCMA and TACI in multiple myeloma. *Blood* (2018) 131(7):746–58. doi:10.1182/blood-2017-05-781351
65. Claudio JO, Masih-Khan E, Tang H, Goncalves J, Voralia M, Li ZH, et al. A molecular compendium of genes expressed in multiple myeloma. *Blood* (2002) 100(6):2175–86. doi:10.1182/blood-2002-01-0008
66. Seckinger A, Delgado JA, Moser S, Moreno L, Neuber B, Grab A, et al. Target expression, generation, preclinical activity, and pharmacokinetics of the BCMA-T cell bispecific antibody EM801 for multiple myeloma treatment. *Cancer Cell* (2017) 31(3):396–410. doi:10.1016/j.ccell.2017.02.002
67. Ghermezi M, Li M, Vardanyan S, Harutyunyan NM, Gottlieb J, Berenson A, et al. Serum B-cell maturation antigen: a novel biomarker to predict outcomes for multiple myeloma patients. *Haematologica* (2017) 102(4):785–95. doi:10.3324/haematol.2016.150896
68. Sanchez E, Li M, Kitto A, Li J, Wang CS, Kirk DT, et al. Serum B-cell maturation antigen is elevated in multiple myeloma and correlates with disease status and survival. *Br J Haematol* (2012) 158(6):727–38. doi:10.1111/j.1365-2141.2012.09241.x
69. Sanchez E, Smith EJ, Yashar MA, Patil S, Li M, Porter AL, et al. The role of B-cell maturation antigen in the biology and management of, and as a potential therapeutic target in, multiple myeloma. *Target Oncol* (2018) 13(1):39–47. doi:10.1007/s11523-017-0538-x
70. Bellucci R, Alyea EP, Chiaretti S, Wu CJ, Zorn E, Weller E, et al. Graft-versus-tumor response in patients with multiple myeloma is associated with antibody response to BCMA, a plasma-cell membrane receptor. *Blood* (2005) 105(10):3945–50. doi:10.1182/blood-2004-11-4463
71. Fragioudaki M, Boula A, Tsirakis G, Psarakis F, Spanoudakis M, Papadakis IS, et al. B cell-activating factor: its clinical significance in multiple myeloma patients. *Ann Hematol* (2012) 91(9):1413–8. doi:10.1007/s00277-012-1470-x
72. Day ES, Cachero TG, Qian F, Sun Y, Wen D, Pelletier M, et al. Selectivity of BAFF/BLyS and APRIL for binding to the TNF family receptors BAFFR/BR3 and BCMA. *Biochemistry* (2005) 44(6):1919–31. doi:10.1021/bi048227k
73. Wallweber HJ, Compaan DM, Starovasnik MA, Hymowitz SG. The crystal structure of a proliferation-inducing ligand, APRIL. *J Mol Biol* (2004) 343(2):283–90. doi:10.1016/j.jmb.2004.08.040
74. Moreaux J, Sprynski AC, Dillon SR, Mahtouk K, Jourdan M, Ythier A, et al. APRIL and TACI interact with syndecan-1 on the surface of multiple myeloma cells to form an essential survival loop. *Eur J Haematol* (2009) 83(2):119–29. doi:10.1111/j.1600-0609.2009.01262.x
75. Tai YT, Anderson KC. Targeting B-cell maturation antigen in multiple myeloma. *Immunotherapy* (2015) 7(11):1187–99. doi:10.2217/imt.15.77
76. Hengeveld PJ, Kersten MJ. B-cell activating factor in the pathophysiology of multiple myeloma: a target for therapy? *Blood Cancer J* (2015) 5:e282. doi:10.1038/bcj.2015.3
77. Matthes T, McKee T, Dunand-Sauthier I, Manfroi B, Park S, Passweg J, et al. Myelopoiesis dysregulation associated to sustained APRIL production in multiple myeloma-infiltrated bone marrow. *Leukemia* (2015) 29(9):1901–8. doi:10.1038/leu.2015.68
78. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, et al. T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. *Blood* (2016) 128(13):1688–700. doi:10.1182/blood-2016-04-711903
79. Cohen AD, Popat R, Trude S, Richardson PG, Libby IIIEN, Lendvai N, et al. First in human study with GSK2857916, an antibody drug conjugated to

microtubule-disrupting agent directed against b-cell maturation antigen (BCMA) in patients with relapsed/refractory multiple myeloma. *Blood* (2016) 128(22):1148.
80. Trudel S, Lendvai N, Popat R, Voorhees PM, Reeves B, Libby EN, et al. Deep and durable responses in patients (Pts) with relapsed/refractory multiple myeloma (MM) treated with monotherapy GSK2857916, an antibody drug conjugate against B-cell maturation antigen (BCMA): preliminary results from Part 2 of Study BMA117159. *Blood* (2017) 130(Suppl 1):741.
81. Hechler T, Palfi A, Müller C, Lutz C, Pahl A, Kulke M. Preclinical evaluation of HDP-101, an anti-BCMA antibody-drug conjugate. *Cancer Res* (2017) 77(13 Suppl):77. doi:10.1158/1538-7445.AM2017-77
82. Ko J, Breunig C, Figueroa V, Lehners N, Baumann A, Pálfi A, et al. Preclinical evaluation of Hdp-101, a novel anti-Bcma antibody-drug conjugate, in multiple myeloma. *Blood* (2017) 130(Suppl 1):3070.
83. Kinneer K, Meekin J, Varkey R, Xiao X, Zhong H, Breen S, et al. Preclinical evaluation of MEDI2228, a BCMA-targeting pyrrolobenzodiazepine-linked antibody drug conjugate for the treatment of multiple myeloma. *Blood* (2017) 130(Suppl 1):3153.
84. Hipp S, Tai YT, Blanset D, Deegen P, Wahl J, Thomas O, et al. A novel BCMA/CD3 bispecific T-cell engager for the treatment of multiple myeloma induces selective lysis in vitro and in vivo. *Leukemia* (2017) 31(8):1743–51. doi:10.1038/leu.2016.388
85. Goyos A, Li CM, Deegen P, Bogner P, Thomas O, Matthias K, et al. Generation of half-life extended anti-BCMA Bite® antibody construct compatible with once-weekly dosing for treatment of multiple myeloma (MM). *Blood* (2017) 130:5389.
86. Brudno J, Lam N, Wang M, Stroncek D, Maric I, Stetler-Stevenson M, et al. T cells genetically modified to express an anti-B-cell maturation antigen chimeric antigen receptor with a CD28 costimulatory moiety cause remissions of poor-prognosis relapsed multiple myeloma. *Blood* (2017) 130(Suppl 1):524.
87. Berdeja JG, Lin Y, Raje NS, Siegel DSD, Munshi NC, Liedtke M, et al. First-in-human multicenter study of bb2121 anti-BCMA CAR T-cell therapy for relapsed/refractory multiple myeloma: updated results. *J Clin Oncol* (2017) 35(15 Supplal):3010.
88. Fan F, Zhao W, Liu J, He A, Chen Y, Cao X, et al. Durable remissions with BCMA-specific chimeric antigen receptor (CAR)-modified t cells in patients with refractory/relapsed multiple myeloma. *J Clin Oncol* (2017) 35 (18 Supplal):LBA3001. doi:10.1200/JCO.2017.35.18_suppl.LBA3001
89. Dolgin E. CAR-Ts move beyond B-cell cancers to myeloma. *Nat Biotechnol* (2017) 35(7):599–601. doi:10.1038/nbt0717-599
90. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT, et al. B-cell maturation antigen (BCMA)-specific chimeric antigen receptor T cells (CART-BCMA) for multiple myeloma (MM): initial safety and efficacy from a Phase I Study. *Blood* (2016) 128(22):1147.
91. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT, et al. Safety and efficacy of B-cell maturation antigen (BCMA)-specific chimeric antigen receptor T cells (CART-BCMA) with cyclophosphamide conditioning for refractory multiple myeloma (MM). *Blood* (2017) 130(Suppl 1):505.
92. Adams GB, Feng J, Ghogha A, Mardiros A, Murakami J, Phung T, et al. Development of KITE-585: a fully human BCMA CAR T-cell therapy for the treatment of multiple myeloma. *Cancer Res* (2017) 77(13 Suppl):4979. doi:10.1158/1538-7445.AM2017-4979
93. Boldajipour B, Galetto R, Sommer C, Pertel T, Valton J, Park Y, et al. Preclinical evaluation of allogeneic anti-bcma chimeric antigen receptor t cells with safety switch domains and lymphodepletion resistance for the treatment of multiple myeloma. *Blood* (2016) 128(22):381.
94. Barnett BE, Wang X, Hermanson DL, Tan Y, Osertag EM, Shedlock DJ. Development of novel non-immunoglobulin centyrin-based cars (CARTyrins) targeting human Bcma. *Blood* (2016) 128(22):4557.
95. Hermanson DL, Barnett BE, Rengarajan S, Codde R, Wang X, Tan Y, et al. A novel BCMA-specific, centyrin-based CAR-T product for the treatment of multiple myeloma. *Blood* (2016) 128(22):2127.
96. Mathur R, Barnett BE, Hermanson D, He J, Zhang Z, Rengarajan S, et al. B-cell maturation antigen (BCMA)-specific, Centyrin™-based, PiggyBac™-transposed CAR-T memory stem cells are effective against $p53^{-/-}$ and patient-derived multiple myeloma tumors. *Blood* (2017) 130(Suppl 1):3068.
97. Lam N, Alabanza L, Trinklein N, Buelow B, Kochenderfer JN. T cells expressing anti-B-cell maturation antigen (BCMA) chimeric antigen receptors with antigen recognition domains made up of only single human heavy chain variable domains specifically recognize Bcma and eradicate tumors in mice. *Blood* (2017) 130(Suppl 1):504.
98. Lin L, Xing L, Acharya CM, Wen K, Liu J, Hsieh P, et al. CD8+ anti-Bcma mRNA CAR T-cells effectively kill human multiple myeloma cells in vitro and in vivo. *Blood* (2017) 130(Suppl 1):3067.
99. Wang X, Barnett BE, Martin C, Hermanson D, Li X, Smith J, et al. Production of universal anti-Bcma CAR-T cells with reduced alloreactivity, but potent effector function for the treatment of multiple myeloma. *Blood* (2017) 130(Suppl 1):503.
100. Panowski SH, Kuo T, Chen A, Geng T, Van Blarcom TJ, Lindquist K, et al. Preclinical evaluation of a potent anti-BCMA CD3 bispecific molecule for the treatment of multiple myeloma. *Blood* (2016) 128(22):383.
101. Moreno L, Zabaleta A, Alignani D, Lasa M, Maiso P, Jelinek T, et al. New insights into the mechanism of action (MoA) of first-in-class IgG-based Bcma T-cell bispecific antibody (TCB) for the treatment of multiple myeloma (MM). *Blood* (2016) 128(22):2096.
102. Pillarisetti K, Baldwin E, Babich A, Majewski N, Barone L, Li Y, et al. Development of a new BCMAxCD3 Duobody ® antibody for multiple myeloma. *Blood* (2016) 128(22):2116.
103. Gantke T, Reusch U, Ellwanger K, Fucek I, Weichel M, Treder M. AFM26 – a novel CD16a-directed bispecific TandAb targeting bcma for multiple myeloma. *Cancer Res* (2017) 77(13 Suppl):5671. doi:10.1158/1538-7445.AM2017-5671
104. Gantke T, Reusch U, Kellner C, Klausz K, Haneke T, Knackmuss S, et al. AFM26 – targeting B cell maturation antigen (BCMA) for NK cell-mediated immunotherapy of multiple myeloma. *Blood* (2017) 130(Suppl 1):3082.
105. Buelow B, Pham D, Choudhry P, Dang K, Pratap P, Clarke S, et al. T Cell engagement without cytokine storm: a novel Bcma x CD3 antibody killing myeloma cells with minimal cytokine secretion. *Blood* (2017) 130(Suppl 1):501.
106. Gantke T, Weichel M, Reusch U, Ellwanger K, Fucek I, Griep R, et al. Trispecific antibodies for selective CD16A-directed NK-cell engagement in multiple myeloma. *Blood* (2016) 128(22):4513.
107. McCombs JR, Owen SC. Antibody drug conjugates: design and selection of linker, payload and conjugation chemistry. *AAPS J* (2015) 17(2):339–51. doi:10.1208/s12248-014-9710-8
108. Rudd MD, Luse DS. Amanitin greatly reduces the rate of transcription by RNA polymerase II ternary complexes but fails to inhibit some transcript cleavage modes. *J Biol Chem* (1996) 271(35):21549–58. doi:10.1074/jbc.271.35.21549
109. Bhakta S, Raab H, Junutula JR. Engineering THIOMABs for site-specific conjugation of thiol-reactive linkers. *Methods Mol Biol* (2013) 1045:189–203. doi:10.1007/978-1-62703-541-5_11
110. Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. Site-specific antibody drug conjugates for cancer therapy. *MAbs* (2014) 6(1):34–45. doi:10.4161/mabs.27022
111. Baeuerle PA, Reinhardt C. Bispecific T-cell engaging antibodies for cancer therapy. *Cancer Res* (2009) 69(12):4941–4. doi:10.1158/0008-5472.CAN-09-0547
112. Brischwein K, Parr L, Pflanz S, Volkland J, Lumsden J, Klinger M, et al. Strictly target cell-dependent activation of T cells by bispecific single-chain antibody constructs of the BiTE class. *J Immunother* (2007) 30(8):798–807. doi:10.1097/CJI.0b013e318156750c
113. Offner S, Hofmeister R, Romaniuk A, Kufer P, Baeuerle PA. Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells. *Mol Immunol* (2006) 43(6):763–71. doi:10.1016/j.molimm.2005.03.007
114. Klein JS, Gnanapragasam PN, Galimidi RP, Foglesong CP, West AP Jr, Bjorkman PJ. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. *Proc Natl Acad Sci U S A* (2009) 106(18):7385–90. doi:10.1073/pnas.0811427106
115. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* (2008) 8(4):299–308. doi:10.1038/nrc2355
116. Yan L, Shang J, Kang L, Shi X, Zhou J, Jin S, et al. Combined infusion of CD19 and Bcma-specific chimeric antigen receptor T cells for RRMM: initial safety and efficacy report from a Clinical Pilot Study. *Blood* (2017) 130(Suppl 1):506.
117. Guadagnoli M, Kimberley FC, Phan U, Cameron K, Vink PM, Rodermond H, et al. Development and characterization of APRIL antagonistic monoclonal

antibodies for treatment of B-cell lymphomas. *Blood* (2011) 117(25): 6856–65. doi:10.1182/blood-2011-01-330852
118. Dulos J, Driessen L, van Zandvoort P, van de Crommert J, Skoble J, Nair N, et al. Bion-1301, a first-in-class APRIL neutralizing antibody for the treatment of multiple myeloma: preclinical safety, and analysis of pharmacokinetics – pharmacodynamics relationship. *Blood* (2017) 130(Suppl 1):1827.
119. Ryan MC, Hering M, Peckham D, McDonagh CF, Brown L, Kim KM, et al. Antibody targeting of B-cell maturation antigen on malignant plasma cells. *Mol Cancer Ther* (2007) 6(11):3009–18. doi:10.1158/1535-7163.MCT-07-0464
120. Berdeja JG, Lin Y, Raje N, Munshi N, Siegel D, Liedtke M, et al. Durable clinical responses in heavily pretreated patients with relapsed/refractory multiple myeloma: updated results from a Multicenter Study of bb2121 Anti-Bcma CAR T cell therapy. *Blood* (2017) 130(Suppl 1):740.
121. Mi J-Q, Fan X, Xu J, Liu Y, Zhuang Y, Yang S, et al. Effective treatment of relapsed/refractory multiple myeloma including extramedullary involvement by BCMA-specific chimeric antigen receptor-modified T cells. *Blood* (2017) 130(Suppl 1):3115.
122. Ruella M, Barrett DM, Kenderian SS, Shestova O, Hofmann TJ, Perazzelli J, et al. Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. *J Clin Invest* (2016) 126(10):3814–26. doi:10.1172/JCI87366
123. Zah E, Lin MY, Silva-Benedict A, Jensen MC, Chen YY. T cells expressing CD19/CD20 bispecific chimeric antigen receptors prevent antigen escape by malignant B cells. *Cancer Immunol Res* (2016) 4(6):498–508. doi:10.1158/2326-6066.CIR-15-0231
124. Hegde M, Mukherjee M, Grada Z, Pignata A, Landi D, Navai SA, et al. Tandem CAR T cells targeting HER2 and IL13Ralpha2 mitigate tumor antigen escape. *J Clin Invest* (2016) 126(8):3036–52. doi:10.1172/JCI83416
125. Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood* (2016) 127(26):3321–30. doi:10.1182/blood-2016-04-703751
126. Teachey DT, Lacey SF, Shaw PA, Melenhorst JJ, Maude SL, Frey N, et al. Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discov* (2016) 6(6):664–79. doi:10.1158/2159-8290.CD-16-0040
127. Teachey DT, Rheingold SR, Maude SL, Zugmaier G, Barrett DM, Seif AE, et al. Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. *Blood* (2013) 121(26):5154–7. doi:10.1182/blood-2013-02-485623
128. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) 371(16):1507–17. doi:10.1056/NEJMoa1407222
129. Quintarelli C, Vera JF, Savoldo B, Giordano Attianese GM, Pule M, Foster AE, et al. Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. *Blood* (2007) 110(8): 2793–802. doi:10.1182/blood-2007-02-072843
130. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* (2011) 365(18):1673–83. doi:10.1056/NEJMoa1106152
131. Ramos CA, Asgari Z, Liu E, Yvon E, Heslop HE, Rooney CM, et al. An inducible caspase 9 suicide gene to improve the safety of mesenchymal stromal cell therapies. *Stem Cells* (2010) 28(6):1107–15. doi:10.1002/stem.433
132. Fedorov VD, Themeli M, Sadelain M. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. *Sci Transl Med* (2013) 5(215):215ra172. doi:10.1126/scitranslmed.3006597
133. Juillerat A, Marechal A, Filhol JM, Valton J, Duclert A, Poirot L, et al. Design of chimeric antigen receptors with integrated controllable transient functions. *Sci Rep* (2016) 6:18950. doi:10.1038/srep18950
134. Wu CY, Roybal KT, Puchner EM, Onuffer J, Lim WA. Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. *Science* (2015) 350(6258):aab4077. doi:10.1126/science.aab4077
135. Murray BC, Peterson MT, Fecik RA. Chemistry and biology of tubulysins: antimitotic tetrapeptides with activity against drug resistant cancers. *Nat Prod Rep* (2015) 32(5):654–62. doi:10.1039/c4np00036f
136. Prota AE, Bargsten K, Diaz JF, Marsh M, Cuevas C, Liniger M, et al. A new tubulin-binding site and pharmacophore for microtubule-destabilizing anticancer drugs. *Proc Natl Acad Sci U S A* (2014) 111(38):13817–21. doi:10.1073/pnas.1408124111
137. Wang J, Xiao H, Qian ZG, Zhong JJ. Bioproduction of antibody-drug conjugate payload precursors by engineered cell factories. *Trends Biotechnol* (2017) 35(5):466–78. doi:10.1016/j.tibtech.2017.03.001
138. Beck A, Goetsch L, Dumontet C, Corvaia N. Strategies and challenges for the next generation of antibody-drug conjugates. *Nat Rev Drug Discov* (2017) 16(5):315–37. doi:10.1038/nrd.2016.268
139. Gerber HP, Sapra P, Loganzo F, May C. Combining antibody-drug conjugates and immune-mediated cancer therapy: what to expect? *Biochem Pharmacol* (2016) 102:1–6. doi:10.1016/j.bcp.2015.12.008
140. Klinger M, Brandl C, Zugmaier G, Hijazi Y, Bargou RC, Topp MS, et al. Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab. *Blood* (2012) 119(26):6226–33. doi:10.1182/blood-2012-01-400515
141. Topp MS, Kufer P, Gokbuget N, Goebeler M, Klinger M, Neumann S, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol* (2011) 29(18):2493–8. doi:10.1200/JCO.2010.32.7270
142. Teicher BA, Chari RV. Antibody conjugate therapeutics: challenges and potential. *Clin Cancer Res* (2011) 17(20):6389–97. doi:10.1158/1078-0432.CCR-11-1417
143. Suen H, Brown R, Yang S, Weatherburn C, Ho PJ, Woodland N, et al. Multiple myeloma causes clonal T-cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* (2016) 30(8):1716–24. doi:10.1038/leu.2016.84
144. Zelle-Rieser C, Thangavadivel S, Biedermann R, Brunner A, Stoitzner P, Willenbacher E, et al. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. *J Hematol Oncol* (2016) 9(1):116. doi:10.1186/s13045-016-0345-3
145. Maia S, Pelletier M, Ding J, Hsu YM, Sallan SE, Rao SP, et al. Aberrant expression of functional BAFF-system receptors by malignant B-cell precursors impacts leukemia cell survival. *PLoS One* (2011) 6(6):e20787. doi:10.1371/journal.pone.0020787
146. Elsawa SF, Novak AJ, Grote DM, Ziesmer SC, Witzig TE, Kyle RA, et al. B-lymphocyte stimulator (BLyS) stimulates immunoglobulin production and malignant B-cell growth in Waldenstrom macroglobulinemia. *Blood* (2006) 107(7):2882–8. doi:10.1182/blood-2005-09-3552
147. Deshayes F, Lapree G, Portier A, Richard Y, Pencalet P, Mahieu-Caputo D, et al. Abnormal production of the TNF-homologue APRIL increases the proliferation of human malignant glioblastoma cell lines via a specific receptor. *Oncogene* (2004) 23(17):3005–12. doi:10.1038/sj.onc.1207350

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REVIEW
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Osteoclast Immunosuppressive Effects in Multiple Myeloma: Role of Programmed Cell Death Ligand 1

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Immunomodulatory drugs and monoclonal antibody-based immunotherapies have significantly improved the prognosis of the patients with multiple myeloma (MM) in the recent years. These new classes of reagents target malignant plasma cells (PCs) and further modulate the immune microenvironment, which prolongs anti-MM responses and may prevent tumor occurrence. Since MM remains an incurable cancer for most patients, there continues to be a need to identify new tumor target molecules and investigate alternative cellular approaches using gene therapeutic strategies and novel treatment mechanisms. Osteoclasts (OCs), as critical multi-nucleated large cells responsible for bone destruction in >80% MM patients, have become an attractive cellular target for the development of novel MM immunotherapies. In MM, OCs are induced and activated by malignant PCs in a reciprocal manner, leading to osteolytic bone disease commonly associated with this malignancy. Significantly, bidirectional interactions between OCs and MM cells create a positive feedback loop to promote MM cell progression, increase angiogenesis, and inhibit immune surveillance *via* both cell–cell contact and abnormal production of multiple cytokines/chemokines. Most recently, hyper-activated OCs have been associated with activation of programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway, which impairs T cell proliferation and cytotoxicity against MM cells. Importantly, therapeutic anti-CD38 monoclonal antibodies and checkpoint inhibitors can alleviate OC-induced immune suppression. Furthermore, a proliferation-inducing ligand, abundantly secreted by OCs and OC precursors, significantly upregulates PD-L1 expression on MM cells, in addition to directly promoting MM cell proliferation and survival. Coupled with increased PD-L1 expression in other immune-suppressive cells, i.e., myeloid-derived suppressor cells and tumor-associated macrophages, these results strongly suggest that OCs contribute to the immunosuppressive MM BM microenvironment. Based on these findings and ongoing osteoimmunology studies, therapeutic interventions targeting OC number and function are under development to diminish both MM bone disease and related immune suppression. In this review, we discuss the classical and novel roles of OCs in the patho-immunology of MM. We also describe novel therapeutic strategies simultaneously targeting OCs and MM interactions, including PD-1/PD-L1 axis, to overcome the immune-suppressive microenvironment and improve patient outcome.

Keywords: multiple myeloma, osteoclast, bone marrow microenvironment, osteoblast, programmed cell death 1, programmed cell death ligand 1, immunotherapy

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INTRODUCTION

Multiple myeloma (MM), a malignancy of plasma cells (PCs), is defined by abnormal growth of malignant PCs within the bone marrow (BM), resulting in excessive monoclonal immunoglobulin in the blood and urine, impaired renal function, and repeated infections in patients (1). Moreover, osteolytic bone disease is a central hallmark of MM, which severely impacts quality of life in >80% of patients (2, 3). Specifically, osteoclast (OC)-mediated lytic bone destruction remains a cause of major morbidity in MM. In the past two decades, the introduction of autologous stem-cell transplantation and the availability of novel agents with different mechanisms of action including proteasome inhibitors (e.g., bortezomib, carfilzomib, ixazomib) and immunomodulatory drugs (IMiDs) (e.g., thalidomide, lenalidomide, pomalidomide) have revolutionized the therapeutic strategies for MM and significantly prolonged overall survival of patients (4–7). However, cure is rarely achieved due to the development of drug resistance and persistence of minimal residual disease. Thus, there is unmet need for innovative treatment modalities to eradicate residual tumor clones and effectively prevent disease relapses, as well as enhance overall anti-MM immunity.

Recently, immunotherapies have showed significant clinical activities not only against malignant, PCs but also potentially relieving the immunocompromised status in MM. Currently, a variety of immunotherapeutic strategies are under intensive preclinical and clinical development, including monoclonal antibodies (mAbs), chimeric antigen receptor T (CAR T) cells, immune checkpoint inhibitors, and as well as cancer vaccines (8). Following the approval of the first two mAbs daratumumab targeting CD38 and elotuzumab targeting SLAMF7 by FDA in late 2015 for the treatment in relapse and refractory MM (RRMM), multiple combination trials of these two mAbs are ongoing (8, 9). Excitingly, daratumumab has also shown clinical responses in newly diagnosed MM patients (9). Another therapeutic anti-CD38 mAb isatuximab, unlike daratumumab, can directly kill MM cells with p53 mutations and in the absence of effector natural killer (NK) cells *in vitro* (10). Indeed, isatuximab, when combined with lenalidomide or pomalidomide plus dexamethasone, also demonstrated significant activity in heavily treated RRMM (11, 12). Isatuximab is currently undergoing studies for the treatment of relapsed and previously untreated MM patients, pursuing FDA approval. Most importantly, more than a dozen targeted immunotherapies besides CD38 and SLAMF7 mAbs, alone or in combinations with current or emerging anti-MM therapies with different mechanisms of actions, have already entered clinical investigations.

Accumulating data for the past two decades has confirmed that the BM microenvironment plays a crucial role in the pathogenesis and recurrence of MM (13, 14). Malignant PCs in the MM BM are in close contact with non-myeloma cells, including bone marrow stromal cells (BMSCs) (13, 15), osteoclasts (OCs) (16–20), myeloid-derived suppressor cells (MDSCs) (21, 22), tumor-associated macrophages (TAMs) (23), regulatory T-cells (Treg) (21, 24, 25), plasmacytoid dendritic cells (pDC) (26), and regulatory B-cells (Breg) (27). These BM accessory cells, alone or in collaboration with others, support the initiation, progression, and re-occurrence of MM. They further influence treatment responses and may promote clonal evolution of malignant PC clones to adapt to the immune microenvironment and escape immune surveillance. For example, MM cells increase their proliferation upon adherence to BMSCs and become resistant to dexamethasone treatment (13, 28). Cytotoxic effects of some conventional drugs, i.e., dexamethasone, melphalan, as well as antibody-mediated cellular cytotoxicity against MM cells are reduced in the presence of BMSCs (13, 29).

Among other abovementioned cells, hyperactive OCs cause osteolytic bone diseases affecting almost every MM patient, thereby making them a potential novel cellular target for novel therapeutics. OCs, critical mediators of bone absorption, are large cells with multiple nuclei derived from CD14+ lineage myeloid cells (i.e., monocyte, macrophage) under the influence of several OC-activating cytokines produced by multiple BM accessory cells. Among many OC-stimulating cytokines, macrophage-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) are two essential OC-differentiation factors during osteoclastogenesis. Traditionally, OCs are known to play a vital role in maintenance of bone metabolism by counteracting osteoblasts (OBs). In contrast to OBs, which produce and secrete matrix proteins and transport mineral into the matrix for bone formation, OCs are responsible for bone degradation by breaking down tissues. In addition to inducing growth and survival of MM cells, OCs are capable of regulating growth of other BM cells, such as hematopoietic stem cells and B cell progenitors (30–32). Moreover, a close crosstalk exists between skeletal and immune systems, termed osteoimmunology, since several regulatory molecules are shared by these two systems (33–35). Most recently, OCs have been further associated with maintenance of immunosuppressive MM BM microenvironment *via* induction and secretion of several immune checkpoint proteins from OCs in close contact with MM cells (20) (**Figure 1**).

Programmed cell death ligand 1, also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1), is a 40 kDa type 1 transmembrane protein encoded by the CD274 gene located in the 9p24.1 region with the full length of cDNA 870 bp in man (36, 37). Following binding to its cognate receptor programmed cell death protein 1 (PD-1) (CD279) expressed on activated T cells, B cells, NK cells, and monocytes, the PD-1/PD-L1 pathway inhibits immune activation by triggering the phosphatases that deactivate signals emanating from the T cell receptor (38–40). Specifically, the engagement of PD-L1 with PD-1 on activated T cell leads to T cell dysfunction, exhaustion, neutralization, and production of interleukin-10 (IL-10) (41, 42). PD-L1 also interacts with B7-1 (CD80) on activated T cells, which in turn downregulates T cell activity (43). This important checkpoint pathway has been associated with autoimmune disease, infection, and cancer (37, 44–46).

In the tumor microenvironment, PD-1/PD-L1 pathway performs a vital role in tumor progression and survival by escaping tumor neutralizing immune surveillance. PD-L1 is expressed on various tumor cells and antigen-presenting cells (APCs) (41). PD-L1 overexpression on tumor cells is further associated with higher risk of cancer progression and poor clinical outcome

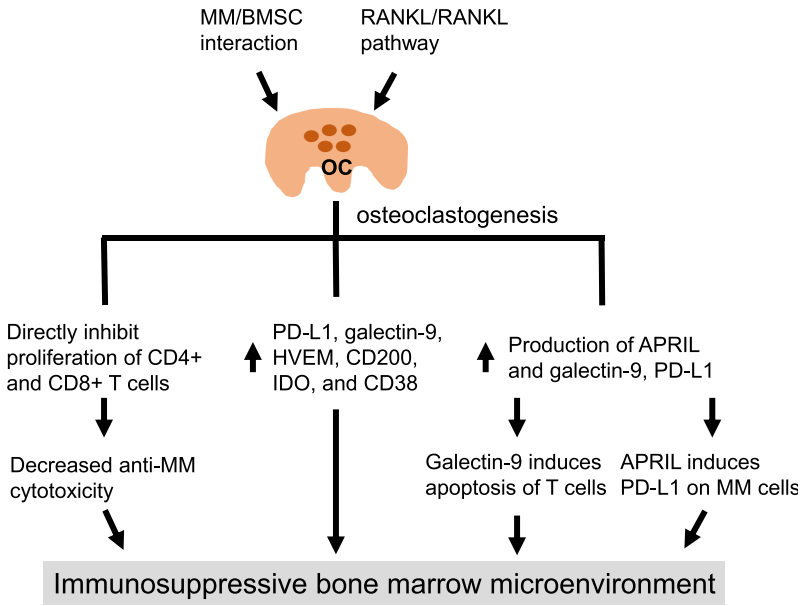

FIGURE 1 | Osteoclasts create an immunosuppressive microenvironment in multiple myeloma (MM). In MM, the interaction of MM cells and bone marrow stromal cells induces production of various cytokines and growth factors, as well as activates RANK/receptor activator of nuclear factor-κB (NF-κB) ligand pathway, to promote the differentiation and expansion of OCs from CD14+ OC precursors. OCs can directly inhibit proliferation of activated CD4+ and CD8+ effector T cells, thereby reducing their numbers and leading to decreased MM cell lysis. The expression of multiple immune checkpoint molecules on OCs is increased during osteoclastogenesis. Furthermore, the secretion of galectin-9 and APRIL is significantly augmented during OC formation, resulting in apoptosis of T cells, i.e., mediated by galectin-9, and enhanced programmed cell death ligand 1 expression on MM cells, i.e., mediated by APRIL, IL-6. APRIL, a proliferation-inducing ligand; BMSC, bone marrow stromal cell; HVEM, herpesvirus entry mediator; IDO, indoleamine 2, 3-dioxygenase.

(47–49). Importantly, immune checkpoint inhibitors targeting PD-1/PD-L1 have generated groundbreaking and durable responses in a broad spectrum of advanced solid tumors (50) and blood cancers including B-cell lymphomas (51, 52). In MM, PD-1/PD-L1 is also activated and associated with immunocompromised status and drug resistance (53, 54), supporting the development of new treatments targeting this pathway in MM (55). Despite inconclusive early clinical reports (51, 55), this important immune checkpoint pathway may still represent one of the novel strategies with potential anti-MM activities targeting defective immune effector cells, when combined with current and emerging therapies for MM.

We here summarized mechanisms of myeloma bone diseases and the novel functional characterization of OCs in the immunosuppressive BM microenvironment in MM *via* PD-1/PD-L1 pathway. Also included are effects of various current and emerging anti-MM treatments on OCs, other cellular subtypes associated the MM bone disease, and immune cells in the BM. Finally, we discuss the novel strategies for immune-therapies targeting OC function and PD-1/PD-L1 pathway in combination with other MM treatments to further overcome OC-induced immune suppression and prolong overall treatment responses.

MYELOMA BONE DISEASE: CLINICAL MANIFESTATION

The cells in skeletal system including OBs, OCs, and osteocytes closely communicate with each other to maintain the balance of bone metabolism. OBs provide essential signals, M-CSF, RANKL, and other co-stimulatory factors, to promote the differentiation of myeloid lineage precursors of OCs (56). However, this balance is significantly disturbed in the majority of MM patients, in whom OCs are highly activated accompanied with little or no OB activity (2). Eventually, increased bone-degrading effects accelerate osteoporosis and the development of lytic bone lesions, shown as characteristic "punched-out" lesions on skeletal X-ray (57, 58).

Clinically, approximately 80% of MM patients have radiologic evidence of bone involvement, and 90% have osteolytic manifestations including generalized osteopenia or discrete lytic lesions over the course of disease (16, 59). The most commonly involved sites include vertebral bodies (49%), skull (35%), pelvis (34%), and ribs (33% of patients) (2, 3, 60). Patients with MM bone disease may suffer from skeletal-related events (SREs) including pain, pathological fractures, spinal cord compression, and hypercalcemia. Furthermore, these SREs may increase mortality, decrease quality of life, and result in an adverse outcome (58, 61).

MYELOMA BONE DISEASE: MAJOR CELLULAR AND MOLECULAR MECHANISMS

The mechanisms of MM-related bone disease involve overactivation of OCs and inhibition of OBs *via* complicated interactions between various BM cells and cytokines secreted by them (2). The contact between MM cells and BMSCs significantly increases

activity and accelerates differentiation of OCs, while inhibiting the growth of OBs (15, 62). For example, the binding of surface VLA-4 ($\alpha_4\beta_1$ integrin) on MM cells to VCAM-1 on BMSCs induces production of cytokines, which favor bone absorption including: RANKL, M-CSF, IL-1, and IL-6 by BMSCs; and OC-activating factors including macrophage inflammatory protein-1α/β (MIP-1α/β), IL-3, stromal-derived factor-1α, and tumor necrosis factor α (TNF-α) by MM cells(63–70). In addition, adhesion between MM cells and BMSCs promotes secretion of B-cell activation factor (BAFF), which also promote growth of MM cells (71, 72) and RANKL-independent proliferation of OCs (72). In parallel, p38 mitogen-activated protein kinase signaling pathway is activated upon MM cell adherence to BMSCs, leading to more secretion of MM cell-supportive factors IL-6 and vascular endothelial growth factor (VEGF), in addition to induction of OC-activating factors (i.e., IL-11, RANKL, MIP-1α) (73). Moreover, IL-6 secretion by BMSC enhances expression and secretion of matrix metalloproteinase-13 (MMP-13) in MM cells (74). MMP-13, in turn, promotes fusion of OCs and bone absorption. Simultaneously, activated OCs support proliferation of MM cells by secreting more factors including annexin-II, osteopontin (OPN), IL-6, IL-10, insulin growth factor-1, BAFF, and a proliferation-inducing ligand (APRIL) (13, 20, 75–78).

In contrast, the expansion and activation of OBs is significantly blocked in MM bone disease due to increased secretion of OB inhibitory factors including: dickkopf-1 (Dkk-1), soluble frizzled-related protein 2 (sFRP2), sFRP3, IL-3, IL-7, growth factor independence-1 (gfi1), hepatocyte growth factor, activin A, sclerostin, and TNF-α (2, 62, 79–84). These factors directly and indirectly block proliferation and differentiation of OBs, impairing mineral deposition and bone regeneration. In addition, osteoprotegerin (OPG), a soluble decoy receptor of RANKL, is produced by OBs and inhibits OC activation under normal physiological conditions. OPG levels are significantly decreased in MM bone disease (85), associated with reduced OB number. Defective bone formation due to decreased proliferation and differentiation of OBs induced by MM cells, along with reduced levels of OC inhibitory cytokines produced by OBs, further augments OC formation and induction of osteolytic bone destruction.

In terms of signaling transduction cascades, the RANK/RANKL pathway critically regulates MM-induced bone lesions since several of the abovementioned OC-activating factors are induced *via* this pathway. RANKL is detected on the surface of MM cells and elevated in MM patients compared with health individuals and patients with monoclonal gammopathy of undetermined significance (MGUS) (86, 87). Concurrently, increased OCs induced by RANKL activate dormant MM cells (32). In fact, higher RANKL expression is associated with more severe bone disease and poorer clinical outcome (86, 88). In addition, MM cells express mRNA encoding the isoform of soluble RANKL (sRANKL), which directly promotes activation of OCs (89). Significantly, sRANKL is elevated in MM patients and closely related to generalized bone loss (90, 91).

Further studies on OC-gene expression profiling identify genes coding for 4 CCR2-targeting chemokines and genes coding for MM growth factors to be highly expressed by MM OCs (92). Specifically, higher CCR2 expression in MM cells is correlated with increased bone lesions, and CCR2 chemokines activate mitogen-activated protein kinase (MEK) pathway to support growth of MM cells (92). These results implicate the MEK1/2 signaling cascade (93), which is significantly induced by M-CSF and RANKL, in the pathogenesis of MM bone disease(17, 18, 94).

OCs IN THE MM BM MICROENVIRONMENT

The suppression of the host immune system is a critical step in the progression of many cancers, including MM. The interaction of MM cells and surrounding cells promotes production of immunosuppressive cytokines, growth of immune-suppressive cell populations, and suppression of the anti-MM ability of normal immune cells. For example, IL-6 and IL-10 levels are increased in the serum samples of MM patients, and both cytokines promote MM cell growth and survival in an autocrine and paracrine fashion. These two cytokines are also critical in MM-related immunosuppression, since IL-10 has potent immunosuppressive ability by inhibiting production of pro-inflammatory interferon-γ (IFN-γ) and TNF-α in immune effector cells (95), and IL-6 has been linked to impaired NK cell activity (96). Furthermore, the pro-osteoclastogenic LIGHT/TNFSF14 was recently linked to MM-bone disease (97). At the cellular level, inhibitory immune T regulatory cells (Tregs), B regulatory cells, and pDCs are significantly increased in the BM of the patients with active MM (24, 26, 27). In parallel, MM cells induce the development of myeloid-derived suppressor cells (MDSCs), which in turn support proliferation of MM cells by promoting proliferation of Tregs and suppressing T-cell-mediated immune responses (22, 98). Importantly, MDSCs induced by MM cells can further differentiate into mature OCs capable of inducing bone lysis, which further links immune suppression and hyper-active bone lysis activity of MDSCs in MM progression (99). Furthermore, the increased percentage of circulating pre-OCs have been described in MM (100, 101).

The MM BM microenvironment is also characterized by increased angiogenesis, which further suppresses anti-MM immunity. Specifically, contact of MM cells and OCs enhances angiogenesis and production of angiogenic factors (VEGF and OPN), which in turn promote the expansion of OCs by vascular endothelial cells (102). Both VEGF and OPN have been shown to directly induce proliferation of MM cells. In addition, increased OC formation by stimulation of RANKL or parathyroid hormone-related protein promotes angiogenesis *via* induction of MMP-9, a potent angiogenic factor secreted by OC mediating RANKL-induced angiogenesis. In contrast, OPG inhibits formation of OCs and decreases formation of new vessels (103).

Most recently, OCs have been shown to significantly block T cell proliferation and cytotoxicity in MM cells (**Figure 2**). The expression of several immune checkpoint molecules on OCs, including PD-L1, galectin-9, herpesvirus entry mediator, CD200, T-cell metabolism regulators indoleamine 2, 3-dioxygenase (IDO), and CD38, is significantly enhanced during OC formation *in vitro* (20) (**Figure 1**). Meanwhile, the secretion of galectin-9

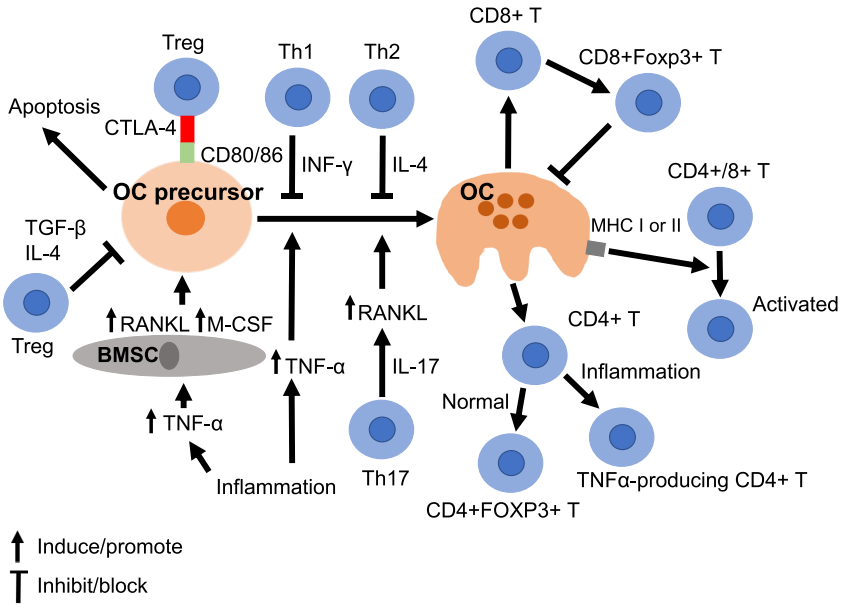

FIGURE 2 | Osteoclasts crosstalk with immune cells. The differentiation of OCs from its precursor (OC precursor) is mediated by multiple cytokines. For example, inflammation induces production of tumor necrosis factor α (TNFα), which activates OC formation directly or indirectly *via* BMSC. Another immune cell, the Th17 cell, which produces IL-17, also stimulate OCs *via* upregulation of receptor activator of nuclear factor-κB (NF-κB) ligand. The process of OC differentiation can be inhibited by INF-γ and IL4 produced by Th1 and Th2 cells, respectively. In parallel, T regulatory cells (Tregs) can inhibit OC precursors by secretion of TGF-β and IL-4. CTLA 4 expressed on Tregs can bind to CD80/86 on OC precursors and further influence the fate of OC precursors. OCs can activate several immune cells. First, OCs induce formation of CD8+FOXP3+ T cells, which in turn inhibit OCs. Second, OCs can act as antigen-presenting cells to promote immune response of CD4 or CD8 T cells. Third, OCs can induce differentiation of CD4+ T cell to TNFα-producing cells or CD4+FOXP3+ T cells, dependent on the surrounding microenvironment. BMSC, bone marrow stromal cell; IL, interleukin; INF, interferon; M-CSF, macrophage-colony-stimulating-factor; TGF, tumor growth factor; TNF, tumor necrosis factor; Treg, regulatory T cell.

and APRIL by OCs is significantly increased. Galectin-9 significantly induces apoptosis of T cells, and APRIL further induces expression of PD-L1 on MM cells mainly *via* MEK/ERK pathway. Significantly higher expression of PD-L1 was observed on OCs than MM cells, which was linked to profound inhibition of T cell activation to lyse MM cells. Importantly, the inhibition of T cell activation can be repaired using blocking PD-L1 or anti-CD38 monoclonal antibody (20), suggesting potential clinical development of these mAbs, alone and in combination, to overcome the immunosuppressive MM BM milieu.

OCs IN OSTEOIMMUNOLOGY

The skeletal and immune systems closely interact, since cytokines produced by lymphocytes significantly affect bone homeostasis. Among cells in these two systems, OCs significantly regulate intricate cytokine and cellular networks, as described above and in **Figure 2**. OCs interact not only with various BM cytokines but also control differentiation and expansion of multiple immune subsets. For example, inflammation or immune-related cytokines like TNF-α, IL-1, and IL-6, are associated with bone absorption (104–107). In autoimmune diseases like rheumatoid arthritis, production of cytokines (TNF-α, IL-1, IL-6, IL-17) is significantly increased in synovium and pannus, which may directly affect bone by upregulating OC activities at sites of articular erosion (108). In fact, TNF-α induces activation of OCs indirectly by enhancing the expression of RANKL and M-CSF in BMSCs or directly by interacting with OC precursors (109).

As for the interaction between OCs and immune cells, activated CD3+ or CD4+ T cells with RANKL expression support differentiation of OC *in vitro* (110, 111). A subset of CD4+ T cells (Th17), which produces IL-17 could upregulate RANKL and promote differentiation of OCs by the effect of IL-17 on BMSCs and OCs (112). T cells also produce IL-7, which can promote formation of OCs by upregulating RANKL (113). In addition, activated T cells secrete soluble RANKL (sRANKL), which is correlated with the formation of OCs and bone loss (114, 115). On the other hand, the activation of OCs can be downregulated by IFNγ and IL-4 secreted by Th1 and Th2 cells, respectively. IFN-γ produced by T cells significantly suppresses differentiation of OCs by interfering with the RANKL–RANK pathway, including degradation of downstream molecules such as tumor necrosis factor receptor-associated factor 6 (TRAF6) (116).

On the other hand, human OCs can function as APCs by expressing class I and II MHC molecules and co-stimulatory molecules to in turn activate both CD4+ and CD8+ T cells (117). In a mouse model study, expression of RANKL was detected on the surface of activated CD4+ and CD8+ T cells (118). Conversely, inhibition by a RANKL inhibitor suppresses activation of T cells, suggesting the role of RANK/RANKL pathway in T cell activation. Meanwhile, OCs are capable of inducing differentiation of CD8+ T cells into FoxP3+ CD8+ Tregs, which not only decrease

antigen-specific T cell proliferation but also suppress bone resorption by forming a negative feedback loop (119–123). In a similar fashion, adoptive transfer of CD4+CD25+ Tregs into T-cell deficient mice enhances bone mass formation accompanied by decreased OC numbers, partially mediated by IL-4 and IL-10 (124). In addition, isolated human Tregs suppress OC differentiation *via* the secretion of TGF-β and IL-4 (125). CD4+CD25+Foxp3+ Treg can also inhibit differentiation of OCs by cytotoxic T lymphocyte antigen 4 (CTLA4) in a cell-to-cell contact-dependent manner (126, 127). Specifically, CTLA4 on Tregs downregulates proliferation of OCs by binding to CD80/86 on OC precursors (128). The engagement of CD80/86 by CTLA-4 in OC precursors activates IDO, which in turn further degrades tryptophan and induces apoptosis of OC precursors.

A recent study in a mouse model showed that OCs derived from normal BM can induce CD4+FoxP3+ regulatory T cells (129). On the contrary, OCs can induce TNFα-producing CD4+ T cells in an inflammatory bowel disease mouse model (129). All these findings suggest that OCs not only play a role in immune suppression, but also serve as true APCs depending on the origin and environment.

PD-L1 EXPRESSION ON MM CELLS AND OCs

Programmed cell death protein 1/PD-L1 pathway contributes to tumor progression and survival by escaping tumor neutralizing immune surveillance in the tumor microenvironment (130). PD-L1 has been linked to the maintenance of Tregs, which are associated with suppression of antitumor immune response (131). The expression of PD-L1 on tumor cells can be enhanced by IFNγ secreted by activated cytotoxic T cells in the tumor microenvironment, thereby downregulating antitumor immunity (132). In addition, PD-L1 expression can be altered by extrinsic factors like inflammatory cytokines, which induce signaling cascades including MEK/ERK, PTEN, mTOR, or PI3K pathways (133–135).

In MM, PD-L1 is expressed on PCs isolated from patients with MM and MM cell lines, but not on normal PCs (20, 133, 136–138). The percentages of PD-L1 + PCs are higher on MM and smoldering MM than MGUS (133). Increased PD-L1 levels in MGUS patients is further linked to a higher risk of progression to clinical MM (139). PD-L1 expression on MM cells is enhanced following stimulation of IFNγ *via* activation of MYD88, TRAF6, and MEK/ERK signaling pathways; conversely, MEK1/2 inhibitors partially block IFNγ-induced PD-L1 upregulation (20, 133). BMSCs also induce expression of PD-L1 on MM cells by production of IL-6 *via* signal transducer and activator of transcription 3, MEK1/2, or Janus kinase 2 (140, 141). In addition, MM cells with PD-L1 expression are correlated with higher proliferation rate and higher expression of BCL-2 and FasL than MM cells without PD-L1 expression. Moreover, the interaction between PD-L1 on MM cells and PD-1 not only inhibited tumor-specific cytotoxic T cells but also promoted drug resistance in myeloma cells through the PI3K/AKT signaling cascade (53). Importantly, higher serum level of soluble PD-L1 in MM patients is associated with shorter progression-free survival (142).

Programmed cell death ligand 1 is expressed on multiple immune cell subsets in the MM BM microenvironment, including pDCs (137, 143), MDSCs (141), and OCs (20). Specifically, PD-L1 on pDCs is overexpressed in 81% of cases (143). Expression of PD-L1 is significantly higher on the CD141+ subset, which regulates immune response of CD8+ T cells, than on the CD141-negative CD4+ T cells. PD-L1 on immunosuppressive MDSCs is increased in patients with RRMM compared with newly diagnosed MM (141). Significantly, blockade of PD-1/PD-L1 pathway inhibits MDSC-mediated growth of MM cells. Furthermore, BM mesenchymal stem cells promote proliferation and reduce apoptosis of MM cells by suppressing T-cell immune responses *via* PD-1/PD-L1 pathway (144).

Furthermore, OCs induce expression of PD-L1 on MM cells in an APRIL-dependent manner *via* binding of two APRIL receptors (BCMA and TACI), which are highly expressed on MM cells (20, 145) (**Figure 3**). Since OCs are the key physiological source of APRIL production in the BM microenvironment, these results further provide evidence of a positive feedback loop between OCs and MM cells in promoting PD-L1-mediated immunosuppression in MM. Meanwhile, increased PD-L1 expression on OCs further enhances immunosuppression by promoting the binding of PD-1 on T cells and inducing dysfunction and apoptosis of effector T cells (20).

Some current and emerging anti-MM agents can affect the expression of PD-L1 on MM cells (**Table 1**). For example, proteasome inhibitors, oncolytic reovirus, and a histone deacetylase inhibitor 6 (HDAC) inhibitor have been shown to enhance PD-L1 expression on MM cells (146–148). On the other hand, lenalidomide and MEK1/2 inhibitors, as well as APRIL blocking reagents, reduce PD-L1 induction on MM cells (20, 133, 141, 145, 149). These findings support further investigations targeting PD-L1 in MM.

TARGETING PD-1/PD-L1 PATHWAY WITH VARIOUS CURRENT AND NOVEL MM TREATMENTS

Preclinical Studies

The combination of hematopoietic stem cell transplantation with whole-cell vaccination and PD-L1 blockade significantly improves the survival of MM-bearing mice (136). In another study where anti-MM activity is mainly mediated by pre-activated T cells, the combination of anti-PD-L1 inhibitor plus lymphodepletion by sublethal dose of radiation augments T-cell-mediated anti-MM effect and significantly improves survival of mice (54).

A combination of PD-1/PD-L1 blockade with IMiDs was also investigated in a study where NK cells or T cells were cocultured with CD138+ tumor cells isolated from MM patients and treated with PD-1or PD-L1 inhibitor, alone or together, and with lenalidomide (141). The immune checkpoint blockade by PD-1 or PD-L1, or PD-1/PD-L1 inhibitor combination, induced effector cell-mediated anti-MM cytotoxicity (137, 141). The expression of PD-1 and PD-L1 on effector cells and MM cells was downregulated by lenalidomide. Lenalidomide further augments anti-MM cytotoxicity mediated by checkpoint

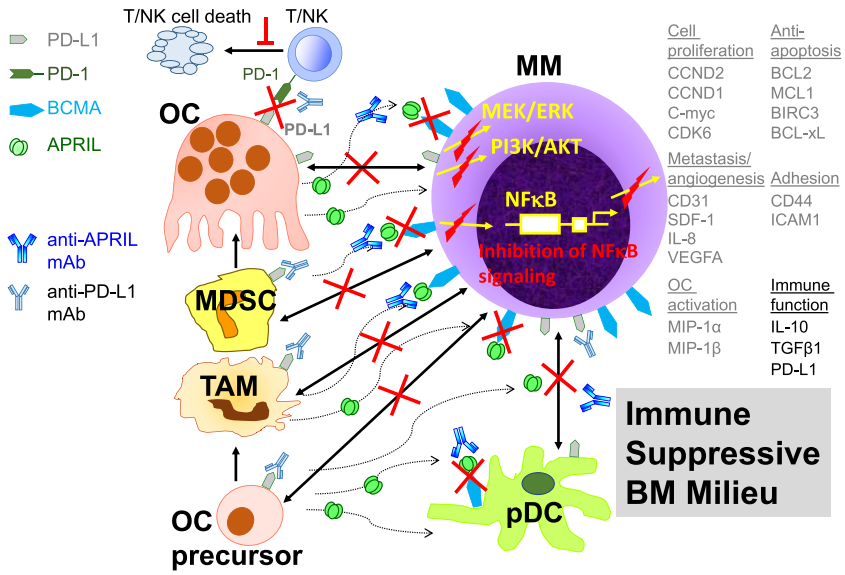

FIGURE 3 | Targeting a proliferation-inducing ligand (APRIL) and programmed cell death ligand 1 (PD-L1) to overcome OC-mediated immune suppression in the multiple myeloma (MM) BM milieu. APRIL is secreted by myeloid lineage cells including OCs, OC precursors, tumor-associated macrophages, and MDSCs, in the BM. MDSCs induced by MM cells further differentiate into functional OCs. Besides the induction of critical downstream targets (listed in gray on the right), APRIL induces PD-L1 on MM cells *via* BCMA, a specific MM antigen. This positive feedback loop between MM cells and MM-supporting cells, coupled with increased PD-L1 expression, further inhibits effector-mediated MM cell lysis *via* binding to programmed cell death protein 1 (PD-1) on activated T and natural killer (NK) effector cells. Blocking PD-1/PD-L1 and APRIL monoclonal antibodies prevent these effects and may mitigate immune suppression in MM. Adapted from Ref. (20, 145). TAM, tumor-associated antigen; MDSC, myeloid-derived suppressor cells; BCMA, B cell maturation antigen; T/NK, immune effector cells; pDC, plasmacytoid dendritic cell. Red lines indicate inhibition in the presence of these blocking monoclonal antibodies; double arrow lines depict interactions.

blockade dependent on NK and T effector cells. In addition, PD-1 inhibitor enhances production of INF-γ and granzyme B from NK cells against MM cells. Treatment with lenalidomide further upregulates PD-1 inhibitor's enhancement of NK-cell lysis of MM cells (150).

When combined with HDAC6 inhibitor, anti-PD-L1 antibody can trigger even higher MM cell killing mediated by NK and cytotoxic T cells, compared with killing in the presence of either agent alone (148). In addition, oncolytic reovirus enhances expression of PD-L1 on MM cells and augments the anti-MM effect of anti-PD-L1 inhibitor (147). Furthermore, since T-cell dependent bispecific antibody (TDB) induces the expression of PD-1 on CD8+ T cells following the engagement of T cells and target MM cells, treatment with PD-L1 inhibitor could enhance anti-MM activity of MM targeted TDB, as recently shown using an anti-FcRH5/CD3 TDB (151).

Clinical Studies

In a phase 1b study, monotherapy with PD-1 inhibitor nivolumab was administered in RRMM patients (152); however, no obvious disease regression was observed. The preliminary data from a phase 1 study, which investigated anti-PD1 antibody pembrolizumab in combination with lenalidomide and low-dose dexamethasone in patients with RRMM showed high response rate (76%) (153). Another phase 2 trial combining pembrolizumab, pomalidomide, and dexamethasone in RRMM patient also showed high response rate (60%) (154). This study further showed that higher PD-L1 expression on MM is linked to better progression-free survival. Importantly, however, there were more deaths in phase III trials in the cohorts comparing lenalidomide or pomalidomide with dexamethasone together with pembrolizumab than in patients treated with lenalidomide or pomalidomide with dexamethasone, which has curtailed the development of IMiD pembrolizumab combinations.

Regarding clinical trials of PD-L1 antibodies, single agent durvalumab or the combination of durvalumab with lenalidomide (NCT02685826) is being evaluated in patients with newly diagnosed MM. Durvalumab, alone or combined with pomalidomide (NCT02616640); as well as durvalumab in combination with daratumumab or in combination with pomalidomide, dexamethasone, and daratumumab (NCT02807454) are also clinical trials in RRMM patients. However, these trials are currently not actively recruiting patients for the time being due to the above-mentioned safety concern. Nonetheless clinical trials of another anti-PD-L1 antibody, atezolizumab are ongoing in patients with RRMM (NCT02431208).

In addition to direct blockade of PD-1/PD-L1 by PD-1 or PD-L1 inhibitor, novel therapeutic interventions, which modulate the expression of PD-L1 on MM cells are under clinical evaluation in RRMM patients, including the combination of oncolytic reovirus with lenalidomide or pomalidomide (NCT03015922), or oncolytic reovirus with bortezomib and dexamethasone (NCT02514382). Moreover, HDAC6 or MEK inhibitors are also under clinical investigation to potentially modulate expression pattern of PD-1 and PD-L1. The studies of PD-L1 inhibitors or PD-L1 modulators are listed in **Table 1**.

TABLE 1 | Summary of trials of programmed cell death ligand 1 (PD-L1) inhibitors and treatment, which modulates PD-L1 expression.

Agents	Effect on PD-L1 in multiple myeloma (MM) cells	Clinical trials in MM
Atezolizumab	Direct inhibition	1. Alone or in combination with an IMiD and/or daratumumab in relapse and refractory MM (RRMM) patients, phase 1 (NCT02431208). Status: recruiting
Durvalumab	Direct inhibition	1. Monotherapy or in combination with pomalidomide with or without dexamethasone in RRMM patients, phase 1 (NCT02616640). Status: active, not recruiting 2. Combination of durvalumab with lenalidomide with or without dexamethasone in newly diagnosed MM patients, phase 1 (NCT02685826). Status: active, not recruiting 3. Combination of durvalumab with daratumumab with or without pomalidomide and dexamethasone in RRMM patients, phase 2 (NCT02807454). Status: active, not recruiting
Proteasome inhibitor	Upregulation	1. Bortezomib with oncolytic reovirus and dexamethasone in RRMM patients (NCT02514382). Status: recruiting
Oncolytic reovirus	Upregulation	1. Bortezomib with oncolytic reovirus and dexamethasone in RRMM patients (NCT02514382). Status: recruiting 2. Combined with lenalidomide or pomalidomide in RRMM patients, phase 1 (NCT03015922). Status: recruiting
HDAC6 inhibitors	Upregulation in MM cells	Ricolinostat (ACY-241): 1. Combination with pomalidomide and dexamethasone in RRMM patients, phase 1b/2 (NCT01997840). Status: active, not recruiting
MEK1/2 inhibitor	Downregulation	1. Binimetinib with encorafenib in RRMM patients with BRAFV600 E or BRAFV600K mutation, phase 2 (NCT02834364). Status: recruiting
BTK inhibitor	Downregulation	1. Ibrutinib with carfilzomib and dexamethasone in RRMM patients, phase 1/2 (NCT01962792). Status: active, not recruiting 2. Ibrutinib with pomalidomide, and dexamethasone in RRMM patients: phase 1/2 (NCT02548962). Status: active, not recruiting 3. Ibrutinib, bortezomib, and dexamethasone in RRMM patients, phase 2 (NCT02902965). Status: active, not recruiting
A proliferation-inducing ligand (APRIL) inhibitor	Downregulation	BION-1301 in RRMM patients, phase 1/2 (NCT03340883). Status: recruiting
APRIL CAR T cells	Downregulation	RRMM patients, phase 1/2 (NCT03287804). Status: recruiting
BCMA CAR T cells	Downregulation	1. bb2121 in RRMM patients, phase 1 (NCT02658929). Status: active, not recruiting. 2. bb2121 in RRMM patients, phase 2 (NCT03361748). Status: recruiting. 3. Anti-BCMA CAR-T for heavily pretreated MM patients, phase 1 (NCT02215967). Status: active, not recruiting. 4. Combination of anti-BCMA CAR-T with lenalidomide in RRMM patients, phase 1 (NCT03070327). Status: recruiting.

THERAPEUTIC INTERVENTIONS TARGETING OCs IN MM THERAPIES

Many novel agents have been under evaluation not only for their direct anti-MM activity but also their abilities to abrogate MM-supporting activities in the BM microenvironment, including targeting of OCs. They include bortezomib and IMiDs, which are already standard anti-MM therapies, as well as several novel agents showing promising results in preclinical studies (**Table 2**).

Bortezomib, as a proteasome inhibitor, not only has direct anti-MM activity, but also targets cells associated with MM bone disease. Bortezomib induces dose-dependent growth inhibition and apoptosis, as well as blocks differentiation, of OCs. It further decreases the resorption capacity of mature OCs, reduces the total number of functional OCs, and increases differentiation of OBs (155–157). In addition to the induction of differentiation and growth of OBs, therapeutic proteasome inhibitors bortezomib and carfilzomib promote bone nodule formation, associated with reduced levels of DKK-1 and RANKL (158–160). Bortezomib preferentially induces differentiation of mesenchymal stem/progenitor cells to OBs by regulating expression of the bone-specifying transcription factor runt-related transcription factor 2 in a mouse model (161).

Immunomodulatory drugs inhibit formation of OCs by inhibiting PU.1 and pERK (2, 162). Cathepsin K, an important molecule in bone collagen matrix resorption, and the serum level of RANKL and RANKL/OPG ratio are significantly reduced in MM patients receiving lenalidomide treatment. Furthermore, lenalidomide and pomalidomide normalize RANKL/OPG ratio and inhibit upregulation of RANKL by downregulating adhesion molecules on MM cells (163).

Bisphosphonate is routinely used in MM bone disease treatment to reduce risk of skeletal events (164, 165). Bisphosphonate has high affinity for bone mineral surfaces at sites of active bone remodeling by OCs. It induces apoptosis of OCs while protecting OBs from apoptosis, in addition to blocking differentiation and maturation of OCs (2, 166, 167).

TABLE 2 | Summary of therapeutic agents targeting osteoclasts (OCs) and other cells associated with multiple myeloma bone disease.

Agents	Mechanisms	Reference
Proteasome inhibitor	1. Induce apoptosis and block differentiation of OCs 2. Increase differentiation of OB 3. Reduced level of DKK-1 and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)	(94, 155, 157, 159, 160)
Immunomodulatory drugs	1. Targeting PU.1 and pERK to inhibit formation of OC 2. Normalize RANKL/osteoprotegerin ratio	(162, 163)
Bisphosphonate	1. Induce OC apoptosis but protect OB from apoptosis 2. Block differentiation and maturation of OC	(166, 167)
RANKL inhibitor	1. Decrease OC formation and activity. 2. Minimal or stimulatory effects on OB.	(18, 168, 169)
BTK inhibitor	1. Suppress bone resorption and differentiation of OCs 2. Inhibit secretion of multiple cytokines and chemokines from OCs and bone marrow stromal cells	(93, 94)
Anti-CD38 antibody	1. Inhibition of OC formation and bone resorption 2. Overcome the inhibition of T-cell proliferation blocked by OCs 3. Inhibit immune checkpoint molecules on OCs	(20, 173)
Programmed cell death protein 1/programmed cell death ligand 1 antibody	Block OC-mediated inhibition in T-cell activation and proliferation	(20)

Denosumab (AMG165), a fully human monoclonal antibody (IgG2), blocks the binding of RANKL to its receptor expressed on OCs and their precursors, leading to decreased OC activity and inhibition of bone resorption, followed by increased bone mass and strength (168, 169). Denosumab reduces bone resorption, increases mass of cortical and cancellous bone, and improves the microstructure of trabecular bone (170). A phase 3 clinical trial in MM has shown that denosumab is not inferior to zoledronic acid, the bisphosphonate most commonly used to reduce skeletal-related event in newly diagnosed MM patients (3, 171).

The development and integration of anti-CD38 monoclonal antibody is an important milestone in MM immunotherapy. In addition to MM cells, CD 38 is also expressed on normal PCs, NK cells, monocytes, early OC progenitors, and OCs, but not on the surface of stromal and osteoblastic cells (172, 173). Daratumumab inhibits OC formation and bone resorption (173). The inhibition of T-cell proliferation caused by OCs is partially overcome by anti-CD38 monoclonal antibody isatuximab (20) *via* inhibition of multiple immune checkpoint molecules expressed on OC. Since anti-PD-L1 partially overcomes inhibitory effects of OCs on T-cell activation and proliferation, these results suggest potential therapeutic benefit of combining CD38 and PD-1/PD-L1 mAbs to block OC-induced immunosuppression in MM.

PERSPECTIVES AND CONCLUSION

Programmed cell death protein 1/PD-L1 pathway plays a critical role in the immunosuppressive tumor microenvironment in MM. As PD-L1 is overexpressed in MM patient cells and other cells associated with immunosuppression including OCs, MDSCs, TAMs, Tregs, and pDCs, blockade of PD-1/PD-L1 pathway may confer an anti-MM effect by restoring the immune dysfunction. Early phase clinical trials in MM showed that blockade of PD-1/PD-L1 pathway alone does not achieve responses. Although combining PD-1 inhibitor with IMiDs (lenalidomide and pomalidomide) showed higher response rates in RRMM patients, clinical trials combining PD-1 inhibitors with IMiDs in MM are currently put on hold due to safety concerns.

On the other hand, anti-PD-L1 mAbs also show promising clinical benefit with acceptable safety profile in clinical trials of various solid tumors, leading to increasing interest in targeting PD-L1 in MM (174). Preclinical studies showed that treatment with anti-PD-L1 antibody induces no direct MM killing, but significantly restores the anti-MM activity of cytotoxic T cells or NK cells, suggesting that PD-L1 inhibitor might be a therapeutic partner with other anti-MM agents. Several combinations of molecules which either upregulate or downregulate expression of PD-L1 in combination with anti-MM agents are under evaluation (**Table 1**). Early phase clinical trials conducted with BCMA CAR-T therapy, HDAC6 inhibitors, and oncolytic reovirus in RRMM patients have shown preliminary promising results (175–177). Novel strategies targeting immune checkpoints and the OC-related pathway have also shown impressive results in preclinical studies. For example, the combination of RANKL and CTLA4 antibody enhances antitumor effect of lymphocytes (178). Blockade of RANKL pathway also augments the antitumor effect of PD1-PD-L1 blockade or dual PD1-PD-L1 and CTLA4 blockade in an animal model (179). Since RANKL inhibitor is now used in MM patients with bone disease, combinations with above agent represent potential novel therapeutic strategies. Finally, preclinical data combining CD38 with PD-1 and/or PD-L1 mAbs provides the rationale for clinical evaluation of these combinations. These various combination therapies may overcome primary and acquired resistance to anti-PD-1/PD-L1 therapies in MM.

An effective anti-MM immunotherapy not only relies on effective killing of MM cells themselves, but also on successfully restoring anticancer immune function. Immunotherapy targeting PD-1/PD-L1 pathway has revolutionized the treatment in several progressive solid tumors but is accompanied by

immune-related adverse events in some patients. For anti-PD-1/PD-L1 immunotherapy to proceed in MM, it will be critical to investigate both the direct effects on tumor cells, as well as the impact on cellular- and cytokine-mediated immunosuppression in the MM microenvironment. Moreover, delineating molecular mechanisms regulating PD-L1 and PD-1 expression in the MM BM milieu will identify novel targets for potential therapeutic application.

AUTHOR CONTRIBUTIONS

Y-TT and S-FC review literatures and design and write this paper. Y-TT and KA critically review and edit the paper.

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REFERENCES

1. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med* (2011) 364(11): 1046–60. doi:10.1056/NEJMra1011442
2. Raje N, Roodman GD. Advances in the biology and treatment of bone disease in multiple myeloma. *Clin Cancer Res* (2011) 17(6):1278–86. doi:10.1158/1078-0432.CCR-10-1804
3. Anderson K, Ismaila N, Flynn PJ, Halabi S, Jagannath S, Ogaily MS, et al. Role of bone-modifying agents in multiple myeloma: American Society of Clinical Oncology Clinical Practice Guideline Update. *J Clin Oncol* (2018) 36(8):812–8. doi:10.1200/JCO.2017.76.6402
4. Rajkumar SV, Blood E, Vesole D, Fonseca R, Greipp PR; Eastern Cooperative Oncology G. Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. *J Clin Oncol* (2006) 24(3):431–6. doi:10.1200/JCO.2005.03.0221
5. San Miguel JF, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, Kropff M, et al. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. *N Engl J Med* (2008) 359(9):906–17. doi:10.1056/NEJMoa0801479
6. Richardson PG, Weller E, Lonial S, Jakubowiak AJ, Jagannath S, Raje NS, et al. Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. *Blood* (2010) 116(5): 679–86. doi:10.1182/blood-2010-02-268862
7. Benboubker L, Dimopoulos MA, Dispenzieri A, Catalano J, Belch AR, Cavo M, et al. Lenalidomide and dexamethasone in transplant-ineligible patients with myeloma. *N Engl J Med* (2014) 371(10):906–17. doi:10.1056/NEJMoa1402551
8. Kumar SK, Anderson KC. Immune therapies in multiple myeloma. *Clin Cancer Res* (2016) 22(22):5453–60. doi:10.1158/1078-0432.CCR-16-0868
9. Mateos MV, Dimopoulos MA, Cavo M, Suzuki K, Jakubowiak A, Knop S, et al. Daratumumab plus bortezomib, melphalan, and prednisone for untreated myeloma. *N Engl J Med* (2018) 378(6):518–28. doi:10.1056/NEJMoa1714678
10. Jiang H, Acharya C, An G, Zhong M, Feng X, Wang L, et al. SAR650984 directly induces multiple myeloma cell death via lysosomal-associated and apoptotic pathways, which is further enhanced by pomalidomide. *Leukemia* (2016) 30(2):399–408. doi:10.1038/leu.2015.240
11. Martin T, Baz R, Benson DM, Lendvai N, Wolf J, Munster P, et al. A phase 1b study of isatuximab plus lenalidomide and dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 129(25):3294–303. doi:10.1182/blood-2016-09-740787
12. Richardson PG, Attal M, Campana F, Le-Guennec S, Hui AM, Risse ML, et al. Isatuximab plus pomalidomide/dexamethasone versus pomalidomide/dexamethasone in relapsed/refractory multiple myeloma: ICARIA phase III study design. *Future Oncol* (2018) 14(11):1035–47. doi:10.2217/fon-2017-0616
13. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer* (2002) 2(12):927–37. doi:10.1038/nrc952
14. Kawano Y, Moschetta M, Manier S, Glavey S, Gorgun GT, Roccaro AM, et al. Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev* (2015) 263(1):160–72. doi:10.1111/imr.12233
15. Fowler JA, Mundy GR, Lwin ST, Edwards CM. Bone marrow stromal cells create a permissive microenvironment for myeloma development: a new stromal role for Wnt inhibitor Dkk1. *Cancer Res* (2012) 72(9):2183–9. doi:10.1158/0008-5472.CAN-11-2067
16. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* (2004) 350(16):1655–64. doi:10.1056/NEJMra030831
17. Tai YT, Fulciniti M, Hideshima T, Song W, Leiba M, Li XF, et al. Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis. *Blood* (2007) 110(5):1656–63. doi:10.1182/blood-2007-03-081240
18. Tai YT, Landesman Y, Acharya C, Calle Y, Zhong MY, Cea M, et al. CRM1 inhibition induces tumor cell cytotoxicity and impairs osteoclastogenesis in multiple myeloma: molecular mechanisms and therapeutic implications. *Leukemia* (2014) 28(1):155–65. doi:10.1038/leu.2013.115
19. Raje N, Woo SB, Hande K, Yap JT, Richardson PG, Vallet S, et al. Clinical, radiographic, and biochemical characterization of multiple myeloma patients with osteonecrosis of the jaw. *Clin Cancer Res* (2008) 14(8):2387–95. doi:10.1158/1078-0432.CCR-07-1430
20. An G, Acharya C, Feng X, Wen K, Zhong M, Zhang L, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. *Blood* (2016) 128(12):1590–603. doi:10.1182/blood-2016-03-707547
21. Brimnes MK, Vangsted AJ, Knudsen LM, Gimsing P, Gang AO, Johnsen HE, et al. Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand J Immunol* (2010) 72(6):540–7. doi:10.1111/j.1365-3083.2010.02463.x
22. Gorgun GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood* (2013) 121(15):2975–87. doi:10.1182/blood-2012-08-448548
23. De Beule N, De Veirman K, Maes K, De Bruyne E, Menu E, Breckpot K, et al. Tumour-associated macrophage-mediated survival of myeloma cells through STAT3 activation. *J Pathol* (2017) 241(4):534–46. doi:10.1002/path.4860
24. Braga WM, da Silva BR, de Carvalho AC, Maekawa YH, Bortoluzzo AB, Rizzatti EG, et al. FOXP3 and CTLA4 overexpression in multiple myeloma bone marrow as a sign of accumulation of CD4(+) T regulatory cells. *Cancer Immunol Immunother* (2014) 63(11):1189–97. doi:10.1007/s00262-014-1589-9
25. Feng X, Zhang L, Acharya C, An G, Wen K, Qiu L, et al. Targeting CD38 suppresses induction and function of T regulatory cells to mitigate immunosuppression in multiple myeloma. *Clin Cancer Res* (2017) 23(15):4290–300. doi:10.1158/1078-0432.CCR-16-3192
26. Chauhan D, Singh AV, Brahmandam M, Carrasco R, Bandi M, Hideshima T, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell* (2009) 16(4):309–23. doi:10.1016/j.ccr.2009.08.019
27. Zhang L, Tai YT, Ho M, Xing L, Chauhan D, Gang A, et al. Regulatory B cell-myeloma cell interaction confers immunosuppression and promotes their survival in the bone marrow milieu. *Blood Cancer J* (2017) 7(3):e547. doi:10.1038/bcj.2017.24

28. Roodman GD. Role of the bone marrow microenvironment in multiple myeloma. *J Bone Miner Res* (2002) 17(11):1921–5. doi:10.1359/jbmr.2002.17.11.1921
29. Tai YT, Dillon M, Song W, Leiba M, Li XF, Burger P, et al. Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood* (2008) 112(4):1329–37. doi:10.1182/blood-2007-08-107292
30. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* (2006) 12(6):657–64. doi:10.1038/nm1417
31. Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med* (2012) 209(3):537–49. doi:10.1084/jem.20110994
32. Lawson MA, McDonald MM, Kovacic N, Hua Khoo W, Terry RL, Down J, et al. Osteoclasts control reactivation of dormant myeloma cells by remodelling the endosteal niche. *Nat Commun* (2015) 6:8983. doi:10.1038/ncomms9983
33. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* (2007) 7(4):292–304. doi:10.1038/nri2062
34. Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. *Endocr Rev* (2008) 29(4):403–40. doi:10.1210/er.2007-0038
35. Charles JF, Nakamura MC. Bone and the innate immune system. *Curr Osteoporos Rep* (2014) 12(1):1–8. doi:10.1007/s11914-014-0195-2
36. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* (1999) 5(12):1365–9. doi:10.1038/70932
37. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest* (2015) 125(9):3384–91. doi:10.1172/JCI80011
38. Nurieva R, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, et al. T-cell tolerance or function is determined by combinatorial costimulatory signals. *EMBO J* (2006) 25(11):2623–33. doi:10.1038/sj.emboj.7601146
39. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* (2000) 192(7):1027–34. doi:10.1084/jem.192.7.1027
40. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev* (2009) 229(1):114–25. doi:10.1111/j.1600-065X.2009.00767.x
41. Chen DS, Irving BA, Hodi FS. Molecular pathways: next-generation immunotherapy – inhibiting programmed death-ligand 1 and programmed death-1. *Clin Cancer Res* (2012) 18(24):6580–7. doi:10.1158/1078-0432.CCR-12-1362
42. Sun Z, Fourcade J, Pagliano O, Chauvin JM, Sander C, Kirkwood JM, et al. IL10 and PD-1 cooperate to limit the activity of tumor-specific CD8+ T cells. *Cancer Res* (2015) 75(8):1635–44. doi:10.1158/0008-5472.CAN-14-3016
43. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* (2007) 27(1):111–22. doi:10.1016/j.immuni.2007.05.016
44. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* (2003) 198(1):63–9. doi:10.1084/jem.20022125
45. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) 236:219–42. doi:10.1111/j.1600-065X.2010.00923.x
46. Lazar-Molnar E, Gacser A, Freeman GJ, Almo SC, Nathenson SG, Nosanchuk JD. The PD-1/PD-L costimulatory pathway critically affects host resistance to the pathogenic fungus *Histoplasma capsulatum*. *Proc Natl Acad Sci U S A* (2008) 105(7):2658–63. doi:10.1073/pnas.0711918105
47. Wu P, Wu D, Li L, Chai Y, Huang J. PD-L1 and survival in solid tumors: a meta-analysis. *PLoS One* (2015) 10(6):e0131403. doi:10.1371/journal.pone.0131403
48. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A* (2007) 104(9):3360–5. doi:10.1073/pnas.0611533104
49. Huang B, Chen L, Bao C, Sun C, Li J, Wang L, et al. The expression status and prognostic significance of programmed cell death 1 ligand 1 in gastrointestinal tract cancer: a systematic review and meta-analysis. *Onco Targets Ther* (2015) 8:2617–25. doi:10.2147/OTT.S91025
50. Errico A. Immunotherapy: PD-1-PD-L1 axis: efficient checkpoint blockade against cancer. *Nat Rev Clin Oncol* (2015) 12(2):63. doi:10.1038/nrclinonc.2014.221
51. Pianko MJ, Moskowitz AJ, Lesokhin AM. Immunotherapy of lymphoma and myeloma: facts and hopes. *Clin Cancer Res* (2018) 24(5):1002–10. doi:10.1158/1078-0432.CCR-17-0539
52. Xu-Monette ZY, Zhou J, Young KH. PD-1 expression and clinical PD-1 blockade in B-cell lymphomas. *Blood* (2018) 131(1):68–83. doi:10.1182/blood-2017-07-740993
53. Ishibashi M, Tamura H, Sunakawa M, Kondo-Onodera A, Okuyama N, Hamada Y, et al. Myeloma drug resistance induced by binding of myeloma B7-H1 (PD-L1) to PD-1. *Cancer Immunol Res* (2016) 4(9):779–88. doi:10.1158/2326-6066.CIR-15-0296
54. Kearl TJ, Jing W, Gershan JA, Johnson BD. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. *J Immunol* (2013) 190(11):5620–8. doi:10.4049/jimmunol.1202005
55. Rosenblatt J, Avigan D. Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality? *Blood* (2017) 129(3):275–9. doi:10.1182/blood-2016-08-731885
56. Teitelbaum SL. Bone resorption by osteoclasts. *Science* (2000) 289(5484):1504–8. doi:10.1126/science.289.5484.1504
57. Bataille R, Chappard D, Marcelli C, Dessauw P, Sany J, Baldet P, et al. Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. *J Clin Oncol* (1989) 7(12):1909–14. doi:10.1200/JCO.1989.7.12.1909
58. Dimopoulos MA, Moulopoulos LA, Datseris I, Weber D, Delasalle K, Gika D, et al. Imaging of myeloma bone disease – implications for staging, prognosis and follow-up. *Acta Oncol* (2000) 39(7):823–7. doi:10.1080/028418600750063578
59. Dimopoulos M, Terpos E, Comenzo RL, Tosi P, Beksac M, Sezer O, et al. International myeloma working group consensus statement and guidelines regarding the current role of imaging techniques in the diagnosis and monitoring of multiple myeloma. *Leukemia* (2009) 23(9):1545–56. doi:10.1038/leu.2009.89
60. Kyle RA. Multiple myeloma: review of 869 cases. *Mayo Clin Proc* (1975) 50(1):29–40.
61. Sonmez M, Akagun T, Topbas M, Cobanoglu U, Sonmez B, Yilmaz M, et al. Effect of pathologic fractures on survival in multiple myeloma patients: a case control study. *J Exp Clin Cancer Res* (2008) 27:11. doi:10.1186/1756-9966-27-11
62. D'Souza S, del Prete D, Jin S, Sun Q, Huston AJ, Kostov FE, et al. Gfi1 expressed in bone marrow stromal cells is a novel osteoblast suppressor in patients with multiple myeloma bone disease. *Blood* (2011) 118(26):6871–80. doi:10.1182/blood-2011-04-346775
63. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* (2001) 97(2):346–51. doi:10.1182/blood.V97.2.346
64. Gunn WG, Conley A, Deininger L, Olson SD, Prockop DJ, Gregory CA. A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma. *Stem Cells* (2006) 24(4):986–91. doi:10.1634/stemcells.2005-0220
65. Giuliani N, Colla S, Rizzoli V. New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). *Exp Hematol* (2004) 32(8):685–91. doi:10.1016/j.exphem.2004.03.015
66. Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD, et al. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood* (2000) 96(2):671–5.
67. Lee JW, Chung HY, Ehrlich LA, Jelinek DF, Callander NS, Roodman GD, et al. IL-3 expression by myeloma cells increases both osteoclast formation

and growth of myeloma cells. *Blood* (2004) 103(6):2308–15. doi:10.1182/blood-2003-06-1992
68. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. *Blood* (1993) 82(12):3712–20.
69. Zannettino AC, Farrugia AN, Kortesidis A, Manavis J, To LB, Martin SK, et al. Elevated serum levels of stromal-derived factor-1alpha are associated with increased osteoclast activity and osteolytic bone disease in multiple myeloma patients. *Cancer Res* (2005) 65(5):1700–9. doi:10.1158/0008-5472.CAN-04-1687
70. Colucci S, Brunetti G, Rizzi R, Zonno A, Mori G, Colaianni G, et al. T cells support osteoclastogenesis in an in vitro model derived from human multiple myeloma bone disease: the role of the OPG/TRAIL interaction. *Blood* (2004) 104(12):3722–30. doi:10.1182/blood-2004-02-0474
71. Tai YT, Li XF, Breitkreutz I, Song W, Neri P, Catley L, et al. Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* (2006) 66(13):6675–82. doi:10.1158/0008-5472.CAN-06-0190
72. Hemingway F, Taylor R, Knowles HJ, Athanasou NA. RANKL-independent human osteoclast formation with APRIL, BAFF, NGF, IGF I and IGF II. *Bone* (2011) 48(4):938–44. doi:10.1016/j.bone.2010.12.023
73. Nguyen AN, Stebbins EG, Henson M, O'Young G, Choi SJ, Quon D, et al. Normalizing the bone marrow microenvironment with p38 inhibitor reduces multiple myeloma cell proliferation and adhesion and suppresses osteoclast formation. *Exp Cell Res* (2006) 312(10):1909–23. doi:10.1016/j.yexcr.2006.02.026
74. Fu J, Li S, Feng R, Ma H, Sabeh F, Roodman GD, et al. Multiple myeloma-derived MMP-13 mediates osteoclast fusogenesis and osteolytic disease. *J Clin Invest* (2016) 126(5):1759–72. doi:10.1172/JCI80276
75. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood* (2004) 104(8):2484–91. doi:10.1182/blood-2003-11-3839
76. Moreaux J, Cremer FW, Reme T, Raab M, Mahtouk K, Kaukel P, et al. The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. *Blood* (2005) 106(3):1021–30. doi:10.1182/blood-2004-11-4512
77. D'Souza S, Kurihara N, Shiozawa Y, Joseph J, Taichman R, Galson DL, et al. Annexin II interactions with the annexin II receptor enhance multiple myeloma cell adhesion and growth in the bone marrow microenvironment. *Blood* (2012) 119(8):1888–96. doi:10.1182/blood-2011-11-393348
78. Sprynski AC, Hose D, Caillot L, Reme T, Shaughnessy JD Jr, Barlogie B, et al. The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. *Blood* (2009) 113(19):4614–26. doi:10.1182/blood-2008-07-170464
79. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med* (2003) 349(26):2483–94. doi:10.1056/NEJMoa030847
80. Standal T, Abildgaard N, Fagerli UM, Stordal B, Hjertner O, Borset M, et al. HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma. *Blood* (2007) 109(7):3024–30. doi:10.1182/blood-2006-07-034884
81. Jost E, Gezer D, Wilop S, Suzuki H, Herman JG, Osieka R, et al. Epigenetic dysregulation of secreted Frizzled-related proteins in multiple myeloma. *Cancer Lett* (2009) 281(1):24–31. doi:10.1016/j.canlet.2009.02.002
82. Vallet S, Mukherjee S, Vaghela N, Hideshima T, Fulciniti M, Pozzi S, et al. Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease. *Proc Natl Acad Sci U S A* (2010) 107(11):5124–9. doi:10.1073/pnas.0911929107
83. Terpos E, Christoulas D, Katodritou E, Bratengeier C, Gkotzamanidou M, Michalis E, et al. Elevated circulating sclerostin correlates with advanced disease features and abnormal bone remodeling in symptomatic myeloma: reduction post-bortezomib monotherapy. *Int J Cancer* (2012) 131(6):1466–71. doi:10.1002/ijc.27342
84. Giuliani N, Rizzoli V, Roodman GD. Multiple myeloma bone disease: pathophysiology of osteoblast inhibition. *Blood* (2006) 108(13):3992–6. doi:10.1182/blood-2006-05-026112
85. Pearse RN, Sordillo EM, Yaccoby S, Wong BR, Liau DF, Colman N, et al. Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. *Proc Natl Acad Sci U S A* (2001) 98(20):11581–6. doi:10.1073/pnas.201394498
86. Jakob C, Goerke A, Terpos E, Sterz J, Heider U, Kuhnhardt D, et al. Serum levels of total-RANKL in multiple myeloma. *Clin Lymphoma Myeloma* (2009) 9(6):430–5. doi:10.3816/CLM.2009.n.085
87. Farrugia AN, Atkins GJ, To LB, Pan B, Horvath N, Kostakis P, et al. Receptor activator of nuclear factor-kappaB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. *Cancer Res* (2003) 63(17):5438–45.
88. Heider U, Langelotz C, Jakob C, Zavrski I, Fleissner C, Eucker J, et al. Expression of receptor activator of nuclear factor kappaB ligand on bone marrow plasma cells correlates with osteolytic bone disease in patients with multiple myeloma. *Clin Cancer Res* (2003) 9(4):1436–40.
89. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* (2000) 275(3):768–75. doi:10.1006/bbrc.2000.3379
90. Terpos E, Szydlo R, Apperley JF, Hatjiharissi E, Politou M, Meletis J, et al. Soluble receptor activator of nuclear factor kappaB ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. *Blood* (2003) 102(3):1064–9. doi:10.1182/blood-2003-02-0380
91. Buckle CH, De Leenheer E, Lawson MA, Yong K, Rabin N, Perry M, et al. Soluble rank ligand produced by myeloma cells causes generalised bone loss in multiple myeloma. *PLoS One* (2012) 7(8):e41127. doi:10.1371/journal.pone.0041127
92. Moreaux J, Hose D, Kassambara A, Reme T, Moine P, Requirand G, et al. Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration. *Blood* (2011) 117(4):1280–90. doi:10.1182/blood-2010-04-279760
93. Tai YT, Chang BY, Kong SY, Fulciniti M, Yang G, Calle Y, et al. Bruton tyrosine kinase inhibition is a novel therapeutic strategy targeting tumor in the bone marrow microenvironment in multiple myeloma. *Blood* (2012) 120(9):1877–87. doi:10.1182/blood-2011-12-396853
94. Eda H, Santo L, Cirstea DD, Yee AJ, Scullen TA, Nemani N, et al. A novel Bruton's tyrosine kinase inhibitor CC-292 in combination with the proteasome inhibitor carfilzomib impacts the bone microenvironment in a multiple myeloma model with resultant antimyeloma activity. *Leukemia* (2014) 28(9):1892–901. doi:10.1038/leu.2014.69
95. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol* (2008) 180(9):5771–7. doi:10.4049/jimmunol.180.9.5771
96. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, et al. Restoring natural killer cell immunity against multiple myeloma in the era of new drugs. *Front Immunol* (2017) 8:1444. doi:10.3389/fimmu.2017.01444
97. Brunetti G, Rizzi R, Oranger A, Gigante I, Mori G, Taurino G, et al. LIGHT/TNFSF14 increases osteoclastogenesis and decreases osteoblastogenesis in multiple myeloma-bone disease. *Oncotarget* (2014) 5(24):12950–67. doi:10.18632/oncotarget.2633
98. Favaloro J, Brown R, Aklilu E, Yang S, Suen H, Hart D, et al. Myeloma skews regulatory T and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. *Leuk Lymphoma* (2014) 55(5):1090–8. doi:10.3109/10428194.2013.825905
99. Zhuang J, Zhang J, Lwin ST, Edwards JR, Edwards CM, Mundy GR, et al. Osteoclasts in multiple myeloma are derived from Gr-1+CD11b+myeloid-derived suppressor cells. *PLoS One* (2012) 7(11):e48871. doi:10.1371/journal.pone.0048871
100. Tucci M, Ciavarella S, Strippoli S, Brunetti O, Dammacco F, Silvestris F. Immature dendritic cells from patients with multiple myeloma are prone to osteoclast differentiation in vitro. *Exp Hematol* (2011) 39(7):773–83.e1. doi:10.1016/j.exphem.2011.04.006
101. Bolzoni M, Ronchetti D, Storti P, Donofrio G, Marchica V, Costa F, et al. IL21R expressing CD14(+)CD16(+) monocytes expand in multiple myeloma patients leading to increased osteoclasts. *Haematologica* (2017) 102(4):773–84. doi:10.3324/haematol.2016.153841

102. Tanaka Y, Abe M, Hiasa M, Oda A, Amou H, Nakano A, et al. Myeloma cell-osteoclast interaction enhances angiogenesis together with bone resorption: a role for vascular endothelial cell growth factor and osteopontin. *Clin Cancer Res* (2007) 13(3):816–23. doi:10.1158/1078-0432.CCR-06-2258
103. Cackowski FC, Anderson JL, Patrene KD, Choksi RJ, Shapiro SD, Windle JJ, et al. Osteoclasts are important for bone angiogenesis. *Blood* (2010) 115(1):140–9. doi:10.1182/blood-2009-08-237628
104. Lee YM, Fujikado N, Manaka H, Yasuda H, Iwakura Y. IL-1 plays an important role in the bone metabolism under physiological conditions. *Int Immunol* (2010) 22(10):805–16. doi:10.1093/intimm/dxq431
105. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, et al. IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol* (1990) 145(10):3297–303.
106. Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J Biol Chem* (2000) 275(7):4858–64. doi:10.1074/jbc.275.7.4858
107. Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* (2004) 15(1):49–60. doi:10.1016/j.cytogfr.2003.10.005
108. Karmakar S, Kay J, Gravallese EM. Bone damage in rheumatoid arthritis: mechanistic insights and approaches to prevention. *Rheum Dis Clin North Am* (2010) 36(2):385–404. doi:10.1016/j.rdc.2010.03.003
109. Kitaura H, Kimura K, Ishida M, Kohara H, Yoshimatsu M, Takano-Yamamoto T. Immunological reaction in TNF-alpha-mediated osteoclast formation and bone resorption in vitro and in vivo. *Clin Dev Immunol* (2013) 2013:181849. doi:10.1155/2013/181849
110. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. *Biochem Biophys Res Commun* (1999) 265(1):144–50. doi:10.1006/bbrc.1999.1623
111. Choi Y, Woo KM, Ko SH, Lee YJ, Park SJ, Kim HM, et al. Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8(+) T cells. *Eur J Immunol* (2001) 31(7):2179–88. doi:10.1002/1521-4141(200107)31:7<2179::AID-IMMU2179>3.0.CO;2-X
112. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) 203(12):2673–82. doi:10.1084/jem.20061775
113. Weitzmann MN, Cenci S, Rifas L, Brown C, Pacifici R. Interleukin-7 stimulates osteoclast formation by up-regulating the T-cell production of soluble osteoclastogenic cytokines. *Blood* (2000) 96(5):1873–8.
114. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* (1998) 93(2):165–76. doi:10.1016/S0092-8674(00)81569-X
115. Giuliani N, Colla S, Sala R, Moroni M, Lazzaretti M, La Monica S, et al. Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. *Blood* (2002) 100(13):4615–21. doi:10.1182/blood-2002-04-1121
116. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* (2000) 408(6812):600–5. doi:10.1038/35046102
117. Li H, Hong S, Qian J, Zheng Y, Yang J, Yi Q. Cross talk between the bone and immune systems: osteoclasts function as antigen-presenting cells and activate CD4+ and CD8+ T cells. *Blood* (2010) 116(2):210–7. doi:10.1182/blood-2009-11-255026
118. Senthilkumar R, Lee HW. CD137L- and RANKL-mediated reverse signals inhibit osteoclastogenesis and T lymphocyte proliferation. *Immunobiology* (2009) 214(2):153–61. doi:10.1016/j.imbio.2008.05.001
119. Kiesel JR, Buchwald ZS, Aurora R. Cross-presentation by osteoclasts induces FoxP3 in CD8+ T cells. *J Immunol* (2009) 182(9):5477–87. doi:10.4049/jimmunol.0803897
120. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* (2007) 445(7129):771–5. doi:10.1038/nature05543
121. Buchwald ZS, Kiesel JR, DiPaolo R, Pagadala MS, Aurora R. Osteoclast activated FoxP3+ CD8+ T-cells suppress bone resorption in vitro. *PLoS One* (2012) 7(6):e38199. doi:10.1371/journal.pone.0038199
122. Buchwald ZS, Kiesel JR, Yang C, DiPaolo R, Novack DV, Aurora R. Osteoclast-induced Foxp3+ CD8 T-cells limit bone loss in mice. *Bone* (2013) 56(1):163–73. doi:10.1016/j.bone.2013.05.024
123. Shashkova EV, Trivedi J, Cline-Smith AB, Ferris C, Buchwald ZS, Gibbs J, et al. Osteoclast-primed Foxp3+ CD8 T cells induce T-bet, eomesodermin, and IFN-gamma to regulate bone resorption. *J Immunol* (2016) 197(3):726–35. doi:10.4049/jimmunol.1600253
124. Zaiss MM, Sarter K, Hess A, Engelke K, Bohm C, Nimmerjahn F, et al. Increased bone density and resistance to ovariectomy-induced bone loss in FoxP3-transgenic mice based on impaired osteoclast differentiation. *Arthritis Rheum* (2010) 62(8):2328–38. doi:10.1002/art.27535
125. Kim YG, Lee CK, Nah SS, Mun SH, Yoo B, Moon HB. Human CD4+CD25+ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells. *Biochem Biophys Res Commun* (2007) 357(4):1046–52. doi:10.1016/j.bbrc.2007.04.042
126. Axmann R, Herman S, Zaiss M, Franz S, Polzer K, Zwerina J, et al. CTLA-4 directly inhibits osteoclast formation. *Ann Rheum Dis* (2008) 67(11):1603–9. doi:10.1136/ard.2007.080713
127. Zaiss MM, Axmann R, Zwerina J, Polzer K, Guckel E, Skapenko A, et al. Treg cells suppress osteoclast formation: a new link between the immune system and bone. *Arthritis Rheum* (2007) 56(12):4104–12. doi:10.1002/art.23138
128. Bozec A, Zaiss MM, Kagwiria R, Voll R, Rauh M, Chen Z, et al. T cell costimulation molecules CD80/86 inhibit osteoclast differentiation by inducing the IDO/tryptophan pathway. *Sci Transl Med* (2014) 6(235):235ra60. doi:10.1126/scitranslmed.3007764
129. Ibanez L, Abou-Ezzi G, Ciucci T, Amiot V, Belaid N, Obino D, et al. Inflammatory osteoclasts prime TNFalpha-producing CD4(+) T cells and express CX3 CR1. *J Bone Miner Res* (2016) 31(10):1899–908. doi:10.1002/jbmr.2868
130. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: mechanisms, response biomarkers, and combinations. *Sci Transl Med* (2016) 8(328):328rv4. doi:10.1126/scitranslmed.aad7118
131. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* (2009) 206(13):3015–29. doi:10.1084/jem.20090847
132. Abiko K, Matsumura N, Hamanishi J, Horikawa N, Murakami R, Yamaguchi K, et al. IFN-gamma from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *Br J Cancer* (2015) 112(9):1501–9. doi:10.1038/bjc.2015.101
133. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* (2007) 110(1):296–304. doi:10.1182/blood-2006-10-051482
134. Sanmamed MF, Chen L. Inducible expression of B7-H1 (PD-L1) and its selective role in tumor site immune modulation. *Cancer J* (2014) 20(4):256–61. doi:10.1097/PPO.0000000000000061
135. Tremblay-LeMay R, Rastgoo N, Chang H. Modulating PD-L1 expression in multiple myeloma: an alternative strategy to target the PD-1/PD-L1 pathway. *J Hematol Oncol* (2018) 11(1):46. doi:10.1186/s13045-018-0589-1
136. Hallett WH, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. *Biol Blood Marrow Transplant* (2011) 17(8):1133–45. doi:10.1016/j.bbmt.2011.03.011
137. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29(6):1441–4. doi:10.1038/leu.2015.11
138. Yousef S, Marvin J, Steinbach M, Langemo A, Kovacsovics T, Binder M, et al. Immunomodulatory molecule PD-L1 is expressed on malignant plasma cells and myeloma-propagating pre-plasma cells in the bone marrow of multiple myeloma patients. *Blood Cancer J* (2015) 5:e285. doi:10.1038/bcj.2015.7
139. Dhodapkar MV, Sexton R, Das R, Dhodapkar KM, Zhang L, Sundaram R, et al. Prospective analysis of antigen-specific immunity, stem-cell antigens, and immune checkpoints in monoclonal gammopathy. *Blood* (2015) 126(22):2475–8. doi:10.1182/blood-2015-03-632919
140. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells,

generating aggressive characteristics in multiple myeloma. *Leukemia* (2013) 27(2):464–72. doi:10.1038/leu.2012.213

141. Gorgun G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res* (2015) 21(20):4607–18. doi:10.1158/1078-0432.CCR-15-0200
142. Wang L, Wang H, Chen H, Wang WD, Chen XQ, Geng QR, et al. Serum levels of soluble programmed death ligand 1 predict treatment response and progression free survival in multiple myeloma. *Oncotarget* (2015) 6(38): 41228–36. doi:10.18632/oncotarget.5682
143. Sponaas AM, Moharrami NN, Feyzi E, Standal T, Holth Rustad E, Waage A, et al. PDL1 expression on plasma and dendritic cells in myeloma bone marrow suggests benefit of targeted anti PD1-PDL1 therapy. *PLoS One* (2015) 10(10):e0139867. doi:10.1371/journal.pone.0139867
144. Chen D, Tang P, Liu L, Wang F, Xing H, Sun L, et al. Bone marrow-derived mesenchymal stem cells promote cell proliferation of multiple myeloma through inhibiting T-cell immune responses via PD-1/PD-L1 pathway. *Cell Cycle* (2018) 17(7):858–67. doi:10.1080/15384101.2018.1442624
145. Tai YT, Acharya C, An G, Moschetta M, Zhong MY, Feng X, et al. APRIL and BCMA promote human multiple myeloma growth and immunosuppression in the bone marrow microenvironment. *Blood* (2016) 127(25): 3225–36. doi:10.1182/blood-2016-01-691162
146. Driscoll J, Aslam I, Malek E. Eosinophils upregulate PD-L1 and PD-L2 expression to enhance the immunosuppressive microenvironment in multiple myeloma. *Blood* (2017) 130(Suppl 1):4417.
147. Kelly KR, Espitia CM, Zhao W, Wu K, Visconte V, Anwer F, et al. Oncolytic reovirus sensitizes multiple myeloma cells to anti-PD-L1 therapy. *Leukemia* (2018) 32(1):230–3. doi:10.1038/leu.2017.272
148. Ray A, Das DS, Song Y, Hideshima T, Tai YT, Chauhan D, et al. Combination of a novel HDAC6 inhibitor ACY-241 and anti-PD-L1 antibody enhances anti-tumor immunity and cytotoxicity in multiple myeloma. *Leukemia* (2018) 32(3):843–6. doi:10.1038/leu.2017.322
149. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116(13):2286–94. doi:10.1182/blood-2010-02-271874
150. Giuliani M, Janji B, Berchem G. Activation of NK cells and disruption of PD-L1/PD-1 axis: two different ways for lenalidomide to block myeloma progression. *Oncotarget* (2017) 8(14):24031–44. doi:10.18632/oncotarget.15234
151. Li J, Stagg NJ, Johnston J, Harris MJ, Menzies SA, DiCara D, et al. Membrane-proximal epitope facilitates efficient T cell synapse formation by anti-FcRH5/CD3 and is a requirement for myeloma cell killing. *Cancer Cell* (2017) 31(3):383–95. doi:10.1016/j.ccell.2017.02.001
152. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib study. *J Clin Oncol* (2016) 34(23):2698–704. doi:10.1200/JCO.2015.65.9789
153. Mateos MV, Orlowski RZ, Siegel DS, Reece DE, Moreau P, Ocio EM, et al. Pembrolizumab in combination with lenalidomide and low-dose dexamethasone for relapsed/refractory multiple myeloma. *J Clin Oncol* (2016) 34(15):Abstract8010.
154. Badros A, Hyjek E, Ma N, Lesokhin A, Dogan A, Rapoport AP, et al. Pembrolizumab, pomalidomide, and low-dose dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 130(10):1189–97. doi:10.1182/blood-2017-03-775122
155. von Metzler I, Krebbel H, Hecht M, Manz RA, Fleissner C, Mieth M, et al. Bortezomib inhibits human osteoclastogenesis. *Leukemia* (2007) 21(9): 2025–34. doi:10.1038/sj.leu.2404806
156. Hongming H, Jian H. Bortezomib inhibits maturation and function of osteoclasts from PBMCs of patients with multiple myeloma by downregulating TRAF6. *Leuk Res* (2009) 33(1):115–22. doi:10.1016/j.leukres.2008.07.028
157. Qiang YW, Hu B, Chen Y, Zhong Y, Shi B, Barlogie B, et al. Bortezomib induces osteoblast differentiation via Wnt-independent activation of beta-catenin/TCF signaling. *Blood* (2009) 113(18):4319–30. doi:10.1182/blood-2008-08-174300
158. Giuliani N, Morandi F, Tagliaferri S, Lazzaretti M, Bonomini S, Crugnola M, et al. The proteasome inhibitor bortezomib affects osteoblast differentiation in vitro and in vivo in multiple myeloma patients. *Blood* (2007) 110(1):334–8. doi:10.1182/blood-2006-11-059188
159. Oyajobi BO, Garrett IR, Gupta A, Flores A, Esparza J, Munoz S, et al. Stimulation of new bone formation by the proteasome inhibitor, bortezomib: implications for myeloma bone disease. *Br J Haematol* (2007) 139(3):434–8. doi:10.1111/j.1365-2141.2007.06829.x
160. Terpos E, Heath DJ, Rahemtulla A, Zervas K, Chantry A, Anagnostopoulos A, et al. Bortezomib reduces serum dickkopf-1 and receptor activator of nuclear factor-kappaB ligand concentrations and normalises indices of bone remodelling in patients with relapsed multiple myeloma. *Br J Haematol* (2006) 135(5):688–92. doi:10.1111/j.1365-2141.2006.06356.x
161. Mukherjee S, Raje N, Schoonmaker JA, Liu JC, Hideshima T, Wein MN, et al. Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. *J Clin Invest* (2008) 118(2):491–504. doi:10.1172/JCI33102
162. Breitkreutz I, Raab MS, Vallet S, Hideshima T, Raje N, Mitsiades C, et al. Lenalidomide inhibits osteoclastogenesis, survival factors and bone-remodeling markers in multiple myeloma. *Leukemia* (2008) 22(10):1925–32. doi:10.1038/leu.2008.174
163. Bolzoni M, Storti P, Bonomini S, Todoerti K, Guasco D, Toscani D, et al. Immunomodulatory drugs lenalidomide and pomalidomide inhibit multiple myeloma-induced osteoclast formation and the RANKL/OPG ratio in the myeloma microenvironment targeting the expression of adhesion molecules. *Exp Hematol* (2013) 41(4):387–97.e1. doi:10.1016/j.exphem.2012.11.005
164. Berenson JR, Lichtenstein A, Porter L, Dimopoulos MA, Bordoni R, George S, et al. Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. Myeloma Aredia Study Group. *N Engl J Med* (1996) 334(8):488–93. doi:10.1056/NEJM199602223340802
165. Rosen LS, Gordon D, Kaminski M, Howell A, Belch A, Mackey J, et al. Zoledronic acid versus pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial. *Cancer J* (2001) 7(5):377–87.
166. Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, et al. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. *J Bone Miner Res* (1995) 10(10):1478–87. doi:10.1002/jbmr.5650101008
167. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* (1999) 104(10):1363–74. doi:10.1172/JCI6800
168. Dempster DW, Lambing CL, Kostenuik PJ, Grauer A. Role of RANK ligand and denosumab, a targeted RANK ligand inhibitor, in bone health and osteoporosis: a review of preclinical and clinical data. *Clin Ther* (2012) 34(3):521–36. doi:10.1016/j.clinthera.2012.02.002
169. Hanley DA, Adachi JD, Bell A, Brown V. Denosumab: mechanism of action and clinical outcomes. *Int J Clin Pract* (2012) 66(12):1139–46. doi:10.1111/ijcp.12022
170. Kostenuik PJ, Nguyen HQ, McCabe J, Warmington KS, Kurahara C, Sun N, et al. Denosumab, a fully human monoclonal antibody to RANKL, inhibits bone resorption and increases BMD in knock-in mice that express chimeric (murine/human) RANKL. *J Bone Miner Res* (2009) 24(2):182–95. doi:10.1359/jbmr.081112
171. Raje N, Terpos E, Willenbacher W, Shimizu K, Garcia-Sanz R, Durie B, et al. Denosumab versus zoledronic acid in bone disease treatment of newly diagnosed multiple myeloma: an international, double-blind, double-dummy, randomised, controlled, phase 3 study. *Lancet Oncol* (2018) 19(3): 370–81. doi:10.1016/S1470-2045(18)30072-X
172. Atanackovic D, Steinbach M, Radhakrishnan SV, Luetkens T. Immunotherapies targeting CD38 in multiple myeloma. *Oncoimmunology* (2016) 5(11): e1217374. doi:10.1080/2162402X.2016.1217374
173. Costa F, Toscani D, Chillemi A, Quarona V, Bolzoni M, Marchica V, et al. Expression of CD38 in myeloma bone niche: a rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation. *Oncotarget* (2017) 8(34):56598–611. doi:10.18632/oncotarget.17896
174. Khunger M, Rakshit S, Pasupuleti V, Hernandez AV, Mazzone P, Stevenson J, et al. Incidence of pneumonitis with use of programmed death 1 and programmed death-ligand 1 inhibitors in non-small cell lung cancer: a systematic review and meta-analysis of trials. *Chest* (2017) 152(2):271–81. doi:10.1016/j.chest.2017.04.177
175. Berdeja JG, Lin Y, Raje N, Munshi N, Siegel D, Liedtke M, et al. Durable clinical responses in heavily pretreated patients with relapsed/refractory

multiple myeloma: updated results from a multicenter study of bb2121 anti-Bcma CAR T cell therapy. *Blood* (2017) 130(Suppl 1):740.

176. Vogl DT, Raje N, Jagannath S, Richardson P, Hari P, Orlowski R, et al. Ricolinostat, the first selective histone deacetylase 6 inhibitor, in combination with bortezomib and dexamethasone for relapsed or refractory multiple myeloma. *Clin Cancer Res* (2017) 23(13):3307–15. doi:10.1158/1078-0432.CCR-16-2526
177. Kelly KR, Wu K, Tsao-Wei D, Groshen S, Triche TJ, Mohrbacher A, et al. Oncolytic reovirus immune priming: a phase 1b study of reolysin with bortezomib and dexamethasone in patients with relapsed/refractory multiple myeloma. *Blood* (2016) 128(22):4507.
178. Ahern E, Harjunpaa H, Barkauskas D, Allen S, Takeda K, Yagita H, et al. Co-administration of RANKL and CTLA4 antibodies enhances lymphocyte-mediated antitumor immunity in mice. *Clin Cancer Res* (2017) 23(19):5789–801. doi:10.1158/1078-0432.CCR-17-0606
179. Ahern E, Harjunpää H, O'Donnell JS, Allen S, Dougall WC, Teng MWL, et al. RANKL blockade improves efficacy of PD1-PD-L1 blockade or dual PD1-PD-L1 and CTLA4 blockade in mouse models of cancer. *Oncoimmunology* (2018) 7:e1431088. doi:10.1080/2162402X.2018.1431088

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REVIEW
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CD38 Antibodies in Multiple Myeloma: Mechanisms of Action and Modes of Resistance

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MM cells express high levels of CD38, while CD38 is expressed at relatively low levels on normal lymphoid and myeloid cells, and in some non-hematopoietic tissues. This expression profile, together with the role of CD38 in adhesion and as ectoenzyme, resulted in the development of CD38 antibodies for the treatment of multiple myeloma (MM). At this moment several CD38 antibodies are at different phases of clinical testing, with daratumumab already approved for various indications both as monotherapy and in combination with standards of care in MM. CD38 antibodies have Fc-dependent immune effector mechanisms, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). Inhibition of ectoenzymatic function and direct apoptosis induction may also contribute to the efficacy of the antibodies to kill MM cells. The CD38 antibodies also improve host-anti-tumor immunity by the elimination of regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells. Mechanisms of primary and/or acquired resistance include tumor-related factors, such as reduced cell surface expression levels of the target antigen and high levels of complement inhibitors (CD55 and CD59). Differences in frequency or activity of effector cells may also contribute to differences in outcome. Furthermore, the microenvironment protects MM cells to CD38 antibody-induced ADCC by upregulation of anti-apoptotic molecules, such as survivin. Improved understanding of modes of action and mechanisms of resistance has resulted in rationally designed CD38-based combination therapies, which will contribute to further improvement in outcome of MM patients.

Keywords: CD38, antibody, daratumumab, isatuximab, MOR202, TAK-079, resistance, mode of action

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INTRODUCTION

CD38 was discovered in 1980 by E.L Reinherz and S. Schlossman, and is a type II transmembrane glycoprotein. CD38 plays a role in regulation of migration, receptor-mediated adhesion by interaction with CD31 or hyaluronic acid, and signaling events (1–3). Furthermore, CD38 also has ectoenzymatic activity and is involved in the generation of nucleotide metabolites, which play a role in the control of intracellular calcium stores (4). Under normal conditions, CD38 is expressed at relatively low levels on myeloid and lymphoid cells and in some non-hematopoietic tissues (1). In contrast, normal plasma cells and multiple myeloma (MM) cells have high levels of CD38 expression, which makes CD38 an interesting target for therapeutic antibodies targeting cell surface molecules in MM.

Currently, daratumumab (fully human; Janssen Pharmaceuticals) is the first CD38-targeting antibody, which is approved as single agent and in combination with several standards of care in MM (4). Additional CD38 antibodies that are under clinical evaluation include isatuximab (chimeric; Sanofi), MOR202 (fully human; Morphosys), and TAK-079 (fully human; Takeda) (5). CD38 antibodies are not only evaluated in relapsed/refractory MM, but also in patients with newly diagnosed MM (6). Furthermore, various preclinical studies, case reports, and clinical trials have already demonstrated promising results of CD38 antibodies in other malignancies such NK/T cell lymphoma, T-cell acute lymphoblastic leukemia, and immunoglobulin light-chain amyloidosis (7–11).

Although, immunotherapy with CD38-targeting antibodies is an attractive approach because of a favorable toxicity profile and high activity of CD38 antibodies alone or in combination with standards of care, there is substantial heterogeneity in quality and duration of response among patients. In this review, we will first describe the different modes of action of CD38 antibodies: Fc-dependent immune effector mechanisms, direct effects, and immunomodulatory effects. This is followed by a discussion of several host- and tumor-related factors that influence daratumumab efficacy. We will also discuss which mechanisms contribute to the development of acquired resistance to CD38 antibodies. An increased understanding of mechanisms underlying variability in sensitivity or acquired resistance to CD38-targeting antibodies, may lead to new strategies to improve the effectiveness of CD38 antibody-based treatment. Our review will not discuss all details of the clinical studies which evaluated CD38 antibodies, and for this topic we refer to several excellent and recently published reviews (5, 12–14).

MECHANISM OF ACTION OF CD38-TARGETING ANTIBODIES

Classic FC-Dependent Immune Effector Mechanisms

CD38 antibodies kill tumor cells via Fc-dependent immune effector mechanisms including complement-dependent cytoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and apoptosis upon secondary cross-linking (4, 5, 15). ADCC, ADCP, and crosslinking, are dependent on the interaction of the Fc region of the antibody with Fcγ receptors (FcγRs) expressed on immune effector cells. Importantly, the CD38-targeting antibodies differ with respect to their potency to induce CDC, ADCC, ADCP, or apoptosis upon secondary cross-linking (16). This may be explained in part by unique epitopes of the different CD38 antibodies.

ADCC

Therapeutic antibody-mediated ADCC results in lysis of antibody-coated tumor cells by effector cells. NK-cells play a critical role in ADCC mediated by therapeutic antibodies. Indeed, depletion of NK-cells markedly reduced the capacity of daratumumab to kill MM cells via ADCC (17). Upon the binding of FcγRs to the Fc tail of the CD38-targeting antibody, NK-cells release toxic proteins including granzymes and perforins, which will kill the target cells (18). In addition, macrophages, neutrophils, eosinophils, and γδ T-cells have also been shown to induce ADCC against tumor cells coated with a therapeutic antibody (19, 20), but their role in CD38 antibody-induced ADCC is currently unknown and requires further investigations.

ADCP

In the process of ADCP, phagocytosis of antibody-opsonized tumor cells occurs via binding of FcγRs (such as FcγRIIA and FcγRIIIA), which are present on monocytes and macrophages. Phagocytosis contributes to the anti-tumor activity of CD38-targeting antibodies (16, 21). Interestingly, individual macrophages have the ability to quickly and sequentially engulf multiple daratumumab-coated tumor cells, indicating that ADCP is an efficient killing mechanism of daratumumab (21).

Uptake of antibody-opsonized cancer cells by antigen-presenting cells, such as macrophages and dendritic cells may also lead to enhanced antigen presentation, which may contribute to the development of tumor antigen-specific $CD4^+$ and $CD8^+$ T-cell immune responses (22, 23). This has been demonstrated for several therapeutic antibodies (24), but additional investigations are required to analyze to what extent FcγR-mediated enhancement of antigen presentation contributes to the anti-MM activity of CD38-targeting antibodies.

CDC

Binding of C1q to the Fc tail of the therapeutic antibody initiates the complement cascade, ultimately resulting in the generation of the membrane attack complex (MAC) and subsequently permeabilization of the cell membrane (25, 26). Deposition of complement components, such as C3b, on the surface of the target cell, is also the consequence of complement activation. These deposited complement components interact with complement receptors on phagocytic cells resulting in the engulfment of the tumor cells. In addition, complement activation also leads to generation of C3a and C5a. C5a increases expression of activating FcγRs, while at the same time reducing inhibitory FcγRs, which leads to enhanced phagocytosis capacity of effector cells. C3a and C5a also recruit immune cells to the tumor. Altogether, this indicates that there may be synergy between complement and the FcγR system in eliminating tumor cells (27, 28).

Daratumumab is the most effective inducer of CDC of all currently available CD38 antibodies (4). Indeed, daratumumab was selected from a panel of 42 antibodies based on its ability to strongly induce CDC (29).

Direct Effects

In an antibody screen, isatuximab was selected for further evaluation based on its ability to directly trigger MM cell death in the absence of cross-linking agents and independently of effector cells, even in cells harboring *p53* mutations (30, 31). These direct effects are independent of Fc fragment binding to Fc receptors (30). Isatuximab-mediated MM cell death is mediated

by the classical caspase-dependent apoptotic pathway, as well as the lysosomal cell death pathway, which is characterized by lysosomal enlargement, lysosomal membrane permeabilization, and cathepsin hydrolase release (30). Isatuximab induces reactive oxygen species production, which occurs downstream of lysosomal activation and contributes to MM cell death (30). Daratumumab and MOR202 lack the ability to directly induce MM cell death (16). In addition, CD38 antibodies also modulate the enzymatic activity of CD38, which may contribute to MM cell death (4, 16).

It is currently unknown whether CD38 antibodies also modulate the activity of key signal transduction pathways that regulate growth and survival, as has been described for other therapeutic antibodies, such as rituximab (32). A better understanding of these potential effects, may lead to improved CD38 antibody-based combinations.

Immunomodulatory Effects

Next to the classic Fc-dependent mechanisms of action, daratumumab has also immunomodulatory effects via the elimination of CD38-positive immune suppressor cells, such as regulatory T cells (Tregs), regulatory B cells, and myeloid-derived suppressor cells (4, 33, 34). The depletion of these suppressor cells in the bone marrow (BM) microenvironment explains the increase in T-cell numbers, T-cell clonality, as well as T-cell activity following the initiation of daratumumab treatment (33, 35). Furthermore, T-cells also contain higher levels of granzyme B after exposure to daratumumab, which indicates that they have improved killing capacity (36, 37). Altogether, this suggests that daratumumab treatment leads to an improved host-anti-tumor immune response, which may be important for sustained disease control (33, 34).

Laboratory experiments showed that isatuximab also has immunomodulatory activity, but at this moment no data are available from isatuximab-treated patients. Isatuximab inhibits the suppressive function of Tregs by reducing their numbers, decreasing immune inhibitory cytokine production including IL-10, and blocking their trafficking. This results in improved NK- and T-cell-mediated anti-tumor immune responses (38).

Interestingly, exhausted T-cells not only express high levels of well-known inhibitory receptors, such as PD-1, but also CD38 (39, 40). Recent findings suggest that the NADase activity of CD38 also contributes to the development of T-cell exhaustion via reducing nicotinamide adenine dinucleotide (NAD+) levels in T-cells, resulting in decreased Sirt1/Foxo1 activity (40). Indeed, elevated levels of NAD+ in T-cells are required for an optimal anti-tumor T-cell immune response (40). Importantly, CD38 inhibition on T-cells by anti-CD38 antibodies improved anti-tumor activity in mouse models by increasing NAD+ levels (40).

MECHANISMS OF RESISTANCE

In a pooled analysis of 148 patients who received daratumumab treatment as single agent at a dose of 16 mg/kg in the first in human phase 1/2 GEN501 study (41) or in the Sirius study (42), at least partial response (PR) was achieved in 31% of the patients including at least very good partial response (VGPR) in 13.5% and complete response (CR) in 4.7% (43). These patients were heavily pretreated with a median of five prior lines of therapy with 86% double-refractory to a proteasome inhibitor and an immunomodulatory drug (IMiD) (43). The median duration of response was 7.6 months. The median progression-free survival (PFS) and median overall survival (OS) were 4.0 and 20.1 months, respectively. This indicates that daratumumab induces durable responses in heavily pretreated patients. However, the majority of the responding patients develop progressive disease during daratumumab monotherapy. In addition, more than half of the patients does not respond to single agent daratumumab. Importantly, the other CD38-targeting antibodies, isatuximab and MOR202, induce similar response rates with similar response duration, when compared to daratumumab in a heavily pretreated patient population (44–46).

To improve these results, various CD38-based combinations were evaluated. Preclinical studies showed enhanced anti-MM activity when IMiDs or proteasome inhibitors were added to CD38-targeting antibodies (17, 47). IMiDs improve CD38 antibody-mediated ADCC, ADCP, direct effects, as well-immunomodulatory activity (additional details are given below) (17, 30, 36, 48). It is currently less clear why proteasome inhibitors combine well with CD38 antibodies, but this is probably related to the pleiotropic effects of proteasome inhibitors on both the MM cells and the tumor microenvironment (49). Based on these preclinical data, CD38 antibodies were combined with several standards of care for the treatment of relapsed/refractory MM patients. Adding daratumumab to lenalidomide-dexamethasone (DRd) or bortezomib-dexamethasone (DVd), led to significant improvements in clinical outcome: higher response rate, higher frequency of minimal-residual disease negativity, and improved PFS (50, 51). Based on these results, DRd and DVd were approved by both FDA and EMA for the treatment of MM patients with at least one prior line of therapy (4). The FDA also approved daratumumab in combination with pomalidomide-dexamethasone (DPd), while in Europe the results of the phase 3 APOLLO study (DPd vs. pomalidomide-dexamethasone) are required for approval of this combination. Isatuximab and MOR202 can also be effectively combined with IMiDs and proteasome inhibitors (44, 52–54).

In the following section, we will describe what is currently known about mechanisms of primary and acquired resistance to CD38-targeting antibodies. At this time, the majority of information about modes of resistance is derived from preclinical and clinical studies which evaluated daratumumab.

Effect of Prior Treatment

Daratumumab as monotherapy was tested in heavily pretreated MM patients (43), but not in untreated newly diagnosed MM patients. However, laboratory studies performed with BM aspirates from MM patients, containing tumor cells and autologous effector cells, showed that the efficacy of daratumumab to induce CDC or ADCC was very heterogeneous, but without a significant difference in ADCC or CDC between samples from patients with newly diagnosed MM or relapsed/refractory disease (55). Also, in the subgroup of

patients with lenalidomide- and bortezomib- (double) refractory MM, the activity of daratumumab was comparable to that observed in samples obtained from newly diagnosed patients or relapsed/refractory patients with less prior treatments (55). Data generated from these preclinical studies indicates that resistance to steroids, anthracyclins, alkylators, IMiDs, and proteasome inhibitors is not associated with reduced sensitivity to ADCC and CDC mediated by daratumumab (55).

Daratumumab is also being evaluated in patients with intermediate-risk and high-risk smoldering MM (SMM) (56). In these patients with a premalignant asymptomatic precursor disease at high risk of progression to symptomatic disease, daratumumab was evaluated in three different treatment schedules: short (16 mg/kg; one 8-weeks cycle with daratumumab administered once weekly), intermediate (16 mg/kg, one 8-weeks cycle with daratumumab administered once weekly, followed by daratumumab once every 8 weeks during cycle 2–20), and long (16 mg/kg, one 8-weeks cycle with daratumumab once weekly, then eight infusions every 2 weeks, followed by eight infusions every 4 weeks, and then infusions every 8 weeks during cycle 8–20) (56). At least PR was achieved in 38%, 54%, and 56% and at least VGPR in 15%, 24%, and 29% in the short, intermediate, and long treatment schedules, respectively. This is a higher response rate when compared to the efficacy of daratumumab in highly pretreated MM. Possible explanations for a better response in SMM include increased genetic instability from SMM to MM, altered interactions with the BM microenvironment during disease progression, and impairment of the host immune system during evolution from SMM to MM.

Interestingly, it was recently demonstrated that reintroduction of a previously failed IMiD in daratumumab-refractory patients while continuing daratumumab as a backbone, can be active in heavily pretreated MM patients (57). Similarly, the combination of pomalidomide-dexamethasone and daratumumab induces a 33% response rate in patients previously demonstrated to be refractory to both pomalidomide and daratumumab (58). In addition, 52% of heavily-pretreated lenalidomide-refractory MM patients achieve at least PR with the combination of isatuximab plus lenalidomide-dexamethasone, which is higher than what would be expected with isatuximab as a single agent (52). Altogether, this suggests that the synergistic effects between IMiDs and daratumumab, such as enhanced NK-cell and T-cell activity, potentially overcome refractoriness to both anti-MM agents.

Cytogenetic Abnormalities

The presence of high-risk cytogenetic abnormalities, such as del(17p), *t*(4;14) and *t*(14;16) is associated with a impaired survival of MM patients. High-risk MM patients benefit from CD38 antibodies, but the poor risk cytogenetic abnormalities still have a negative impact on clinical outcome in patients treated with CD38-targeting antibodies.

Twenty percent of high-risk patients achieved at least PR in the SIRIUS study (daratumumab monotherapy), while this was 29.4% for standard-risk patients (42). Interestingly, deep sustained response with daratumumab monotherapy in a high-risk patient was associated with profound reduction in Treg frequency and T-cell expansion (59).

In the randomized phase 3 POLLUX and CASTOR studies, the addition of daratumumab to Rd or Vd markedly improved the outcome of high-risk patients, when compared to Rd or Vd only. However, poor-risk conferred by the presence of del(17p), *t*(4;14), or *t*(14;16) was not completely abrogated by adding daratumumab (60). Although overall response rates with the DPd combination were similar for MM patients with standard or high-risk disease, the median PFS was inferior in high-risk patients, when compared to standard risk patients (3.9 vs. 10.3 months), while OS was similar in both groups (61). Also high-risk patients treated with isatuximab plus lenalidomide-dexamethasone or isatuximab plus pomalidomide-dexamethasone had a lower response rate, when compared to standard-risk patients (52, 62).

It is likely that other tumor-related factors, such as mutations in oncogenes and tumor suppressor genes, and activation status of signaling pathways also contribute to the variability in response to therapy with CD38 antibodies, but this requires further investigation. A better understanding of the role of molecular and biochemical mechanisms of resistance may also contribute to new combination treatments that overcome resistance.

CD38 Target Antigen

CD38 and Primary Resistance

Extent of daratumumab-associated ADCC and CDC is associated with expression levels of CD38 on the cell surface (55). Indeed, CD38-overexpressing clones were more susceptible toward ADCC and CDC, when compared to the non-transduced parental cell lines (55). There is also marked heterogeneity in intensity of CD38 expression on primary MM cells without a difference between MM cells from newly diagnosed or relapsed/refractory patients (55). Similar to the observations with cell lines, daratumumab-mediated ADCC and CDC was less effective against MM cells with low CD38 expression (55).

To further understand the heterogeneity in response, we analyzed CD38 cell surface expression levels in 102 patients, who received 16 mg/kg daratumumab as monotherapy in the GEN501 and Sirius studies to analyze the impact of CD38 expression levels on response. In this analysis, MM patients who achieved at least PR had higher baseline CD38 expression levels, when compared to patients who achieved less than PR (63). Because of the substantial overlap in CD38 expression levels between responders and non-responders, selecting patients based on CD38 expression alone does not seem warranted.

Since CD38 expression is a key determinant of susceptibility of MM cells to daratumumab-mediated ADCC and CDC, as well as clinical response, several groups are evaluating agents that increase CD38 protein levels to improve the efficacy of daratumumab. Binding of all-trans retinoic acid (ATRA) to the retinoic acid receptor affects gene expression, which includes increased expression of CD38 (64, 65). This can be explained by the presence of a retinoic acid-responsive element in the first intron of the *CD38* gene (66). Interestingly, ATRA also increased CD38 expression levels on MM cell lines and primary MM cells without having an effect on MM cell viability

(55). ATRA-induced CD38 upregulation markedly enhanced daratumumb-mediated ADCC and CDC against MM cells. Furthermore, ATRA increased the activity of daratumumab in MM cells, which were resistant to daratumumab in the absence of other drugs (55). Also in a humanized mouse model, ATRA and daratumumab showed synergistic anti-MM activity (55). A clinical study is currently evaluating the value of adding ATRA to daratumumab-refractory patients. Furthermore, the histone deacetylase inhibitor panobinostat induces epigenetic modifications that lead to enhanced expression of CD38 (67). The increase in CD38 antigen density by panobinostat resulted in improved daratumumab-mediated ADCC (67).

CD38 and Acquired Resistance

There is a rapid decrease in CD38 expression levels on the MM cell surface during daratumumab-treatment (63, 68). Directly following the first datatumumab infusion an ∼90% reduction in CD38 expression levels is noticed on non-depleted MM cells (68). A similar CD38 reduction is observed at the time of progression during daratumumab therapy. The reduction in CD38 cell surface expression is a transient phenomenon, because CD38 levels are restored to baseline levels on the MM cells ∼6 months after the last daratumumab infusion (63). Daratumumab-mediated CD38 reduction is a general phenomenon, which is also observed on non-tumor cells, such as normal B-cells, T-cells, NK-cells and monocytes (68). Daratumumab reduces CD38 on the cell surface by several mechanisms. First, in responding patients daratumumab may select for MM cells with lower CD38 expression levels, while preferentially killing the MM cells with higher levels of CD38 (68). In addition, recent studies showed that daratumumab treatment results in the clustering of CD38 molecules into distinct polar aggregates, which can subsequently be released as tumor-derived microvesicles (69). Direct internalization may also contribute to loss of CD38. Finally, active transfer of CD38-daratumumab complexes and accompanying cell membrane from MM cells to monocytes and granulocytes also contributes to CD38 reduction (68). This process of trogocytosis is in part FcγR-dependent (68).

Reduced CD38 expression on non-depleted MM cells is associated with protection against ADCC and CDC (63, 68). Reduced daratumumab-mediated ADCC and CDC induced by CD38 loss was also observed in patients with persistent response (68). Interestingly, ATRA also increased CD38 expression, almost to pretreatment values, in these daratumumab-resistant MM cells, leading to improvements in daratumumab-mediated CDC and ADCC.

Importantly, the reduction in CD38 expression levels, which is associated with impaired classic Fc-dependent immune effector mechanisms, was similar in responding and non-responding patients (63). Indeed, CD38 expression is also reduced in patients with sustained high quality response, suggesting that CD38 reduction is not necessarily associated with escape from daratumumab-mediated killing, but indicates that the pressure to keep MM cells in a state of low CD38 expression, may also offer clinical benefit. Reduced CD38 expression may result in impaired adhesion to stromal cells via CD38-CD31 interactions leading to reduced growth and impaired protection against apoptosis (70). Moreover, daratumumab-mediated trogocytosis may also impair the ability of tumor cells to interact with the protective BM microenvironment by reducing expression of several other adhesion molecules (such as CD49d, CD56, and CD138) on MM cells (68). In addition, daratumumab-mediated reduction of CD38 on MM cells may also result in reduced generation of immunosuppressive adenosine molecules (71), and thereby an improved host-anti-tumor immune response (72–74).

Soluble CD38 and Anti-drug Antibodies

Soluble CD38 may neutralize CD38-targeting antibodies and thereby have an impact on pharmacokinetic profile and response. In the GEN501 and Sirius daratumumab monotherapy studies, soluble CD38 was found in only 2 out of 110 patients (63). Both patients achieved a PR with daratumumab treatment. To the best of our knowledge, impact of soluble CD38 levels on clinical outcome was not reported in the studies with MOR202 and isatuxumab (5).

In a similar way, development of anti-drug antibodies may lead to impaired activity of CD38 antibodies. Up till now, anti-daratumumab or anti-isatuximab antibodies have not been detected (41, 42, 50, 75), while development of anti-drug antibodies is a rare event with MOR202 (76).

CDC Resistance

Several fluid phase regulators, as well as membrane-associated complement-inhibitory proteins, such as CD46, CD55 and CD59, protect healthy tissues against accidental complement attack. These complement inhibitors have also been shown to confer protection of tumor cells against several therapeutic antibodies (77–79).

In an analysis of 23 MM and lymphoma cell lines, daratumumab-sensitive cell lines had lower CD59 and CD55 expression, when compared to CDC-resistant cell lines (63). No difference was found for CD46 (63). Removal of the glycosylphosphatidylinositol-anchored CD55 and CD59 molecules from the cell surface with phospholipase-C, rendered cell lines more sensitive to daratumumab-mediated CDC. In contrast, expression levels of these complement inhibitors were not associated with extent of complement-mediated lysis of primary MM cells by daratumumab (63). Similarly, in the GEN501 and Sirius studies (MM patients treated with 16 mg/kg daratumumab as single agent), there were no differences in pretreatment expression levels of CD46, CD55 and CD59 between responding and non-responding patients (63). However, at the time of progression during daratumumab therapy, a marked increase in CD55 and CD59 was observed on both MM cells localized in the BM, as well as on circulating MM cells (63). Interestingly, in some MM tumors there are coexisting subpopulations of tumor cells with markedly different levels of CD55 and CD59 expression. During daratumumab therapy, the selective pressure resulted in selection of daratumumab-resistant MM cells with high expression of complement-inhibitory proteins (63).

ATRA improved CDC to a higher extent than ADCC, which was explained by the reduction of CD55 and CD59 by ATRA, next to its effect on CD38 expression (55). Importantly,

ATRA also reduces CD55 and CD59 expression levels in MM cells obtained from patients with daratumumab-refractory disease, which together with CD38 upregulation, leads to improved daratumumab-mediated CDC (63). Although the histone deacetylase inhibitor, panobinostat, induces a marked increase in CD38 expression on MM cells, CDC was not enhanced, probably as a result of a concomitant increase in CD55 and CD59 expression (67).

ADCC Resistance

NK-Cells

In experiments with patients' samples, daratumumab-mediated ADCC was superior in samples with a high NK-cell to MM cell ratio, when compared to samples with a low ratio (55, 80–83). Similar associations were found between efficacy of daratumumab to kill primary MM cells and frequency of activated NK-cells defined as $CD3^-/CD56^+/CD16^+$ (55).

This indicates that agents that have the ability to induce NK-cell activation may enhance daratumumab-mediated ADCC. Indeed, IMiDs, such as lenalidomide and pomalidomide, induce NK-cell activation and synergize with daratumumab in ADCC assays (17, 47, 84). In preclinical experiments, IMiDs also improve daratumumab-mediated ADCC in case of lenalidomide-refractory MM cells, indicating that the immune system of these patients is still able to respond to the immunomodulatory effects of IMiDs (17). Similarly, lenalidomide also increases anti-MM activity of CD38-targeting antibodies in patients with lenalidomide-refractory MM (52). Blocking the three main inhibitory KIR receptors (KIR2DL1/2/3) on NK cells with the IPH2102 antibody also leads to improved NK-cell activity against tumor cells (85, 86). This monoclonal antibody also enhances the efficacy of daratumumab-induced, NK-cell-mediated ADCC via the modulation of KIR-inhibitory signaling (87). Interestingly, *KIR* and *HLA* genotypes have an impact on the clinical outcome of MM patients receiving treatment with isatuximab plus lenalidomide-dexamethasone (88).

ADCC requires activation of FcγRs, which are present on the cell surface of NK-cells. Allelic variants of FcγRs with different functionality are implicated in differential response to antibody-based therapy in lymphomas and solid tumors (89–91). The FcγRIIA-131H or FcγRIIIA-158V polymorphisms are associated with a higher affinity for IgG, when compared to their allelic counterparts (92, 93). In addition, the FcγRIIB-232T polymorphism is not able to associate with lipid rafts and thereby markedly weaker in its negative regulatory activity (93). In patients treated with daratumumab monotherapy, FcγRIIIA and FcγRIIB variants have a modest impact on response and PFS, but have no significant effect on OS (94).

Although daratumumab-mediated ADCC is enhanced by agents that increase NK-cell activity, CD38 is highly expressed on NK-cells, which explains their rapid reduction in peripheral blood and BM after infusion of daratumumab (95). This reduction in NK-cells may impair tumor cell killing (95, 96). The rapid NK-cell depletion occurs due to daratumumab-mediated NK-cell fratricide via ADCC (NK-mediated cytotoxicity against neighboring NK-cells) (96). As expected, the residual NK-cells have low CD38 cell surface expression levels (68, 96). NK-cell numbers increase again 3–6 months after the last daratumumab infusion (95). Importantly, responding and non-responding patients experience similar reductions in NK-cell frequencies. The multiple mechanisms of action of daratumumab may explain the lack of association between extent of NK-cell depletion and efficacy of treatment. In addition, no relationship was observed between PFS or occurrence of side effects including infections and maximum reduction in NK-cells (95). Outcome following daratumumab therapy may be enhanced by administration of *ex vivo* expanded NK-cells (96). In addition, pretreatment of expanded NK-cells with F(ab)2 fragments of daratumumab to avoid NK-cell fratricide may represent an alternative approach to improve daratumumab-mediated ADCC in patients. However, feasibility and efficacy of this approach should be assessed in clinical trials. At this moment there is no clinical data on NK-cell frequencies available from patients treated with isatuximab or MOR202, but in *ex vivo* assays isatuximab and, to a lesser extent, MOR202, also reduce NK-cell numbers (95).

Bone Marrow Stromal Cells

It is well-known that stromal cells protect MM cells against various anti-MM drugs, such as dexamethasone, doxorubicin, melphalan, lenalidomide, and bortezomib, via soluble factors or cell adhesion (97–100). It was recently shown that stromal cells also confer protection of MM cells against daratumumab-induced ADCC (101). This protection was not mediated via alteration of target expression levels or suppression of NK cell activity, but possibly via upregulation of anti-apoptotic molecules, such as survivin and Mcl-1 (101).

ADCP Resistance

Similar to CDC and ADCC, capacity of daratumumab to induce phagocytosis is in part dependent on CD38 expression levels (21). Furthermore, in *ex vivo* experiments a high monocyte-MM cell ratio resulted in improved killing of MM cells (55). Similar to ADCC, ADCP also requires activation of the FcγR. As described in the previous section, FcγR polymorphisms have a modest impact on efficacy of CD38 antibodies to eliminate tumor cells (94). Interestingly, it was recently shown that CD47 on MM cells inhibits phagocytosis induced by CD38 antibodies via ligation to SIPRα, which is expressed on phagocytes (102). Blockade of CD47-SIPRα "don't eat me" signaling may therefore increase the clinical activity of CD38 antibodies. In addition, low-dose cyclophosphamide potentiates daratumumab-mediated ADCP via enhancing FcγR expression levels on macrophages and reducing CD47 levels on tumor cells (103, 104). IMiDs also enhance the tumoricidal activity of macrophages and promote ADCP (48). Other possible determinants of ADCP efficiency of CD38 antibodies include target cell size and shape (105, 106).

Resistance to Direct Effects

Extent of isatuximb-mediated direct anti-MM activity is in part dependent on CD38 target expression levels. Indeed, CD38-overexpressing cell lines were more sensitive to the direct cytotoxic effects of isatuximab, when compared to the parental cell lines (30). IMiDs enhance the direct apoptotic effects of isatuximab (30). In this respect, pomalidomide was more

potently enhancing direct cytotoxic effects than lenalidomide (30).

Acquired mechanisms of resistance to these direct effects, such as altered activity of signal transduction pathways, are currently unknown, and require further investigations.

Resistance to Immunomodulatory Activity

In patients treated with daratumumab as single agent, the frequency of activated T-cells declines when patients experienced relapse (33). Future studies are needed to evaluate why the number of activated T-cells is reduced at the time of relapse. In addition, single-cell RNA sequencing in patients treated with daratumumab plus IMiD revealed that responding patients are characterized by higher CD28 expression on T cells, a significantly larger cluster of central memory T cells, and a M1 activated macrophage signature, when compared to resistant or progressing patients (107).

It is currently unknown whether tumor-associated factors, such as mutations in the antigen processing and presentation pathways, loss of neoantigen expression, or insensitivity to T-cell effector molecules are associated with primary or acquired resistance to CD38-targeting antibodies (108).

Compensatory upregulation of multiple inhibitory immune checkpoints, which is implicated in the resistance to programmed cell death-1 (PD-1) or programmed death ligand-1 (PD-L1) inhibitors, may also contribute to development of resistance to the immunomodulatory activities of CD38 antibodies (108, 109). Indeed, preclinical data suggest that immunomodulatory activity of CD38 antibodies can be enhanced by combining a CD38 antibody with a PD-1/PD-L1 inhibitor. For example, in MM, lung cancer, and colon adenocarcinoma mouse models targeting the CD38 and PD-1 pathway with the combination of a CD38 antibody and PD-1 antibody resulted in enhanced anti-tumor activity, when compared to targeting either pathway alone (110). This was accompanied by increased T-cell infiltration and T-cell activation in the tumors with combined anti-CD38 and anti-PD-1 treatment (110). In addition, another group showed that CD38 expression is increased following therapy with a PD-L1 inhibitor in a lung cancer mouse model, which was associated with impaired $CD8^+$ T-cell function (111). This suggests that increased CD38 expression is a novel resistance mechanism to PD-1/PD-L1 antibody treatment. As expected, enhanced antitumor activity was observed when a CD38 antibody was combined with a PD-L1 inhibitor in this lung cancer mouse model (111).

Based on these preclinical studies, various clinical trials are evaluating whether the anti-MM activity of CD38 antibodies can be enhanced by immuno-oncology combinations with PD-1 or PD-L1 inhibitors (5). Furthermore, this antibody combination is also tested in other tumors irrespective of expression of CD38 on the tumor cells (5).

Furthermore, IMiDs not only enhance ADCC and ADCP, but also increase CD38 expression levels on Tregs, which leads to enhanced isatuximab-induced inhibition of Tregs in the presence of IMiDs (38). This indicates that IMiDS also enhance the immunomodulatory activity of CD38 antibodies.

CONCLUSIONS AND FUTURE PROSPECTS

CD38-targeting antibodies utilize multiple effector mechanisms including classic Fc-dependent immune effector mechanisms, but also the recently discovered immunomodulatory mode of action contributes to anti-tumor activity. These pleiotropic mechanisms of action explain the high activity of the CD38 antibodies as single agent in heavily pretreated MM patients.

The efficacy of CD38-targeting antibody therapy can be improved by adding a partner drug with a different mode of action. Indeed, addition of an IMiD or proteasome inhibitor to a CD38 antibody leads to markedly improved outcome. Further improvement may be achieved by addition of an agent that has the ability to enhance complement activation, NK-cell-mediated ADCC, macrophage-mediated ADCP and/or host-anti-tumor T-cell immunity. Indeed, a better understanding of mechanisms that contribute to innate and acquired resistance has already resulted in the rational design of several new combinations with daratumumab, which are currently evaluated in clinical trials (**Figure 1**).

At the moment of development of resistance to a CD38 antibody-based treatment, an alternative treatment regimen can be selected based on several patient- and tumor-related factors, such as type of prior therapies, presence of comorbidities, and aggressiveness of relapse (112, 113). Alternatively, patients that develop resistance to a CD38 antibody may benefit from adding another drug, such as ATRA, that reverses resistance to CD38 antibodies. Several trials are currently evaluating such agents in patients who developed CD38 antibody-refractory disease (**Figure 1**). Another approach is to switch to a different CD38 antibody with different mode of action in case of refractoriness to CD38 antibody treatment. However, although functional differences exist between the CD38-targeting antibodies (16), it is currently unclear whether resistance to one CD38-targeting antibody confers resistance to all CD38 antibodies. A phase 1 trial is currently evaluating the value of isatuximab in daratumumab-refractory patients (NCT02514668). Alternatively, resistance to CD38 antibody-based therapy may also be reversed by adding a synergistic partner drug or changing the partner drug, while continuing the CD38 antibody (57).

Development of next generation CD38 antibodies with optimized CDC or ADCC capacity, by using new antibody engineering techniques, may also lead to more effective CD38-targeting antibodies. For example, the ability of the antibody to activate complement can be enhanced by generating targeted single amino acid changes in the Fc region of the antibody, which allows for hexamer formation upon binding to antigens on a cell (25, 26, 114). In addition, Fc glycosylation (glycoengineering) improves the affinity of the antibody for FcγRs. Indeed, the glycoengineered Fc portion

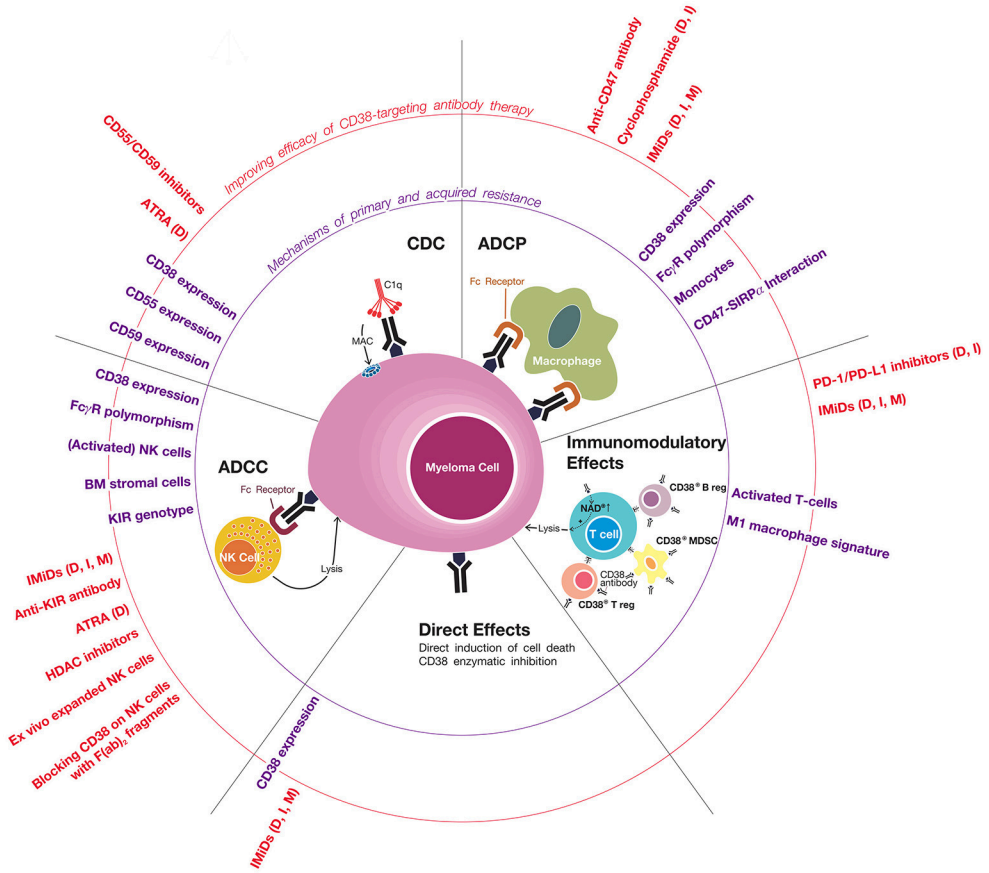

FIGURE 1 | Mechanisms of primary and acquired resistance to CD38 antibodies. CD38-targeting antibodies have Fc-dependent immune effector mechanisms: complement-dependent cytoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells play an important role in CD38 antibody-mediated ADCC, but the possible additional role of other effector cells, such as macrophages, neutrophils, eosinophils, and γδ T-cells, is currently unknown. Daratumumab and isatuximab also have immunomodulatory effects via the eradication of CD38-positive regulatory T-cells, regulatory B-cells, and myeloid-derived suppressor cells, which is associated with $CD4^+$ and $CD8^+$ T-cell expansion, and probably a better host-anti-tumor immune response. In addition, CD38 inhibition on T-cells by anti-CD38 antibodies may also contribute to improved anti-tumor activity by increasing NAD+ levels in T-cells. It is currently unknown whether MOR202 has immunomodulatory effects. In addition, isatuximab also directly induces MM cell death by both the classical caspase-dependent apoptotic pathway and lysosomal cell death pathway. Determinants and mechanisms of primary or acquired resistance to these individual modes of action are indicated (in purple), as well as strategies of how to improve these mechanisms of action in order to improve sensitivity and prevent development of resistance (indicated in red). In case the indicated agents have been tested or are being tested in a clinical trial, we added between brackets the CD38 antibody in the combination regimen (D, daratumumab; I, isatuximab; M, MOR202). General mechanisms of resistance include the presence of high-risk cytogenetic abnormailities and development of anti-drug antibodies. Of note, most data with respect to mechanisms of resistance to CD38 antibodies is derived from studies, which evaluated daratumumab. Additional studies are required for isatuximab and MOR202.

of obinutuzumab enhances the binding affinity to FcγRIIIA, leading to enhanced ADCC and ADCP (115). Furthermore, bispecific antibodies that simultaneously bind to two distinct targets (epitopes on two distinct proteins or two epitopes on a single protein) may offer therapeutic benefit. In this respect, a CD38xCD3 bispecific antibody has been shown to stimulate T-cell-mediated killing of MM cells (116). Moreover, a CD38xCD59 bispecific antibody may have increased CDC activity by simultaneously targeting CD38 and neutralizing CD59 (117).

In conclusion, an increased understanding of host- and tumor-related features that underlie differential therapeutic efficacy and contribute to resistance toward CD38 antibodies, may lead to further optimization and individualization of treatment and a better outcome for MM patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Deaglio S, Mehta K, Malavasi F. Human CD38: a (r)evolutionary story of enzymes and receptors. *Leuk Res.* (2001) 25:1–12. doi: 10.1016/S0145-2126(00)00093-X
2. Deaglio S, Vaisitti T, Billington R, Bergui L, Omede' P, Genazzani AA, et al. CD38/CD19: a lipid raft-dependent signaling complex in human B cells. *Blood* (2007) 109:5390–8. doi: 10.1182/blood-2006-12-061812
3. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, et al. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev.* (2008) 88:841–86. doi: 10.1152/physrev.00035.2007
4. van de Donk NW, Janmaat ML, Mutis T, Lammerts van Bueren JJ, Ahmadi T, Sasser AK, et al. Monoclonal antibodies targeting CD38 in hematological malignancies and beyond. *Immunol Rev.* (2016) 270:95–112. doi: 10.1111/imr.12389
5. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood* (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
6. Mateos MV, Dimopoulos MA, Cavo M, Suzuki K, Jakubowiak A, Knop S, et al. Daratumumab plus Bortezomib, Melphalan, and Prednisone for Untreated Myeloma. *N Engl J Med*. (2018) 378:518–28. doi: 10.1056/NEJMoa1714678
7. Mustafa N, Nee H, Lee X, Jin W, Yu Y, Chen Y. Daratumumab efficiently targets NK/T cell lymphoma with high CD38 expression. *Blood* (2017)130:2814.
8. Bride KL, Vincent TL, Im SY, Aplenc R, Barrett DM, Carroll WL, et al. Preclinical efficacy of daratumumab in T-cell acute lymphoblastic leukemia. *Blood* (2018) 131:995–9. doi: 10.1182/blood-2017-07-794214
9. Kaufman GP, Schrier SL, Lafayette RA, Arai S, Witteles RM, Liedtke M. Daratumumab yields rapid and deep hematologic responses in patients with heavily pretreated AL amyloidosis. *Blood* (2017) 130:900–902. doi: 10.1182/blood-2017-01-763599
10. Sher T, Fenton B, Akhtar A, Gertz MA. First report of safety and efficacy of daratumumab in 2 cases of advanced immunoglobulin light chain amyloidosis. *Blood* (2016) 128:1987–9. doi: 10.1182/blood-2016-06-722496
11. Hari P, Raj RV, Olteanu H. Targeting CD38 in refractory extranodal natural killer cell-T-cell lymphoma. *N Engl J Med*. (2016) 375:1501–2. doi: 10.1056/NEJMc1605684
12. van de Donk NW, Moreau P, Plesner T, Palumbo A, Gay F, Laubach JP, et al. Clinical efficacy and management of monoclonal antibodies targeting CD38 and SLAMF7 in multiple myeloma. *Blood* (2016) 127:681–95. doi: 10.1182/blood-2015-10-646810
13. Plesner T, Krejcik J. Daratumumab for the treatment of multiple myeloma. *Front Immunol*. (2018) 9:1228. doi: 10.3389/fimmu.2018.01228
14. Zamagni E, Tacchetti P, Pantani L, Cavo M. Anti-CD38 and anti-SLAMF7: the future of myeloma immunotherapy. *Exp Rev Hematol.* (2018) 11:423–35. doi: 10.1080/17474086.2018.1456331
15. Overdijk MB, Jansen JH, Nederend M, Lammerts van Bueren JJ, Groen RW, Parren PW, et al. The Therapeutic CD38 monoclonal antibody daratumumab induces programmed cell death via fcgamma receptor-mediated cross-linking. *J Immunol*. (2016) 197:807–13. doi: 10.4049/jimmunol.1501351
16. Lammerts van Bueren J, Jakobs D, Kaldenhoven N, Roza M, Hiddingh S, Meesters J, et al. Direct *in vitro* comparison of daratumumab with surrogate analogs of CD38 antibodies MOR03087, SAR650984 and Ab79. *Blood* (2014) 124:3474.
17. Nijhof IS, Groen RW, Noort WA, van KB, de Jong-Korlaar R, Bakker J, et al. Preclinical evidence for the therapeutic potential of CD38-targeted immuno-chemotherapy in multiple myeloma patients refractory to Lenalidomide and Bortezomib. *Clin Cancer Res.* (2015) 21:2802–10. doi: 10.1158/1078-0432.CCR-14-1813
18. Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. *J Hematol Oncol*. (2013) 6:1. doi: 10.1186/1756-8722-6-1
19. Braza MS, Klein B, Fiol G, Rossi JF. Gammadelta T-cell killing of primary follicular lymphoma cells is dramatically potentiated by GA101, a type II glycoengineered anti-CD20 monoclonal antibody. *Haematologica* (2011) 96:400–7. doi: 10.3324/haematol.2010.029520
20. Yeap WH, Wong KL, Shimasaki N, Teo EC, Quek JK, Yong HX, et al. CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes. *Sci Rep*. (2016) 6:34310. doi: 10.1038/srep34310
21. Overdijk MB, Verploegen S, Bogels M, van Egmond M, Lammerts van Bueren JJ, Mutis T, et al. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs* (2015) 7:311–21. doi: 10.1080/19420862.2015.1007813
22. van de Donk NW, Kamps S, Mutis T, Lokhorst HM. Monoclonal antibody-based therapy as a new treatment strategy in multiple myeloma. *Leukemia* (2012) 26:199–213. doi: 10.1038/leu.2011.214
23. Rafiq K, Bergtold A, Clynes R. Immune complex-mediated antigen presentation induces tumor immunity. *J Clin Invest.* (2002) 110:71–9. doi: 10.1172/JCI0215640
24. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* (2010) 18:160–70. doi: 10.1016/j.ccr.2010.06.014
25. Taylor RP, Lindorfer MA. Cytotoxic mechanisms of immunotherapy: harnessing complement in the action of anti-tumor monoclonal antibodies. *Semin Immunol.* (2016) 28:309–16. doi: 10.1016/j.smim.2016.03.003
26. Melis JP, Strumane K, Ruuls SR, Beurskens FJ, Schuurman J, Parren PW. Complement in therapy and disease: regulating the complement system with antibody-based therapeutics. *Mol Immunol*. (2015) 67:117–30. doi: 10.1016/j.molimm.2015.01.028
27. Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in autoimmunity. *Immunol Lett.* (2005) 100:56–67. doi: 10.1016/j.imlet.2005.06.022
28. Shushakova N, Skokowa J, Schulman J, Baumann U, Zwirner J, Schmidt RE, et al. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcgammaRs in immune complex-induced lung disease. *J Clin Invest*. (2002) 110:1823–30. doi: 10.1172/JCI16577
29. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J. Immunol*. (2011) 186:1840–8. doi: 10.4049/jimmunol.1003032
30. Jiang H, Acharya C, An G, Zhong M, Feng X, Wang L, et al. SAR650984 directly induces multiple myeloma cell death via lysosomal-associated and apoptotic pathways, which is further enhanced by pomalidomide. *Leukemia* (2016) 30:399–408. doi: 10.1038/leu.2015.240
31. Deckert J, Wetzel MC, Bartle LM, Skaletskaya A, Goldmacher VS, Vallee F, et al. SAR650984, a novel humanized CD38-targeting antibody, demonstrates potent antitumor activity in models of multiple myeloma and other $CD38^+$ hematologic malignancies. *Clin Cancer Res.* (2014) 20:4574–83. doi: 10.1158/1078-0432.CCR-14-0695
32. Jazirehi AR, Huerta-Yepez S, Cheng G, Bonavida B. Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappa}B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. *Cancer Res*. (2005) 65:264–76.
33. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes $CD38^+$ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* (2016) 128:384–94. doi: 10.1182/blood-2015-12-687749
34. van de Donk N. Immunomodulatory effects of CD38-targeting antibodies. *Immunol Lett*. (2018). 199:16–22. doi: 10.1016/j.imlet.2018.04.005
35. Chiu C, Casneuf T, Axel A, Lysaght A, Bald J, Khokar N, et al. Daratumumab in combination with lenalidomide plus dexamethasone induces clonality increase and t-cell expansion: results from a phase 3 randomized study (POLLUX). *Blood* (2016) 128:4531.
36. Van de Donk NW, Adams H, Vanhoof G, Krejcik J, Van der Borcht K, Casneuf T. Daratumumab in combination with lenalidomide plus dexamethasone results in persistent Natural Killer (NK) Cells with a distinct phenotype and expansion of effector memory T-cells in pollux, a phase 3 randomized study. *Blood* (2017) 130:3124.

37. Adams H, Stevenaert F, Krejcik J, van der Borcht K, Casneuf T, Smets T, et al. High-Parameter Mass Cytometry (CyTOF) Evaluation of Relapsed/Refractory Multiple Myeloma (MM) Pts (Pts) treated with daratumumab supports immune modulation as a novel mechanism of action. *Blood* (2016) 128:4521.
38. Feng X, Zhang L, Acharya C, An G, Wen K, Qiu L, et al. Targeting CD38 suppresses induction and function of t regulatory cells to mitigate immunosuppression in multiple myeloma. *Clin Cancer Res.* (2017) 23:4290–300. doi: 10.1158/1078-0432.CCR-16-3192
39. Bengsch B, Ohtani T, Khan O, Setty M, Manne S, O'Brien S, et al. Epigenomic-guided mass cytometry profiling reveals disease-specific features of exhausted CD8 T cells. *Immunity* (2018) 48:1029–45.e5. doi: 10.1016/j.immuni.2018.04.026
40. Chatterjee S, Daenthanasanmak A, Chakraborty P, Wyatt MW, Dhar P, Selvam SP, et al. CD38-NAD(+)axis regulates immunotherapeutic anti-tumor t cell response. *Cell Metab.* (2018) 27:85–100.e8. doi: 10.1016/j.cmet.2017.10.006
41. Lokhorst HM, Plesner T, Laubach JP, Nahi H, Gimsing P, Hansson M, et al. Targeting CD38 with Daratumumab monotherapy in multiple myeloma. *N Engl J Med.* (2015) 373:1207–19. doi: 10.1056/NEJMoa1506348
42. Lonial S, Weiss BM, Usmani SZ, Singhal S, Chari A, Bahlis NJ, et al. Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. *Lancet* (2016) 387:1551–60. doi: 10.1016/S0140-6736(15)01120-4
43. Usmani SZ, Weiss BM, Plesner T, Bahlis NJ, Belch A, Lonial S, et al. Clinical efficacy of daratumumab monotherapy in patients with heavily pretreated relapsed or refractory multiple myeloma. *Blood* (2016) 128:37–44. doi: 10.1182/blood-2016-03-705210
44. Raab M, Chatterjee M, Goldschmidt H, Agis H, Blau I, Einsele H, et al. A Phase I/IIa study of the CD38 antibody MOR202 alone and in combination with pomalidomide or lenalidomide in patients with relapsed or refractory multiple myeloma. *Blood* (2016) 128:1152.
45. Martin TG III, Richter J, Vij R, Cole C, Atanackovic D, Zonder J. A dose finding phase II trial of isatuximab (SAR650984, Anti-CD38 mAb) as a single agent in relapsed/refractory multiple myeloma. *Blood* (2015) 126:509.
46. Richter J, Martin TG III, Vij R, Cole C, Atanackovic D, Zonder J. et al. Updated data from a phase II dose finding trial of single agent isatuximab (SAR650984, anti-CD38 mAb) in relapsed/refractory multiple myeloma (RRMM). *J Clin Oncol.* (2016) 34:abstract 8005. doi: 10.1200/JCO.2016.34.15_suppl.8005
47. d van V, de WM, van KB, Bakker JM, Wittebol S, Parren PW, et al. The therapeutic human CD38 antibody daratumumab improves the anti-myeloma effect of newly emerging multi-drug therapies. *Blood Cancer J.* (2011) 1:e41. doi: 10.1038/bcj.2011.42
48. Bruns H, Jitschin R, Mougiakakos D, Bittenbring J, Nolting J, Buttner M, et al. Lenalidomide enhances MOR202 dependent macrophage-mediated effector functions via the vitamin d pathway. *Blood* (2015) 2203:abstract 2203.
49. de Haart SJ, van de Donk NW, Minnema MC, Huang JH, Aarts-Riemens T, Bovenschen N, et al. Accessory cells of the microenvironment protect multiple myeloma from T-cell cytotoxicity through cell adhesion-mediated immune resistance. *Clin Cancer Res.* (2013) 19:5591–601. doi: 10.1158/1078-0432.CCR-12-3676
50. Palumbo A, Chanan-Khan A, Weisel K, Nooka AK, Masszi T, Beksac M, et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma. *N Engl J Med.* (2016) 375:754–66. doi: 10.1056/NEJMoa1606038
51. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, et al. Daratumumab, Lenalidomide, and Dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:1319–31. doi: 10.1056/NEJMoa1607751
52. Martin T, Baz R, Benson DM, Lendvai N, Wolf J, Munster P, et al. A phase 1b study of isatuximab plus lenalidomide and dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 129:3294–303. doi: 10.1182/blood-2016-09-740787
53. Chatterjee M, Raab M, Goldschmidt H, Agis H, Blau I, Einsele H, et al. A phase I/IIa study of the CD38 antibody MOR202 in combination with pomalidomide or lenalidomide in patients with relapsed or refractory multiple myeloma. *Blood* (2016) 128:1152
54. Martin TG III, Mannis G, Chari A, Munster P, Campana F, Hui A. Phase Ib study of isatuximab and carfilzomib in relapse and refractory multiple myeloma. *Blood* (2016) 128:2111.
55. Nijhof IS, Groen RW, Lokhorst HM, van Kessel B, Bloem AC, van Velzen J, et al. Upregulation of CD38 expression on multiple myeloma cells by all-trans retinoic acid improves the efficacy of daratumumab. *Leukemia* (2015) 29:2039–49. doi: 10.1038/leu.2015.123
56. Hofmeister CC, Chari A, Cohen Y, Spencer A, Voorhees PM, Estell J, et al. Daratumumab monotherapy for patients with intermediate or high-risk Smoldering Multiple Myeloma (SMM): centaurus, a randomized, open-label, multicenter phase 2 study. *Blood* (2017) 130:510.
57. Gavriatopoulou M, Kastritis E, Ntanasis-Stathopoulos I, Fotiou D, Roussou M, Migkou M, et al.The addition of IMiDs for patients with daratumumab-refractory multiple myeloma can overcome refractoriness to both agents. *Blood* (2018) 131:464–7. doi: 10.1182/blood-2017-10-809293
58. Nooka A, Joseph N, Boise L, Gleason C, Kaufman J, Lonial S. Clinical efficacy of daratumumab, pomalidomide and dexamethasone in relapsed, refractory myeloma patients: utility of retreatment with daratumumab among refractory patients. *Blood* (2016) 128:492.
59. Usmani SZ, Khan I, Chiu C, Foureau D, Druhan LJ, Rigby K, et al. Deep sustained response to daratumumab monotherapy associated with T-cell expansion in triple refractory myeloma. *Exp Hematol Oncol.* (2018) 7:3. doi: 10.1186/s40164-018-0096-7
60. Weisel K, San-Miguel J, Cook G, Leiba M, Suzuki K, Kumar S, et al. Efficacy of daratumumab in combination with lenalidomide plus dexamethasone (DRd) or bortezomib plus dexamethasone (DVd) in relapsed or refractory multiple myeloma (RRMM) based on cytogenetic risk status. *J Clin Oncol.* (2017) 35:abstract 8006. doi: 10.1200/jco.2017.35.15_suppl.8006
61. Chari A, Suvannasankha A, Fay JW, Arnulf B, Kaufman JL, Ifthikharuddin JJ, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. *Blood* (2017) 130:974–81. doi: 10.1182/blood-2017-05-785246
62. Mikhael J, Karanes C, Dubin F, Anderson K, Raje N, Vitse O. Final results of a phase 1B study of isatuximab plus pomalidomide and dexamethasone in relapsed/refractory multiple myeloma. *EHA* (2018) S850.
63. Nijhof IS, Casneuf T, van Kessel B, van Velzen J, Axel AE, Syed K, et al. CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma. *Blood* (2016) 128:959–70. doi: 10.1182/blood-2016-03-703439
64. Malavasi F. Editorial: CD38 and retinoids: a step toward a cure. *J Leukoc Biol.* (2011) 90:217–9. doi: 10.1189/jlb.0211069
65. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, Collins SJ, et al. Retinoic acid-induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid receptor-alpha. *Cancer Res.* (1994) 54:1746–52.
66. Kishimoto H, Hoshino S, Ohori M, Kontani K, Nishina H, Suzawa M, et al. Molecular mechanism of human CD38 gene expression by retinoic acid. Identification of retinoic acid response element in the first intron. *J Biol Chem.* (1998) 273:15429–34. doi: 10.1074/jbc.273.25.15429
67. Garcia-Guerrero E, Gogishvili T, Danhof S, Schreder M, Pallaud C, Perez-Simon JA, et al. Panobinostat induces CD38 upregulation and augments the antimyeloma efficacy of daratumumab. *Blood* (2017) 129:3386–8. doi: 10.1182/blood-2017-03-770776
68. Krejcik J, Frerichs KA, Nijhof IS, van Kessel B, van Velzen JF, Bloem AC, et al. Monocytes and granulocytes reduce CD38 expression levels on myeloma cells in patients treated with daratumumab. *Clin Cancer Res.* (2017) 23:7498–511. doi: 10.1158/1078-0432.CCR-17-2027
69. Horenstein AL, Chillemi A, Quarona V, Zito A, Roato I, Morandi F, et al. NAD(+)-Metabolizing ectoenzymes in remodeling tumor-host interactions: the human myeloma model. *Cells* (2015) 4:520–37. doi: 10.3390/cells4030520
70. An G, Jiang H, Acharya C, Zhong MY, Cai T, Yang G, et al. SAR 650984, a Therapeutic Anti-CD38 monoclonal antibody, blocks CD38-CD31 interaction in multiple myeloma. *Blood* (2014) 124:4729.
71. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. *Nat Rev Cancer* (2017) 17:709–24. doi: 10.1038/nrc.2017.86

72. Chillemi A, Zaccarello G, Quarona V, Ferracin M, Ghimenti C, Massaia M, et al. Anti-CD38 antibody therapy: windows of opportunity yielded by the functional characteristics of the target molecule. *Mol Med*. (2018) 19:99–108. doi: 10.2119/molmed.2013.00009
73. Morandi F, Horenstein AL, Chillemi A, Quarona V, Chiesa S, Imperatori A, et al. CD56brightCD16- NK cells produce adenosine through a CD38-mediated pathway and act as regulatory cells inhibiting autologous $CD4^+$ T cell proliferation. *J Immunol*. (2015) 195:965–72. doi: 10.4049/jimmunol.1500591
74. Horenstein AL, Quarona V, Toscani D, Costa F, Chillemi A, Pistoia V, et al. Adenosine generated in the bone marrow niche through a CD38-mediated pathway correlates with progression of human myeloma. *Mol Med*. (2016) 22–694–704. doi: 10.2119/molmed.2016.00198
75. Martin T, Strickland S, Glenn M, Zheng W, Daskalakis N, Mikhael J. SAR650984, a CD38 monoclonal antibody in patients with selected $CD38^+$ hematological malignancies–data from a dose-escalation phase I study. *Blood* (2013) 122:284.
76. Raab M, Goldschmidt H, Agis H, Blau I, Einsele H, Engelhardt M. A phase I/IIa study of the human anti-CD38 antibody MOR202 (MOR03087) in relapsed or refractory multiple myeloma (rrMM). *J Clin Oncol*. (2015) 33:abstract 8574. doi: 10.1200/jco.2015.33.15_suppl.8574
77. Meyer S, Leusen JH, Boross P. Regulation of complement and modulation of its activity in monoclonal antibody therapy of cancer. *MAbs* (2014) 6:1133–44. doi: 10.4161/mabs.29670
78. Tsai PC, Hernandez-Ilizaliturri FJ, Bangia N, Olejniczak SH, Czuczman MS. Regulation of CD20 in rituximab-resistant cell lines and B-cell non-Hodgkin lymphoma. *Clin Cancer Res*. (2012) 18:1039–50. doi: 10.1158/1078-0432.CCR-11-1429
79. Czuczman MS, Olejniczak S, Gowda A, Kotowski A, Binder A, Kaur H, et al. Acquirement of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. *Clin Cancer Res*. (2008) 14:1561–70. doi: 10.1158/1078-0432.CCR-07-1254
80. Ge X, Wu L, Hu W, Fernandes S, Wang C, Li X, et al. rILYd4, a human CD59 inhibitor, enhances complement-dependent cytotoxicity of ofatumumab against rituximab-resistant B-cell lymphoma cells and chronic lymphocytic leukemia. *Clin Cancer Res*. (2011) 17:6702–11. doi: 10.1158/1078-0432.CCR-11-0647
81. Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, et al. CD20 levels determine the *in vitro* susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* (2001) 98:3383–9. doi: 10.1182/blood.V98.12.3383
82. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* (2000) 95:3900–08.
83. Hu W, Ge X, You T, Xu T, Zhang J, Wu G, et al. Human CD59 inhibitor sensitizes rituximab-resistant lymphoma cells to complement-mediated cytolysis. *Cancer Res*. (2011) 71:2298–307. doi: 10.1158/0008-5472.CAN-10-3016
84. van der Veer MS, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. Towards effective immunotherapy of myeloma: enhanced elimination of myeloma cells by combination of lenalidomide with the human CD38 monoclonal antibody daratumumab. *Haematologica* (2011) 96:284–90. doi: 10.3324/haematol.2010.030759
85. Romagne F, Andre P, Spee P, Zahn S, Anfossi N, Gauthier L, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. Blood (2009) 114:2667–77. doi: 10.1182/blood-2009-02-206532
86. Benson D, Bakan C, Zhang S, Alghothani L, Liang J, Hofmeister C, et al. IPH2101, a novel anti-inhibitory kir monoclonal antibody, and lenalidomide combine to enhance the Natural Killer (NK) Cell Versus Multiple Myeloma (MM) Effect. *ASH Annu Meet Abstr*. (2009) 114:3870.
87. Nijhof IS, Lammerts van Bueren JJ, van Kessel B, Andre P, Morel Y, Lokhorst HM, et al. Daratumumab-mediated lysis of primary multiple myeloma cells is enhanced in combination with the human anti-KIR antibody IPH2102 and lenalidomide. *Haematologica* (2015) 100:263–8. doi: 10.3324/haematol.2014.117531
88. Marra J, Du J, Hwang J, Wolf J, Martin TG III, Venstrom J. KIR and HLA genotypes influence clinical outcome in multiple myeloma patients treated with SAR650984 (Anti-CD38) in combination with lenalidomide and dexamethasone. *Blood* (2014) 124:2126.
89. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* (2002) 99:754–8. doi: 10.1182/blood.V99.3.754
90. Dornan D, Spleiss O, Yeh RF, Duchateau-Nguyen G, Dufour A, Zhi J, et al. Effect of FCGR2A and FCGR3A variants on CLL outcome. *Blood* (2010) 116:4212–22. doi: 10.1182/blood-2010-03-272765
91. Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J Clin Oncol*. (2007) 25:3712–8. doi: 10.1200/JCO.2006.08.8021
92. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* (1997) 90:1109–14.
93. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol*. (2008) 8:34–47. doi: 10.1038/nri2206
94. van de Donk NWCJ, Casneuf T, Di Cara A, Parren PW, Zweegman S, van Kessel B, et al. Impact of Fc gamma receptor polymorphisms on efficacy and safety of daratumumab in relapsed/refractory multiple myeloma. *Br J Haematol*. (2018). doi: 10.1111/bjh.15122. [Epub ahead of print].
95. Casneuf T, Xu XS, Adams HC III, Axel AE, Chiu C, Khan I, et al. Effects of daratumumab on natural killer cells and impact on clinical outcomes in relapsed or refractory multiple myeloma. *Blood Adv*. (2017) 1:2105–14. doi: 10.1182%2Fbloodadvances.2017006866
96. Wang Y, Zhang Y, Hughes T, Zhang J, Caligiuri MA, Benson DM, et al. Fratricide of NK cells in daratumumab therapy for multiple myeloma overcome by *ex vivo* expanded autologous NK cells. *Clin Cancer Res*. (2018) 24:4006–17. doi: 10.1158/1078-0432.CCR-17-3117
97. Bjorklund CC, Baladandayuthapani V, Lin HY, Jones RJ, Kuiatse I, Wang H, et al. Evidence of a role for CD44 and cell adhesion in mediating resistance to lenalidomide in multiple myeloma: therapeutic implications. *Leukemia* (2014) 28:373–83. doi: 10.1038/leu.2013.174
98. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* (1999) 93:1658–67.
99. Noborio-Hatano K, Kikuchi J, Takatoku M, Shimizu R, Wada T, Ueda M, et al. Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. *Oncogene* (2009) 28:231–42. doi: 10.1038/onc.2008.385
100. Hao M, Zhang L, An G, Meng H, Han Y, Xie Z, et al. Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression. *Leuk Lymphoma* 52 (2011) 1787–94. doi: 10.3109/10428194.2011.576791
101. de Haart SJ, Holthof L, Noort WA, Minnema MC, Emmelot ME, Aarts-Riemens T, et al. Sepantronium bromide (YM155) improves daratumumab-mediated cellular lysis of multiple myeloma cells by abrogation of bone marrow stromal cell-induced resistance. *Haematologica* (2016) 101:e339–42. doi: 10.3324/haematol.2015.139667
102. van Bommel PE, He Y, Schepel I, Hendriks M, Wiersma VR, van Ginkel RJ, et al. CD20-selective inhibition of CD47-SIRPalpha "don't eat me" signaling with a bispecific antibody-derivative enhances the anticancer activity of daratumumab, alemtuzumab and obinutuzumab. *Oncoimmunology* (2018) 7:e1386361. doi: 10.1080/2162402X.2017.1386361
103. Rigalou A, Ryan A, Natoni A, Chiu C, Sasser AK, O'Dwyer M. Potentiation of anti-myeloma activity of daratumumab with combination of cyclophosphamide, lenalidomide or bortezomib via a tumor secretory response that greatly augments macrophage-induced ADCP. *Blood* (2016) 128:2101.

104. Naicker S, Rigalou A, McEllistrim C, Natoni A, Chiu C, Sasser K. Patient data supports the rationale of low dose cyclophosphamide to potentiate the anti-myeloma activity of daratumumab through augmentation of macrophage-induced ADCP. *Blood* (2017) 130:121.
105. Swanson JA, Hoppe AD, The coordination of signaling during Fc receptor-mediated phagocytosis. *J Leukoc Biol.* 76 (2004) 1093-103. doi: 10.1189/jlb.0804439
106. Paul D, Achouri S, Yoon YZ, Herre J, Bryant CE, Cicuta P. Phagocytosis dynamics depends on target shape. *Biophys J.* (2013) 105:1143–50. doi: 10.1016/j.bpj.2013.07.036
107. Neri P, Maity R, Tagoug I, Duggan P, McCullogh S, Jimenez-Zepeda VH. Single cell resolution profiling defines the innate and adaptive immune repertoires modulated by daratumumab and IMiDs treatment in multiple myeloma (MM). *Blood* (2017) 130:123.
108. Syn NL, Teng MWL, Mok TSK, Soo RA. De-novo and acquired resistance to immune checkpoint targeting. *Lancet Oncol.* (2017) 18:e731–41. doi: 10.1016/S1470-2045(17)30607-1
109. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun.* (2016) 7:10501. doi: 10.1038/ncomms10501
110. Bezman NA, Kinder M, Jhatakia A, Mattson B, Pizutti D, Thompson E, et al. Antitumor activity associated with dual targeting of CD38 and programmed death-1 pathways in preclinical models. *AACR Meeting Abstracts* (2018) 78:4563. doi: 10.1158/1538-7445.AM2018-1727
111. Chen L, Averett Byers L, Ullrich S, Wistuba I, Qin X, Gibbons D. CD38 as a novel immune checkpoint and a mechanism of resistance to the blockade of the PD-1/PD-L1 axis. *J Clin Oncol.* (2017) 35:79.
112. Moreau P, San Miguel J, Sonneveld P, Mateos MV, Zamagni E, Avet-Loiseau H, et al. Multiple myeloma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* (2017) 28:iv52–iv61. doi: 10.1093/annonc/mdx096
113. van de Donk NW, Lokhorst HM, Dimopoulos M, Cavo M, Morgan G, Einsele H, et al. Treatment of relapsed and refractory multiple myeloma in the era of novel agents. *Cancer Treat Rev.* (2011) 37:266–83. doi: 10.1016/j.ctrv.2010.08.008
114. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science* (2014) 343:1260–3. doi: 10.1126/science.1248943
115. Mossner E, Brunker P, Moser S, Puntener U, Schmidt C, Herter S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood* (2010) 115:4393–402. doi: 10.1182/blood-2009-06-225979
116. Chu SY, Miranda Y, Phung S, Chen H, Rashid R, Endo N, et al. Immunotherapy with long-lived Anti-CD38 × Anti-CD3 bispecific antibodies stimulates potent T cell-mediated killing of human myeloma cell lines and CD38^{+} cells in monkeys: a potential therapy for multiple myeloma. *Blood* (2014) 124:4727.
117. Macor P, Secco E, Mezzaroba N, Zorzet S, Durigutto P, Gaiotto T, et al. Bispecific antibodies targeting tumor-associated antigens and neutralizing complement regulators increase the efficacy of antibody-based immunotherapy in mice. *Leukemia* (2015) 29:406–14. doi: 10.1038/leu.2014.185

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Checkpoint Inhibition in Myeloma: Opportunities and Challenges

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Despite major improvements in the treatment landscape, most multiple myeloma (MM) patients eventually succumb to the underlying malignancy. Immunotherapy represents an attractive strategy to achieve durable remissions due to its specificity and capacity for long term memory. Activation of immune cells is controlled by a balance of agonistic and inhibitory signals via surface and intracellular receptors. Blockade of such inhibitory immune receptors (termed as "immune checkpoints") including PD-1/PD-L1 has led to impressive tumor regressions in several cancers. Preclinical studies suggest that these immune checkpoints may also play a role in regulating tumor immunity in MM. Indeed, myeloma was among the first tumors wherein therapeutic efficacy of blockade of PD-1 axis was demonstrated in preclinical models. Expression of PD-L1 on tumor and immune cells also correlates with the risk of malignant transformation. However, early clinical studies of single agent PD-1 blockade have not led to meaningful tumor regressions. Immune modulatory drugs (IMiDs) are now the mainstay of most MM therapies. Interestingly, the mechanism of immune activation by IMiDs also involves release of inhibitory checkpoints, such as Ikaros-mediated suppression of IL-2. Combination of PD-1 targeted agents with IMiDs led to promising clinical activity, including objective responses in some patients refractory to IMiD therapy. However, some of these studies were transiently halted in 2017 due to concern for a possible safety signal with IMiD-PD1 combination. The capacity of the immune system to control MM has been further reinforced by recent success of adoptive cell therapies, such as T cells redirected by chimeric-antigen receptors (CAR-Ts). There remains an unmet need to better understand the immunologic effects of checkpoint blockade, delineate mechanisms of resistance to these therapies and identify optimal combination of agonistic signaling, checkpoint inhibitors as well as other therapies including CAR-Ts, to realize the potential of the immune system to control and prevent MM.

Keywords: myeloma, immunotherapy, immune checkpoint, PD-1, PD-L1

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IMMUNE SYSTEM AS AN EFFECTIVE APPROACH TO TREAT CANCER AND PRINCIPLES OF IMMUNE CHECKPOINT BLOCKADE

The role of immune system in cancer progression has been studied for over a century (1). However, only recently immunotherapy has emerged as an effective strategy to treat several types of cancers with impressive results in terms of tumor regression and durable remissions (2). The concept of immune surveillance and editing of tumors is now well-accepted (3).

Several studies suggest a role for genetic and epigenetic modifications in cancer development and progression (4–6) and some of them correlate with the ability to escape this immunosurveillance (5, 6). Tumor cells can indeed lower their immunogenicity through the down regulation of MHC-mediated neo-antigen presentation, accompanied by deletion of cancer cells expressing T cell targets (immunoediting) (3). The immunoediting process in cancer pathogenesis comprises of three phases: elimination, equilibrium and escape. In the first phase, the innate and adaptive immune systems recognize and eradicate cancer cells through the cytolytic activity of immune cells (i.e., NK cells, NKT cells, $\gamma\delta$ T cells, and $CD8^+$ T cells), antibody-dependent cell-mediated cytotoxicity (ADCC), or complement-dependent cytotoxicity (CDC) mechanisms (7, 8). In the equilibrium phase, a balance between cancer progression and cancer elimination is established through the modulation of control checkpoints (3, 7). However, if cancer persists, it overcomes the immunity response and escapes with further progression and metastasis (3, 7).

Along with the suppression of tumor antigen expression, different mechanisms that involve surface molecules and soluble factors released in the tumor microenvironment, e.g., indoleamine 2,3-dioxygenase (IDO), type I interferons (IFNs) and IFN-γ, galectin-1, have been described in the disruption of immune homeostasis and in the altered balance from effector to regulatory and suppressive cells induced by cancer (7, 9).

In principle, immunotherapy could either enhance the immune response or inhibit tumor suppression (10). The most commonly used approach is the modulation of inhibitory immune receptors (termed as "immune checkpoints") that regulate the balance between immune response and immune tolerance (11). Several studies showed that cancer cells increase the expression of some checkpoint proteins (summarized in **Table 1**), such as programmed cell death ligand-1 (PD-L1), with inhibitory properties on T cell functions, as a mechanism of immune resistance (20). These results lead to the development of monoclonal antibodies (mAbs) directed against such immune checkpoints, further approved for the treatment of several solid tumors as melanoma, renal and lung cancer (21–23).

CTLA-4 is the first immune-checkpoint explored as a clinical target (24). It is normally expressed at low levels on the surface of effector T cells and regulatory T cells (Tregs) and it is involved in the early stages of T cell activation (25). CTLA-4 shares the same ligands of CD28 (CD80 and CD86) expressed on antigen presenting cells (APCs). Once CD28 binds CD80 or CD86 to provide co-stimulation, the inhibitory CTLA-4 molecule is shuttled to the T cell surface where it binds CD80 or CD86 with higher affinity (26) thus counteracting the costimulatory activity of CD28 through the binding of the phosphatases PP2A and SHP-2 (25, 27). CTLA-4 expression also exerts its immunosuppressive functions by other mechanisms, including Treg expansion and induction of immunosuppressive cytokines, such as transforming growth factor (TGF)-β and the enzyme IDO (13, 21). While CTLA4 expression is mostly studied for its expression on lymphoid cells, recent studies suggest that myeloid dendritic cells can secrete $CTLA4^+$ microvesicles that may mediate immune suppression (28). CTLA-4 blockade with mAbs (i.e., ipilimumab) can then enhance the immune response against tumor by inactivating Treg, tumor-infiltrating lymphocytes (TILs) (29) and increasing T helper (Th)1 cell functions (20).

PD-1 is a member of the CD28/CTLA-4 family, with inhibitory properties, mainly expressed on exhausted T cells (dysfunctional T cells classically associated with chronic infection), NK and NKT cells following activation (14). APCs, monocytes and malignant cells express its ligands, PD-L1 and PD-L2, especially under inflammatory conditions (14).

Similarly to CTLA-4, the interaction between PD-1 and PD-L1 interferes with TCR signal transduction, by recruiting the tyrosine phosphatase SHP-2 and subsequent inactivating the PI3 kinase-signaling cascade (30, 31), which leads to reduced cytokine synthesis, cytotoxic functions and blockade of T cell proliferation and survival (14).

In the physiologic setting, this pathway enables the immunologic equilibrium after initial T cell response, preventing over-activation and the possible expansion of auto-reactive T cells (32). Studies on $PD\text{-}L1^{-/-}$ murine models reported an accumulation of effector T cells along with an increased IFN-γ production by $CD8^+$ T cells, suggesting an impaired apoptosis regulation in the absence of PD-L1 (33). Moreover, spontaneous accumulation of $CD8^+$ T cells occurred in the liver even in the absence of "non-self" antigen exposure leading to the development of multiple autoimmune features (33). These data highlighted the importance of PD-L1 in controlling the responses of self-reactive T cells that have escaped into the periphery. In addition, PD-1/PD-L1 axis regulates the dynamic interplay between Tregs and T effector cells. In the presence of inflammatory milieu, PD-L1 expressed on both APC and naïve Tregs induces PD-1 expression on naïve T cells and promotes their differentiation toward a regulatory phenotype and function (induced Tregs) (34). On the other hand, a negative feedback loop occurs to downregulate Treg development and function. This effect is mediated by the increased PTEN expression via PD-1 signaling which in turn reduces PD-L1 expression on Tregs (35). Finally, $PD\text{-}L1^+$ Tregs directly induce a tolerogenic phenotype in APCs that reduces the priming of T effector cells (34). All these results thus confirmed the critical role of the PD-1/PD-L1 pathway in the balance between T cell activation and tolerance.

According to the relevance of PD-1/PD-L1 axis in immune control, tumors seem to highjack this pathway to suppress and escape the activation of an immune response (36). High PD-L1 expression is associated with a poor prognosis in solid tumors, including lung, ovarian or colon cancer, thus supporting the impressive results that PD-1/PD-L1 blockade has led in several cancers (36).

In addition to CTLA-4 and PD-1, other proteins with inhibitory properties, as Lymphocyte-activation gene (LAG)-3, T cell immunoglobulin (TIM)-3, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) are currently under active investigations as potential targets for mAbs (37). LAG-3 is expressed on activated conventional T cells, Tregs, B cells and plasmacytoid dendritic cells (pDCs) (38) and the interaction with its major ligand, Class II MHC, inhibits conventional T cell activity while enhancing the suppressive function of

TABLE 1 | Immune checkpoint distribution and functions.

Checkpoint	Expression	Function	References
CTLA4 (CD80/CD86)	Activated T cells and Tregs	Inhibition of CD28 co-stimulation and T cell activity; Enhancement of Treg functions; Induction of TGF-β and IDO	(12) (13)
PD-1 (PD-L1/PD-L2)	Activated T cells, NK cells, NKT cells, B cells, Monocytes, DCs, MDSCs	T cell exhaustion and apoptosis; inhibition of cytokine production; downregulation of NK and NKT cell activity	(14)
LAG3 (MHCII)	activated T cells, NK cells, B cells, pDCs	Effector T cell inhibition; Increased Treg activity	(15)
TIM3 (Galectin 9, HMGB1)	Exhausted T cells, NK cells, NKT cells, B cells, DCs, Macrophages	Th1 cell apoptosis; Reduced cytokine release; Induction of tolerogenic M2 phenotype	(16) (17)
TIGIT (CD155)	Exhausted cytotoxic T cells, NK cells	Effector T cell inhibition; Reduced NK cell cytotoxicity; Enhanced Treg activity	(18) (19)

DCs, dendritic cells; MDSCs, myeloid derived suppressor cells; NK, natural killer; NKT, NK-like T cells; Th, T helper; Tregs, T regulatory cells.

Tregs (39). LAG-3 blockade in addition to anti-PD-1 strategy showed an additive therapeutic activity in preclinical models of chronic infection and cancer, according to their role as markers of exhaustion (40, 41). TIM-3 is another exhaustion-associated inhibitory receptor that blunts T-cell-effector function and induce T cell apoptosis (17). Mouse models of colon adenocarcinoma, melanoma, and sarcoma demonstrated anti-tumor activity of TIM-3 blockade especially in combination with PD-L1 blockade (42, 43). Moreover, anti-TIM-3 treatment increases the proliferation and cytokine production of $CD8^+$ T cells derived from patients with melanoma (44). Anti-TIM-3 or anti-PD-L1 mAbs in combination with the blockade of TIGIT, a marker of exhausted cytotoxic cells, showed enhance anti-tumor activity in several animal models (45). Recent studies suggest promise for TIGIT blockade in future immunotherapy regimens without adding significant toxicity (19, 46).

In addition to checkpoint blockade, mAbs targeting agonist receptors, such as inducible co-stimulator (ICOS), OX40 and 4-1BB (47), are currently under clinical development, especially in solid tumors like melanoma (37).

The combined strategy to enhance T-cell activity with co-stimulatory mAbs and concurrently restoring T cell cytotoxic functions against cancer cells by blocking inhibitory proteins could be a promising approach (48). Several clinical trials on both solid and hematological malignancies are currently exploring this strategy (49).

REGULATION OF TUMOR IMMUNITY IN MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHIES OF UNDETERMINED SIGNIFICANCE

Multiple myeloma (MM) is a hematological malignancy characterized by clonal expansion of terminally differentiated B cells (plasma cells) in the bone marrow (BM). It is clinically manifested with osteolytic bone disease, infections, renal insufficiency, and BM failure (50). The cross talk between malignant plasma cells (PCs) and the BM microenvironment, including immune cells, bone cells, endothelial cells, mesenchymal stromal cells (MSCs) and extracellular matrix, plays a pivotal role in the proliferation and survival of tumor cells (51).

Of note, "immunoparesis," with a reduction in "uninvolved Igs," is a common feature of MM (52). PC interactions with BM niche cells create a permissive microenvironment that can promote tumor growth and immune escape, through the production of several factors including TGF-β, interleukin (IL)-10, IL-6, and prostaglandin E2, known to have immunosuppressive properties (53). Among immune cells, DCs display an impaired differentiation and maturation in MM patients (54, 55) and their interaction with PCs enhance MM clonogenicity and proliferation through B cell activating factor (BAFF)/a proliferation inducing ligand (APRIL) signals (56, 57). Malignant PCs can in turn prompt DC fusion and trans-differentiation into osteoclasts (OCs) through receptor activator of nuclear factor κB ligand and CD47 pathways (58–60), thus promoting immunosuppression and disease progression. Beside their role in bone remodeling, OCs also show immunosuppressive properties specifically inducing T-cell apoptosis through the up-regulation of immune checkpoint proteins as TIM-3 and the production of IDO and APRIL (61). These factors increase PD-L1 expression in MM cells thus supporting tumor escape from the immune control (61). DCs can also indirectly enhance osteoclastogenesis by promoting the expansion of T helper 17 (Th-17) clones in MM microenvironment (62) and the consequent accumulation of IL-17, known to be a potent pro-osteoclastogenic factor, in MM BM (60). Sponaas AM et al. reported that myeloid DCs also express PD-L1 and correlate with $PD\text{-}L1^+$ PCs, suggesting that both cell types could contribute to the suppression of the anti-tumor T cell response in MM through PD-1/PD-L1 pathway (63). Furthermore, MM DC differentiation and maturation is inhibited by MSC production of immunosuppressive factors as IDO, IL-6, PTGS2

(64, 65). MSCs also increase PD-L1 expression on MM cells (66) which in turn suppress PD-1$^+$ T cell and NK cell activity (67).

Along with PD-1/PD-L1 axis, a role for other inhibitory pathways, such as CD226 (68), and the induction of T-cell senescence (69) has also been implicated in the suppression of tumor immunity which characterized MM (68, 69). Several studies also reported an accumulation of myeloid derived suppressor cells (MDSCs) and Tregs, along with an unbalanced ratio of Th1/Th2 cells and dysfunctional NK cell cytotoxic activity in MM, compared to patients with monoclonal gammopathy of undetermined significance (MGUS) (70–72). Of note, this loss of function in several immune effector cells is associated with progression to clinical MM (73) and is in part due to the increased expression of suppressive factors, such as ligands of the activating receptor NKG2D (i.e., MHC class I chain-related protein A) from MGUS to MM (74).

More than 10 years ago it was demonstrated that the immune system can detect MGUS pre-neoplastic lesions and potentially control tumor growth (75). Indeed, the presence of CD4$^+$ and CD8$^+$ T cells, functionally active against pre-neoplastic cells and able to recognize a pattern of specific antigens for each patient tumor, was reported in the BM of MGUS patients (73). A further study identified SOX2 embryonal stem cell antigen as a distinct target of immunity in MGUS compared to MM (76). Interestingly, the presence of SOX2-specific T cells and PD-L1 expression on tumor cells and T cells at baseline was then found to be correlated with the risk of progression to MM (77). Of note, T cells against SOX2 were recently found to be implicated in durable response of a MM patient following chimeric-antigen receptor T (CART) cells (78).

Beside these mechanisms, the establishment of a chronic inflammatory status has been described in the evolution of asymptomatic diseases to MM (79), according to the tight correlation between inflammation and cancer development dating back to Virchow's studies in 1863. It is known that BM serum of MM patients is enriched of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-15, IL-17, IL-18, IL-22, IL-23, TNF-α, and IFN-γ (80). Moreover, a recent study from Botta C et al. interestingly defined an 8-genes signature (IL8, IL10, IL17A, CCL3, CCL5, VEGFA, EBI3, and NOS2) able to identify MGUS/smoldering/symptomatic-MM with 84% accuracy and built a prognostic risk score based on six genes (IFNG, IL2, LTA, CCL2, VEGFA, CCL3), validated in three additional independent datasets (79).

In the context of MM inflammatory status, bioactive lipids, typically increased during inflammation, may also play a crucial role in tumor development (81). In the past decade, obesity has indeed emerged as one of the risk factors for MM (82) and recent studies have shown an enrichment of lysophosphatidylcholine (LPC) species in MM patient serum compared to healthy donors (HDs) along with an expansion of CD1d-restricted type II NKT cell subsets, reactive against these lipids (83). These cells secrete high amounts of the immunosuppressive IL-13, thus supporting their role in the progression of the disease (83). On the other hand, a decline as well as dysfunctional activation of type I NKT cells was also reported in MM patients, suggesting the balance between these two cell subsets as a new important immune-regulatory axis in the evolution of myeloma (83, 84). In support of this evidence, another study described that CD1d is highly expressed in premalignant and early MM and its expression decreases with disease progression (85). Dysregulation of lipid-reactive immune cells and a higher number of type II NKT cells, with enhanced capacity to promote PC differentiation, may be involved in the increased risk of gammopathy in Gaucher Disease, a lipid disorder (86–88). The multiplicity of mechanisms behind MM immunosuppression and enhancement of disease progression thus suggests the need of combinatorial approaches in the treatment of MM.

PRECLINICAL STUDIES TARGETING IMMUNE CHECKPOINTS IN MM

The role of PD-1/PD-L1 pathway in mediating immune escape of malignant PCs and the therapeutic efficacy of PD-1/PD-L1 blockade in other hematological malignancies led to an increased interest in the use of anti-PD-1/PD-L1 therapeutic strategies in MM (68). PD-L1 is highly expressed on PCs isolated from patients with MM, but not on normal PCs (66, 89–91). High PD-L1 expression on PCs was associated with disease progression in patients with MGUS and asymptomatic MM (77) and it could play a role in the development of clonal resistance as demonstrated by PD-L1 high levels in relapsed or refractory MM patients (66). Furthermore, PD-L1 upregulation emerged in patients with minimal residual disease, suggesting that residual PD-L1$^+$ myeloma cells have an increased ability to survive and escape immunosurveillance (90). Nevertheless, high variability of PD-1/PD-L1 expression on PCs and BM niche cells was highlighted among patients with the same stage of disease (63, 90).

In vitro studies showed that MM microenvironment could induce PD-L1 expression on PCs; PD-L1 up-regulation indeed occurs in the presence of stromal cells (66) and PD-L1 blockade inhibits stromal cell-mediated PC growth (67). This effect is IL-6 dependent and mediated by STAT3, MEK1/2, and JAK2 pathways (66).

IFN-γ produced by cytotoxic T lymphocytes (CTLs) and NK cells strongly induces PD-L1 expression through the activation of MEK/ERK pathway (89). In addition, myeloid DCs, pDCs and MDSCs express PD-L1 in MM patients (63), with an increased proportion of PD-L1$^+$ MDSCs in MM patients at remission compared to newly diagnosed and relapsed MM (92).

T cells from MM patients also display higher PD-1 expression levels, associated with loss of effector cell function (93) on both circulating T cells and BM CD8$^+$ T and NK cells compared to HDs (67). Moreover, a study from Castella et al. (92) showed that PD-1 expression is already present on the anergic BM Vγ9Vδ2 T cell subset from MGUS patients and remained upregulated in MM after clinical remission (92). In contrast, PD-1 expression is reduced in T cells from patients who achieved minimal disease state following high dose chemotherapy (94).

In vitro studies further demonstrated that PD-1/PD-L1 blockade directly enhances NK and T cell mediated anti–MM responses (67, 93) and restores the capacity of PD-L1$^+$ pDCs to induce cytotoxic activity of T cells and NK cells against MM PCs (95).

The effects of anti-PD-L1 mAb were also tested *in vivo*, on the 5T33 murine MM models, after autologous (syngeneic) stem-cell transplantation plus administration of a cell-based vaccine (96) or after irradiation (97). It was demonstrated that mice with advanced MM expressed higher levels of PD-1 on both CD8$^+$ and CD4$^+$ T cells compared to non-tumor bearing mice and the percentages of PD-1$^+$ T cells correlated with the amount of tumor burden (97). Moreover, PD-1$^+$ CD8$^+$ T cells isolated from these mice showed a defective production of pro-inflammatory cytokines (IFN-γ and IL-2) after *in vitro* stimulation and expressed increased levels of the exhausted T cell marker TIM-3 (97). PD-1 blockade also prolonged the survival in disseminated myeloma-bearing mice (90, 96, 97) and this effect was abrogated by the depletion of CD4$^+$ or CD8$^+$ T cells, thus indicating the main role of both T cell subsets behind this strategy (96). Taken together, these studies supported the potential contribution of PD-1/PD-L1 pathway in the immune escape in MM and suggested that its blockade may be an effective therapeutic strategy against this tumor.

However, current evidences indicate that PD-1 blockade as single agent does not induce clinically meaningful anti-myeloma responses (98). In this regard, it was recently reported that the compromised functions of effector cells in MM may be due to senescence rather than PD-1 mediated exhaustion (69, 98). Exhausted T cells overexpress multiple inhibitory molecules, such as PD-1, CTLA-4, CD160, TIM-3 and LAG-3 and lack of IFN-γ expression (99). However, a PD-1^{low} T cell clonal expansion was observed in 75% of myeloma patients, in contrast to the non-clonal PD-1^{high} T cells (69, 98). This expanded population potentially represented tumor-reactive cells with a senescent phenotype. They indeed showed low levels of LAG-3, TIM-3, PD-1, and CTLA-4 and did not express CD27 and CD28, suggesting a late differentiated phenotype. Moreover, this clone expressed the typical senescent markers CD57, CD160 and KLRG-1 and displayed a secretory profile (69). In addition, it was described that the senescent phenotype was telomere independent as demonstrated by the low levels of p38-mitogen-activated protein kinase, p16 and p21 signaling pathways and it could be potentially reversed by other agents, as immunomodulatory drugs (IMiDs) or histone deacetylase inhibitors (69).

IMMUNOLOGIC EFFECTS OF IMIDS-RELEASING THE IKAROS CHECKPOINT

The development of the IMiDs, thalidomide (Thal) and its analogs lenalidomide (Len) and pomalidomide (Pom), has led to a paradigm shift in the treatment of MM (100). IMiDs exert their immunological functions through several mechanisms, including proliferation and functional enhancement of NK/NKT cells, induction of T-cell co-stimulation and reduction of Treg activity, increased Th1 cytokine production, such as IL-2 and IFN-γ, anti-MM ADCC improvement and enhanced DC maturation and functions (101–103). The main molecular mechanism was recently elucidated showing that IMiDs bind Cereblon, causing a subsequent degradation of the transcriptional factors, Ikaros (IKZF1) and Aiolos (IKZF3) on both MM cells and T cells (104). Aiolos is a known repressor of the IL-2 gene promoter while Ikaros is also involved in the regulation of transcriptional silencing during Th2 differentiation (104–106).

Beside these effects, *in vitro* studies interestingly showed that Len treatment downregulates PD-1 expression on both T cells (93) and NK cells (67), restoring their cytotoxic activity, and decreases PD-L1 expression on malignant PCs and MDSCs (93). These data suggested that Len could enhance the effect of anti PD-1/PD-L1 blockade as further reported by Görgün G et al. *In vitro* studies (67).

Moreover, evaluation of immune function in MM patients treated with Pom demonstrated a poly-functional T-cell activation, with increased proportion of co-inhibitory receptor BTLA$^+$ T cells and TIM-3$^+$ NK cells (107), thus giving a rationale for the use of combination with immune checkpoint inhibitors. Analysis of the molecular mechanism of action revealed that Pom induces depletion of IKZF1 on both T and NK cells; however this effect is dependent on drug exposure and IKZF1 levels return back to baseline, prior to new cycle, with intermittent dosing (107). Interestingly, Pom-mediated immune activation correlated with clinical outcome even in heavily pretreated MM patients; although the baseline expression of Ikaros/Aiolos protein in tumor cells was not predictive of outcome (107).

More recently, a study from Bailur et al. (108) reported that Pom also reduces IKZF1 and IKZF3 levels on innate lymphoid cells (ILCs) and enhances their function, as demonstrated by the increased IFN-γ production both *in vitro* and *in vivo* (108). ILCs are a new subset of innate immune cells known to be involved in the regulation of immunity, inflammation and tissue homeostasis (109). The study also reported that ILCs are increased in BM of MGUS patients compared to HDs and their functions are enhanced in MGUS but decline in patients with asymptomatic MM (108). These results thus provided evidence that ILCs are among the earliest cell subsets enriched in the tumor microenvironment during the evolution of monoclonal gammopathies and represent a possible target to prevent disease progression by acting on their IKZF1 expression. In addition, PD-1 seems to be a negative regulator for ILC function (110) thus supporting the potential for synergy between IMiDs and anti-PD-1 mAbs in the treatment of MM.

EARLY CLINICAL STUDIES OF CHECKPOINT BLOCKADE AND COMBINATIONS IN MM

The preclinical evidence that PD-1/PD-L1 blockade enhances T cell and NK cell anti-MM cytotoxicity encouraged the use

of mAbs against these checkpoints in clinical trials. However, the use of anti PD-1/PD-L1 antibodies as monotherapy has not provided satisfying results. Specifically, a phase Ib clinical trial testing the anti-PD-1 Nivolumab (IgGk, fully human) in monotherapy reported no objective responses in 27 patients with relapsed or refractory MM (RRMM) (111). Similarly, a phase Ib trial of pembrolizumab (IgGk, humanized anti-PD-1) in monotherapy for RRMM (NCT01953692/KEYNOTE-013) described a stable disease in 57% of patients (112). Preliminary results of a phase II trial of pembrolizumab used in monotherapy as consolidation in MM patients (NCT02636010) demonstrated an increased depth of response in only 3 of 14 patients treated. This lack of efficacy could be explained by the low level of infiltrating effector cells that characterize MM, along with a relatively modest mutational burden as compared to solid tumors wherein therapeutic efficacy correlates with the mutational burden (113).

Lack of single agent activity led to studies testing PD-1/PD-L1 blockade as a part of a combined therapeutic strategy, particularly with IMiDs (**Table 2**). Pembrolizumab in combination with Len and dexamethasone (Dex) was evaluated in a phase I dose-escalation in 40 RRMM patients who experienced disease progression after more than two prior therapies (114). The objective response rate (ORR) in the whole population was 50%, with an ORR of 38% in Len-refractory patients (114). Preliminary results from the phase II clinical trial conducted on 48 RRMM patients, previously treated with a median of three regimens, showed an ORR of 60%, including 8% of stringent complete response/complete response, 19% VGPR, and 33% PR, with a median duration of response of 14.7 months (115, 116). Interestingly, a phase II study of Pembrolizumab following ASCT reported a CR rate of 31% at 6 months, including a 67% rate of BM MRD-negative state (117).

These results lead to the development of the phase III studies of pembrolizumab in combination with Len and Dex (KEYNOTE-185, NCT02579863) or Pom and Dex (KEYNOTE-183, NCT02576977) and one phase III study of Pom and Dex vs. nivolumab, Pom, and Dex vs. nivolumab, elotuzumab, Pom, and Dex (CheckMate 602, NCT02726581). However, in June 2017 the US Food and Drug Administration transiently halted the clinical trials of anti-PD-1/PD-L1 mAbs in combination with IMiDs, due to an imbalance of deaths in the Pembrolizumab arms in KEYNOTE-183 and KEYNOTE-185 and no significant differences in terms of objective response (https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm). As these studies have not yet been published in a peer-reviewed format, more details that might shed light on the possible explanations for these observations are lacking. With further review of safety data on ongoing trials, some of the studies of combinations of PD-1/PD-L1 blockade in MM have now been reinitiated. Combinations of PD-1 blockade with other MM therapies are also currently under evaluation. Preliminary results on a phase I trial of the anti PD-1 Nivolumab in combination with other established anti-myeloma agents (Len/Pom, Dex, anti-CD38 Daratumumab, proteasome inhibitors) revealed acceptable safety profile in refractory, heavily pre-treated, high-risk MM patients (118). In addition anti-PD-1 based therapy, clinical trials of mAbs targeting PD-L1 (Atezolizumab and Durvalumab), both alone and in combination with other agents (i.e., Elotuzumab, and Daratumumab) have also been developed.

Together, these studies point to the need for careful evaluation of immune checkpoint strategies and their combinations in MM, with cautious attention to toxicities as well as pharmacodynamics endpoints.

MAJOR UNMET NEEDS AND FUTURE DIRECTIONS

The concept that immune system can regulate the growth of MM cells is now well-established and immune-based approaches carry the promise of long term disease control and even cure without the need for ongoing therapy. Current MM therapies, such as IMiDs and anti-CD38 antibodies can have immunologic effects; newer therapies particularly CAR-T cells and T cell-engaging bi-specifics are in active clinical investigation and showing promising results. However, there remains an unmet need to address the mechanisms operative in the tumor microenvironment that restrict or prevent long term control of tumors.

Further studies are needed to better understand the mechanisms behind the lack of clinical activity of single agent PD-1 blockade in MM. Several mechanistic possibilities exist, including dominance of other inhibitory checkpoints, immune suppressive cells, lack of agonistic signaling, the low number of tumor-specific T cells in the tumor microenvironment, poor antigen presentation, low mutational burden of MM tumors, as well as senescence of tumor-infiltrating T cells. Moreover, MM is not a single disease and it consists of several distinct genetic subtypes; thus, it is likely that immune microenvironment in MM may also differ between patients. This heterogeneity may even be spatial within the same patient, as recently illustrated for solid tumors (120). As MM is a malignancy involving an immune cell, it is also theoretically possible that PD-1 blockade may lead to altered cross-talk with other immune cells and paradoxically promote tumor growth. It is also of interest to identify if there are specific subsets of patients (such as those with high mutational burden on MM cells), who might preferentially benefit from checkpoint blockade.

Some of the possibilities discussed above suggest the chance that the lack of efficacy of PD-1 blocking antibodies as single agents can be reverted by the combination with other agents. This strategy could lead to distinct pharmacodynamics effects as well as toxicity profiles compared to monotherapies (121).

It should be noted that many of the published data involve PD-1 targeted therapies; however, the effects of PD-L1 blockade may differ.

Preclinical studies also suggest a potential efficacy of agonistic antibodies in preclinical models as well. As an example, anti-CD137 Abs were shown to lead to strong tumor immunity in

TABLE 2 | Selected clinical trials of checkpoint inhibitor-based therapies in Multiple Myeloma.

Study	Phase	Clinical trial identifier	References
A study of pembrolizumab (mk-3475) in combination with standard of care treatments in participants with multiple myeloma (MK-3475-023/KEYNOTE-023)	I	NCT02036502	(114)
An investigational immuno-therapy study to determine the safety and effectiveness of nivolumab and daratumumab, with or without pomalidomide and dexamethasone, in patients with multiple myeloma	I	NCT01592370	(118)
Study of lenalidomide/dexamethasone with nivolumab and ipilimumab in patients with newly diagnosed multiple myeloma	I	NCT03283046	–
A study to determine dose and regimen of durvalumab as monotherapy or in combination with pomalidomide with or without dexamethasone in subjects with relapsed and refractory multiple myeloma	I	NCT02616640	–
A study of PVX-410, a cancer vaccine, and durvalumab ± lenalidomide for smoldering MM	I	NCT02886065	–
A study of atezolizumab (anti-programmed death-ligand 1 [PD-L1] antibody) alone or in combination with an immunomodulatory drug and/or daratumumab in participants with multiple myeloma (MM)	Ib	NCT02431208	–
A study of durvalumab in combination with lenalidomide with and without dexamethasone in subjects with newly diagnosed multiple myeloma	Ib	NCT02685826	–
1454GCC: Anti-PD-1 (MK-3475) and IMiD (Pomalidomide) combination immunotherapy in relapsed/refractory multiple myeloma	I/II	NCT02289222	(115, 116)
Pembrolizumab cyclophosphamide and lenalidomide for patients with relapsed multiple myeloma (MUKfourteen)	I/II	NCT03191981	–
Pembrolizumab, lenalidomide, and dexamethasone in treating patients with newly diagnosed multiple myeloma eligible for stem cell transplant	II	NCT02880228	–
Phase 2 multi-center study of anti-pd-1 during lymphopenic state after HDT/ASCT for multiple myeloma	II	NCT02331368	(117)
Pembrolizumab + Lenalidomide post-autologous stem cell transplant (ASCT) in high-risk multiple myeloma (MM)	II	NCT02906332	(119)
Efficacy and safety study of pembrolizumab (MK-3475) in combination with daratumumab in participants with relapsed refractory multiple myeloma (MK-3475-668/KEYNOTE-668)	II	KEYNOTE-668 NCT03221634	–
A study of elotuzumab in combination with pomalidomide and low dose dexamethasone and elotuzumab in combination with nivolumab in patients with multiple myeloma relapsed or refractory to prior treatment with lenalidomide	II	NCT02612779	–
An exploratory study to evaluate the combination of elotuzumab and nivolumab with and without pomalidomide in relapsed refractory multiple myeloma	II	NCT03227432	–
A Phase II trial if nivolumab, lenalidomide and dexamethasone in high risk smoldering myeloma	II	NCT02903381	Based on ClinicalTrials.gov. U.S. National Library of Medicine [https://clinicaltrials.gov/]. Accessed 2 Jan 2018.
A study to determine the safety and efficacy for the combination of durvalumab and daratumumab in relapsed and refractory multiple myeloma (FUSIONMM-003)	II	NCT02807454	–
A study to determine the efficacy of the combination of Daratumumab (DARA) plus Durvalumab (DURVA) (D2) in subjects with Relapsed and Refractory Multiple Myeloma (RRMM) (FUSION-MM-005)	II	NCT03000452	–
Study of pomalidomide and low dose dexamethasone with or without pembrolizumab (MK-3475) in Refractory or Relapsed and Refractory Multiple Myeloma (rrMM) (MK3475-183/KEYNOTE-183)	III	KEYNOTE-183/ NCT02576977	https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm
Study of lenalidomide and dexamethasone with or without pembrolizumab (MK-3475) in participants with newly diagnosed treatment naive multiple myeloma (MK3475-185/KEYNOTE-185)	III	KEYNOTE-185/ NCT02579863	https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm

VKappa-myc MM models (122, 123). Although a small study with this agent in MM was not completed, further evaluation of this pathway particularly in combination may be of interest. T cells in MM lesions also express other inhibitory molecules, such as TIM-3 and LAG-3. Antibodies targeting these molecules are now entering the clinic and the effects of these agents in human MM are awaited. In addition to their effects on T cells, immune regulatory pathways are also operative for innate cells, such as NK-T cells and ILCs. These pathways may also limit the efficacy of engineered T cells, such as CAR-T cells, as well as bispecifics. Future combinations of these strategies to harness immune-mediated MM control are therefore eagerly awaited.

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FC wrote the first draft of the paper; FC, RD, JKB, KD, and MVD reviewed the paper and gave final approval of the submitted publication.

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REFERENCES

1. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). *Proc R Soc Med.* (1910) 3:1–48.
2. Khalil DN, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat Rev Clin Oncol.* (2016) 13:273–90. doi: 10.1038/nrclinonc.2016.25
3. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* (2002) 3:991–8. doi: 10.1038/ni1102-991
4. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* (2000) 100:57–70. doi: 10.1016/S0092-8674(00)81683-9
5. Maio M, Covre A, Fratta E, Di Giacomo AM, Taverna P, Natali PG, et al. Molecular pathways: at the crossroads of cancer epigenetics and immunotherapy. *Clin Cancer Res.* (2015) 21:4040–7. doi: 10.1158/1078-0432.CCR-14-2914
6. Peng D, Kryczek I, Nagarsheth N, Zhao L, Wei S, Wang W, et al. Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* (2015) 527:249–53. doi: 10.1038/nature 15520
7. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* (2011) 331:1565–70. doi: 10.1126/science.1203486
8. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol.* (2011) 29:235–71. doi: 10.1146/annurev-immunol-031210-101324
9. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol.* (2006) 6:836–48. doi: 10.1038/nri1961
10. Finn OJ. A believer's overview of cancer immunosurveillance and immunotherapy. *J Immunol.* (2018) 200:385–91. doi: 10.4049/jimmunol.1701302
11. Dyck L, Mills KHG. Immune checkpoints and their inhibition in cancer and infectious diseases. *Eur J Immunol.* (2017) 47:765–79. doi: 10.1002/eji.201646875
12. Engelhardt JJ, Sullivan TJ, Allison JP. CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. *J Immunol.* (2006) 177:1052–61. doi: 10.4049/jimmunol.177.2.1052
13. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med.* (2009) 206:1717–25. doi: 10.1084/jem.20082492
14. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
15. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. *Immunity* (2004) 21:503–13. doi: 10.1016/j.immuni.2004.08.010
16. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* (2005) 6:1245–52. doi: 10.1038/ni1271
17. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA.* (2010) 107:14733–8. doi: 10.1073/pnas.1009731107
18. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol.* (2009) 10:48–57. doi: 10.1038/ni.1674
19. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol.* (2011) 186:1338–42. doi: 10.4049/jimmunol.1003081
20. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
21. Pico De Coana Y, Choudhury A, Kiessling R. Checkpoint blockade for cancer therapy: revitalizing a suppressed immune system. *Trends Mol Med.* (2015) 21:482–91. doi: 10.1016/j.molmed.2015.05.005
22. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med.* (2015) 372:320–30. doi: 10.1056/NEJMoa1412082
23. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med.* (2015) 372:2521–32. doi: 10.1056/NEJMoa1503093
24. Wolchok JD, Saenger Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist* (2008) 13(Suppl 4):2–9. doi: 10.1634/theoncologist.13-S4-2
25. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol.* (2001) 1:220–8. doi: 10.1038/35105024
26. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* (1994) 1:793–801. doi: 10.1016/S1074-7613(94)80021-9
27. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev.* (2009) 229:12–26. doi: 10.1111/j.1600-065X.2009.00770.x
28. Halpert MM, Konduri V, Liang D, Chen Y, Wing JB, Paust S, et al. Dendritic cell-secreted cytotoxic T-lymphocyte-associated protein-4 regulates the T-cell response by downmodulating bystander surface B7. *Stem Cells Dev.* (2016) 25:774–87. doi: 10.1089/scd.2016.0009
29. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* (2015) 27:450–61. doi: 10.1016/j.ccell.2015.03.001
30. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) 173:945–54. doi: 10.4049/jimmunol.173.2.945

31. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol.* (2005) 25:9543–53. doi: 10.1128/MCB.25.21.9543-9553.2005
32. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
33. Dong H, Zhu G, Tamada K, Flies DB, Van Deursen JM, Chen L. B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. *Immunity* (2004) 20:327–36. doi: 10.1016/S1074-7613(04)00050-0
34. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev.* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
35. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med.* (2007) 13:84–8. doi: 10.1038/nm1517
36. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol.* (2007) 19:813–24. doi: 10.1093/intimm/dxm057
37. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science* (2015) 348:56–61. doi: 10.1126/science.aaa8172
38. Huard B, Gaulard P, Faure F, Hercend T, Triebel F. Cellular expression and tissue distribution of the human LAG-3-encoded protein, an MHC class II ligand. *Immunogenetics* (1994) 39:213–7. doi: 10.1007/BF00241263
39. Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol.* (1995) 25:2718–21. doi: 10.1002/eji.1830250949
40. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of $CD8^+$ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* (2009) 10:29–37. doi: 10.1038/ni.1679
41. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res.* (2012) 72:917–27. doi: 10.1158/0008-5472.CAN-11-1620
42. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med.* (2010) 207:2187–94. doi: 10.1084/jem.20100643
43. Ngiow SF, Von Scheidt B, Akiba H, Yagita H, Teng MW, Smyth MJ. Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. *Cancer Res.* (2011) 71:3540–51. doi: 10.1158/0008-5472.CAN-11-0096
44. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific $CD8^+$ T cell dysfunction in melanoma patients. *J Exp Med.* (2010) 207:2175–86. doi: 10.1084/jem.20100637
45. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
46. Dixon KO, Schorer M, Nevin J, Etminan Y, Amoozgar Z, Kondo T, et al. Functional anti-TIGIT antibodies regulate development of autoimmunity and antitumor immunity. *J Immunol.* (2018) 200:3000–7. doi: 10.4049/jimmunol.1700407
47. Buchan SL, Rogel A, Al-Shamkhani A. The immunobiology of CD27 and OX40 and their potential as targets for cancer immunotherapy. *Blood* (2018) 131:39–48. doi: 10.1182/blood-2017-07-741025
48. Buchan SL, Fallatah M, Thirdborough SM, Taraban VY, Rogel A, Thomas LJ, et al. PD-1 blockade and CD27 stimulation activate distinct transcriptional programs that synergize for CD8(+) T-cell-driven antitumor immunity. *Clin Cancer Res.* (2018) 24:2383–94. doi: 10.1158/1078-0432.CCR-17-3057
49. Naidoo J, Page DB, Wolchok JD. Immune modulation for cancer therapy. *Br J Cancer* (2014) 111:2214–9. doi: 10.1038/bjc.2014.348
50. Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med.* (2004) 351:1860–73. doi: 10.1056/NEJMra041875
51. Anderson KC, Carrasco RD. Pathogenesis of myeloma. *Annu Rev Pathol.* (2011) 6:249–74. doi: 10.1146/annurev-pathol-011110-130249
52. Perez-Persona E, Vidriales MB, Mateo G, Garcia-Sanz R, Mateos MV, De Coca AG, et al. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. *Blood* (2007) 110:2586–92. doi: 10.1182/blood-2007-05-088443
53. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. *Br J Haematol.* (2007) 138:563–79. doi: 10.1111/j.1365-2141.2007.06705.x
54. Ratta M, Fagnoni F, Curti A, Vescovini R, Sansoni P, Oliviero B, et al. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* (2002) 100:230–7. doi: 10.1182/blood.V100.1.230
55. Bahlis NJ, King AM, Kolonias D, Carlson LM, Liu HY, Hussein MA, et al. CD28-mediated regulation of multiple myeloma cell proliferation and survival. *Blood* (2007) 109:5002–10. doi: 10.1182/blood-2006-03-012542
56. Kukreja A, Hutchinson A, Dhodapkar K, Mazumder A, Vesole D, Angitapalli R, et al. Enhancement of clonogenicity of human multiple myeloma by dendritic cells. *J Exp Med.* (2006) 203:1859–65. doi: 10.1084/jem.20052136
57. Chauhan D, Singh AV, Brahmandam M, Carrasco R, Bandi M, Hideshima T, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell* (2009) 16:309–23. doi: 10.1016/j.ccr.2009.08.019
58. Kukreja A, Radfar S, Sun BH, Insogna K, Dhodapkar MV. Dominant role of CD47-thrombospondin-1 interactions in myeloma-induced fusion of human dendritic cells: implications for bone disease. *Blood* (2009) 114:3413–21. doi: 10.1182/blood-2009-03-211920
59. Tucci M, Ciavarella S, Strippoli S, Brunetti O, Dammacco F, Silvestris F. Immature dendritic cells from patients with multiple myeloma are prone to osteoclast differentiation *in vitro*. *Exp Hematol.* (2011) 39:773–83.e1. doi: 10.1016/j.exphem.2011.04.006
60. Tucci M, Stucci S, Strippoli S, Dammacco F, Silvestris F. Dendritic cells and malignant plasma cells: an alliance in multiple myeloma tumor progression? *Oncologist* (2011) 16:1040–8. doi: 10.1634/theoncologist.2010-0327
61. An G, Acharya C, Feng X, Wen K, Zhong M, Zhang L, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. *Blood* (2016) 128:1590–603. doi: 10.1182/blood-2016-03-707547
62. Dhodapkar KM, Barbuto S, Matthews P, Kukreja A, Mazumder A, Vesole D, et al. Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma. *Blood* (2008) 112:2878–85. doi: 10.1182/blood-2008-03-143222
63. Sponaas AM, Moharrami NN, Feyzi E, Standal T, Holth Rustad E, Waage A, et al. PDL1 expression on plasma and dendritic cells in myeloma bone marrow suggests benefit of targeted anti PD1-PDL1 therapy. *PLoS ONE* (2015) 10:e0139867. doi: 10.1371/journal.pone.0139867
64. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* (2007) 110:3499–506. doi: 10.1182/blood-2007-02-069716
65. Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood* (2009) 113:6576–83. doi: 10.1182/blood-2009-02-203943
66. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. *Leukemia* (2013) 27:464–72. doi: 10.1038/leu.2012.213
67. Gorgun G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res.* (2015) 21:4607–18. doi: 10.1158/1078-0432.CCR-15-0200
68. Guillerey C, Ferrari De Andrade L, Vuckovic S, Miles K, Ngiow SF, Yong MC, et al. Immunosurveillance and therapy of multiple myeloma are CD226 dependent. *J Clin Invest.* (2015) 125:2077–89. doi: 10.1172/JCI77181
69. Suen H, Brown R, Yang S, Weatherburn C, Ho PJ, Woodland N, et al. Multiple myeloma causes clonal T-cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* (2016) 30:1716–24. doi: 10.1038/leu.2016.84

70. Brimnes MK, Vangsted AJ, Knudsen LM, Gimsing P, Gang AO, Johnsen HE, et al. Increased level of both $CD4^+FOXP3^+$ regulatory T cells and $CD14^+$HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand J Immunol.* (2010) 72:540–7. doi: 10.1111/j.1365-3083.2010.02463.x
71. Gorgun GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood* (2013) 121:2975–87. doi: 10.1182/blood-2012-08-448548
72. Romano A, Conticello C, Cavalli M, Vetro C, La Fauci A, Parrinello NL, et al. Immunological dysregulation in multiple myeloma microenvironment. *Biomed Res Int.* (2014) 2014:198539. doi: 10.1155/2014/198539
73. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med.* (2003) 197:1667–76. doi: 10.1084/jem.20021650
74. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc Natl Acad Sci USA.* (2008) 105:1285–90. doi: 10.1073/pnas.0711293105
75. Dhodapkar MV. Harnessing host immune responses to preneoplasia: promise and challenges. *Cancer Immunol Immunother.* (2005) 54:409–13. doi: 10.1007/s00262-004-0607-8
76. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. *Blood* (2007) 109:4839–45. doi: 10.1182/blood-2006-10-054221
77. Dhodapkar MV, Sexton R, Das R, Dhodapkar KM, Zhang L, Sundaram R, et al. Prospective analysis of antigen-specific immunity, stem-cell antigens, and immune checkpoints in monoclonal gammopathy. *Blood* (2015) 126:2475–8. doi: 10.1182/blood-2015-03-632919
78. Garfall AL, Stadtmauer EA, Hwang WT, Lacey SF, Melenhorst JJ, Krevvata M, et al. Anti-CD19 CAR T cells with high-dose melphalan and autologous stem cell transplantation for refractory multiple myeloma. *JCI Insight.* (2018) 3:120505. doi: 10.1172/jci.insight.120505
79. Botta C, Di Martino MT, Ciliberto D, Cuce M, Correale P, Rossi M, et al. A gene expression inflammatory signature specifically predicts multiple myeloma evolution and patients survival. *Blood Cancer J.* (2016) 6:e511. doi: 10.1038/bcj.2016.118
80. Musolino C, Allegra A, Innao V, Allegra AG, Pioggia G, Gangemi S. Inflammatory and anti-inflammatory equilibrium, proliferative and antiproliferative balance: the role of cytokines in multiple myeloma. *Mediators Inflamm.* (2017) 2017:1852517. doi: 10.1155/2017/1852517
81. Dhodapkar MV, Richter J. Harnessing natural killer T (NKT) cells in human myeloma: progress and challenges. *Clin Immunol.* (2011) 140:160–6. doi: 10.1016/j.clim.2010.12.010
82. Larsson SC, Wolk A. Body mass index and risk of multiple myeloma: a meta-analysis. *Int J Cancer* (2007) 121:2512–6. doi: 10.1002/ijc.22968
83. Chang DH, Deng H, Matthews P, Krasovsky J, Ragupathi G, Spisek R, et al. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* (2008) 112:1308–16. doi: 10.1182/blood-2008-04-149831
84. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. *Blood* (2013) 121:423–30. doi: 10.1182/blood-2012-06-435503
85. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, et al. Regulation of multiple myeloma survival and progression by CD1d. *Blood* (2009) 113:2498–507. doi: 10.1182/blood-2008-06-161281
86. Nair S, Boddupalli CS, Verma R, Liu J, Yang R, Pastores GM, et al. Type II NKT-TFH cells against Gaucher lipids regulate B-cell immunity and inflammation. *Blood* (2015) 125:1256–71. doi: 10.1182/blood-2014-09-600270
87. Nair S, Branagan AR, Liu J, Boddupalli CS, Mistry PK, Dhodapkar MV. Clonal immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med.* (2016) 374:555–61. doi: 10.1056/NEJMoa1508808
88. Nair S, Sng J, Boddupalli CS, Seckinger A, Chesi M, Fulciniti M, et al. Antigen-mediated regulation in monoclonal gammopathies and myeloma. *JCI Insight* (2018) 3:e98259. doi: 10.1172/jci.insight.98259
89. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* (2007) 110:296–304. doi: 10.1182/blood-2006-10-051482
90. Paiva B, Azpilikueta A, Puig N, Ocio EM, Sharma R, Oyajobi BO, et al. PD-L1/PD-1 presence in the tumor microenvironment and activity of PD-1 blockade in multiple myeloma. *Leukemia* (2015) 29:2110–3. doi: 10.1038/leu.2015.79
91. Yousef S, Marvin J, Steinbach M, Langemo A, Kovacsovics T, Binder M, et al. Immunomodulatory molecule PD-L1 is expressed on malignant plasma cells and myeloma-propagating pre-plasma cells in the bone marrow of multiple myeloma patients. *Blood Cancer J.* (2015) 5:e285. doi: 10.1038/bcj.2015.7
92. Castella B, Foglietta M, Sciancalepore P, Rigoni M, Coscia M, Griggio V, et al. Anergic bone marrow Vgamma9Vdelta2 T cells as early and long-lasting markers of PD-1-targetable microenvironment-induced immune suppression in human myeloma. *Oncoimmunology* (2015) 4:e1047580. doi: 10.1080/2162402X.2015.1047580
93. Benson DMJr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
94. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances *ex vivo* T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother.* (2011) 34:409–18. doi: 10.1097/CJI.0b013e31821ca6ce
95. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29:1441–4. doi: 10.1038/leu.2015.11
96. Kearl TJ, Jing W, Gershan JA, Johnson BD. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. *J Immunol.* (2013) 190:5620–8. doi: 10.4049/jimmunol.1202005
97. Hallett WH, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. *Biol Blood Marrow Transplant.* (2011) 17:1133–45. doi: 10.1016/j.bbmt.2011.03.011
98. Suen H, Brown R, Yang S, Ho PJ, Gibson J, Joshua D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* (2015) 29:1621–2. doi: 10.1038/leu.2015.104
99. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol.* (2013) 25:214–21. doi: 10.1016/j.coi.2012.12.003
100. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
101. Galustian C, Meyer B, Labarthe MC, Dredge K, Klaschka D, Henry J, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. *Cancer Immunol Immunother.* (2009) 58:1033–45. doi: 10.1007/s00262-008-0620-4
102. Luptakova K, Rosenblatt J, Glotzbecker B, Mills H, Stroopinsky D, Kufe T, et al. Lenalidomide enhances anti-myeloma cellular immunity. *Cancer Immunol Immunother.* (2013) 62:39–49. doi: 10.1007/s00262-012-1308-3
103. Costa F, Vescovini R, Bolzoni M, Marchica V, Storti P, Toscani D, et al. Lenalidomide increases human dendritic cell maturation in multiple myeloma patients targeting monocyte differentiation and modulating mesenchymal stromal cell inhibitory properties. *Oncotarget* (2017) 8:53053–67. doi: 10.18632/oncotarget.18085
104. Kronke J, Hurst SN, Ebert BL. Lenalidomide induces degradation of IKZF1 and IKZF3. *Oncoimmunology* (2014) 3:e941742. doi: 10.4161/21624011.2014.941742
105. Thomas RM, Chunder N, Chen C, Umetsu SE, Winandy S, Wells AD. Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in $CD4^+$ T lymphocytes. *J Immunol.* (2007) 179:7305–15. doi: 10.4049/jimmunol.179.11.7305

106. Thomas RM, Chen C, Chunder N, Ma L, Taylor J, Pearce EJ, et al. Ikaros silences T-bet expression and interferon-gamma production during T helper 2 differentiation. *J Biol Chem.* (2010) 285:2545–53. doi: 10.1074/jbc.M109.038794
107. Sehgal K, Das R, Zhang L, Verma R, Deng Y, Kocoglu M, et al. Clinical and pharmacodynamic analysis of pomalidomide dosing strategies in myeloma: impact of immune activation and cereblon targets. *Blood* (2015) 125:4042–51. doi: 10.1182/blood-2014-11-611426
108. Kini Bailur J, Mehta S, Zhang L, Neparidze N, Parker T, Bar N, et al. Changes in bone marrow innate lymphoid cell subsets in monoclonal gammopathy: target for IMiD therapy. *Blood Adv.* (2017) 1:2343–7. doi: 10.1182/bloodadvances.2017012732
109. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* (2016) 17:765–74. doi: 10.1038/ni.3489
110. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates KLRG1(+) group 2 innate lymphoid cells. *J Exp Med.* (2017) 214:1663–78. doi: 10.1084/jem.20161653
111. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib study. *J Clin Oncol.* (2016) 34:2698–704. doi: 10.1200/JCO.2015.65.9789
112. Ribrag V, Avigan DE, Martinelli G, Green DJ, Wise-Draper T, Posada JG, et al. Pembrolizumab monotherapy for relapsed/refractory multiple myeloma: phase 1b keynote-013 study. *Haematologica* (2017) 102:114.
113. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* (2015) 348:69–74. doi: 10.1126/science.aaa4971
114. Mateos MV, Hernandez MT, Giraldo P, De La Rubia J, De Arriba F, Corral LL, et al. Lenalidomide plus dexamethasone versus observation in patients with high-risk smouldering multiple myeloma (QuiRedex): long-term follow-up of a randomised, controlled, phase 3 trial. *Lancet Oncol.* (2016) 17:1127–36. doi: 10.1016/S1470-2045(16)30124-3
115. Wilson L, Cohen AD, Weiss BM, Vogl DT, Garfall AL, Capozzi DL, et al. Pembrolizumab in combination with pomalidomide and dexamethasone (PEMBRO/POM/DEX) for pomalidomide exposed relapsed or refractory multiple myeloma. *Blood* (2016) 128:2119.
116. Badros A, Hyjek E, Ma N, Lesokhin A, Dogan A, Rapoport AP, et al. Pembrolizumab, pomalidomide, and low-dose dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 130:1189–97. doi: 10.1182/blood-2017-03-775122
117. Pawarode A, D'Souza A, Pasquini MC, Johnson B, Braun T, Dhakal B, et al. Phase 2 study of pembrolizumab during lymphodepleted state after autologous hematopoietic cell transplantation in multiple myeloma patients. *Blood* (2017) 130:339.
118. Thanendrarajan S, Puryear J, Schinke CD, van Rhee F, Zangari M, Mathur P, et al. Nivolumab for treatment of advanced, refractory, high-risk multiple myeloma. *Blood* (2017) 130:1858.
119. Biran N, Andrews T, Feinman R, Vesole DH, Richter JR, Zenreich J, et al. A phase II trial of the anti -PD-1 monoclonal antibody pembrolizumab (MK-3475) + lenalidomide plus dexamethasone as post autologous stem cell transplant consolidation in patients with high-risk multiple myeloma. *Blood* (2017) 130:1831.
120. Boddupalli CS, Bar N, Kadaveru K, Krauthammer M, Pornputtapong N, Mai Z, et al. Interlesional diversity of T cell receptors in melanoma with immune checkpoints enriched in tissue-resident memory T cells. *JCI Insight* (2016) 1:e88955. doi: 10.1172/jci.insight.88955
121. Das R, Bar N, Ferreira M, Newman AM, Zhang L, Bailur JK, et al. Early B cell changes predict autoimmunity following combination immune checkpoint blockade. *J Clin Invest.* (2018) 128:715–20. doi: 10.1172/JCI96798
122. Murillo O, Arina A, Hervas-Stubbs S, Gupta A, Mccluskey B, Dubrot J, et al. Therapeutic antitumor efficacy of anti-CD137 agonistic monoclonal antibody in mouse models of myeloma. *Clin Cancer Res.* (2008) 14:6895–906. doi: 10.1158/1078-0432.CCR-08-0285
123. Westwood JA, Matthews GM, Shortt J, Faulkner D, Pegram HJ, Duong CP, et al. Combination anti-CD137 and anti-CD40 antibody therapy in murine myc-driven hematological cancers. *Leuk Res.* (2014) 38:948–54. doi: 10.1016/j.leukres.2014.05.010

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REVIEW
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Role of Osteocytes in Myeloma Bone Disease: Anti-sclerostin Antibody as New Therapeutic Strategy

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Osteocytes are terminally differentiated cells of the osteoblast lineage. They are involved in the regulation of bone remodeling by increasing osteoclast formation or decreasing bone formation by the secretion of the osteoblast inhibitor sclerostin. Monoclonal antibody anti-sclerostin, Romosozumab, has been developed and tested in clinical trials in patients with osteoporosis. In the last years, the role of osteocytes in the development of osteolytic bone lesions that occurs in multiple myeloma, have been underlined. Myeloma cells increase osteocyte death through the up-regulation of both apoptosis and autophagy that, in turn, triggers osteoclast formation, and activity. When compared to healthy controls, myeloma patients with bone disease have higher osteocyte cell death, but the treatment with proteasome inhibitor bortezomib has been shown to maintain osteocyte viability. In preclinical mouse models of multiple myeloma, treatment with blocking anti-sclerostin antibody increased osteoblast numbers and bone formation rate reducing osteolytic bone lesions. Moreover, the combination of anti-sclerostin antibody and the osteoclast inhibitor zoledronic acid increased bone mass and fracture resistance synergistically. However, anti-sclerostin antibody did not affect tumor burden *in vivo* or the efficacy of anti-myeloma drugs *in vitro*. Nevertheless, the combination therapy of anti-sclerostin antibody and the proteasome inhibitor carfilzomib, displayed potent anti-myeloma activity as well as positive effects on bone disease *in vivo*. In conclusion, all these data suggest that osteocytes are involved in myeloma bone disease and may be considered a novel target for the use of antibody-mediated anti-sclerostin therapy also in multiple myeloma patients.

Keywords: osteocytes, multiple myeloma, bone disease, sclerostin, immunotherapy

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INTRODUCTION

Multiple Myeloma (MM) is characterized by uncoupling bone resorption and osteoblast (OB) formation resulting in severe bone formation inhibition leading to osteolytic bone lesions (1). Currently, only osteoclast (OCL) inhibitors such as bisphosphonates (BPs) and the monoclonal antibody anti- receptor activator of nuclear factor-κB ligand (RANKL) denosumab are FDA-approved for the treatment of MM bone disease. To date, studies investigating the bone anabolic effects of anti-MM drugs demonstrated that proteasome inhibitors stimulate osteogenic differentiation of human mesenchymal stromal cells and also improve the viability of osteocytes

reducing apoptosis and autophagic cell death both *in vitro* and *in vivo* (2). Nevertheless, studies investigating new therapeutic targets and approaches that improve bone formation are strongly encouraged.

In recent years, there has been increasing interest in elucidating the role of osteocytes in MM bone disease and in developing new therapeutic strategy that target osteocyte functions. It is a widely accepted notion that osteocytes are involved in the regulation of physiological bone remodeling through the release of molecules that affect OCL and OB function. Moreover, recent studies demonstrated that MM cells induced apoptosis and autophagic cell death in osteocytes contributing to the increased activity of OCLs (2, 3).

Sclerostin (Scl) is a potent Wnt/β-catenin inhibitor secreted by mature osteocytes that control bone formation and resorption (4). Moreover, it has been demonstrated that MM cells increased Scl expression in osteocytes in MM murine models (5, 6) and its levels have been found elevated in MM patients in correlation with abnormal bone remodeling (7).

Indeed, the use of anti-Scl antibody (Scl-Ab) has been explored in experimental animal models of bone disorders demonstrating its efficacy in increasing bone formation and decreasing bone resorption (8, 9). In the clinical setting, the Scl-Abs romosozumab and blosozumab have been efficaciously tested in osteoporotic patients demonstrating potent activity in stimulating bone formation and reducing bone resorption (10, 11). While some research has been carried out on the feasibility of Scl-Ab therapy in MM mouse model, no clinical studies have been yet conducted among MM patients. In this perspective, the notion that Scl-Ab does not affect the activity of currently available anti-MM drugs (8) encourages the use of a combined therapy to treat skeletal disease and tumor progression.

The purpose of this review is to provide an overview of the role of osteocytes in MM bone disease describing the numerous improvements that have been made in this field. We first describe the osteocyte role in physiological bone remodeling as well as the importance of Scl in modulating their activity and functions. Moreover, we discuss the main mechanisms underlie the involvement of osteocytes in MM bone disease and the preclinical use of an immunotherapeutic approach based on Scl-Ab for improving bone disease in patients with MM.

OSTEOCYTES AND BONE REMODELING

Osteocytes are cells belonging to the osteogenic lineage embedded in the bone matrix within the lacuno-canalicular cavities. They are derived from the original rounded OBs through conspicuous morphological and ultrastructural changes, such as reduction in size, in parallel with the formation and elongation of the cytoplasmic processes (12, 13). Osteocytes create an extensive network throughout the skeleton, by means of multiple dendrite-like processes, joining with the other bone cells (OBs/bone lining cells and stromal cells); this functional syncytium, based on interaction through intercellular junctions, is extended from the inner bone to the vascular endothelia (14–16). The bone cells' activity is involved in all bone processes, i.e., bone growth, bone modeling and bone remodeling. Bone remodeling induces bone turnover throughout life, i.e., the continuous skeletal "destruction" and "reconstruction," in a dynamic manner, driven by the activity of osteoclastic and osteogenic cell lineages, thus allowing bone adaptation to both mechanical and metabolic requirements. This process also occurs in repairing skeletal damage, preventing accumulation of brittle hyper-mineralized bone, and maintaining mineral homeostasis by liberating stores of calcium and phosphorus (17). The activities of OCLs and OBs must be strictly regulated to ensure that bone homeostasis is maintained. Osteocytes are considered the key regulators to maintain this balance (18). Recently, signaling pathways by which the osteocyte exerts control over the other bone cells and also the potential ways in which these pathways may be exploited therapeutically have been investigated (19–29). In physiological conditions, the bone remodeling should occur when required. During targeted remodeling, which is the removal of a specific area of old or damaged bone, the initiating signal originates from the osteocytes that use their dendritic network to communicate to other cells (25, 30–33). On the other hand, it has been reported that the osteocyte damage, induced for example through the disruption of bone matrix canaliculi, may lead to release of paracrine factors that increase local angiogenesis and recruitment of OCL and OB precursors (21, 33–35). Other authors have suggested that another possible triggering event of the bone remodeling cycle is osteocyte apoptosis, as the increase of RANKL expression occurs concurrently, thus enhancing the osteoclastogenesis (36–38).

Osteocytes are mechanosensors (39–44) and capable of modulating OCLs and OBs that, together with the capillary blood supply, form the Basic Multicellular Unit (BMU), which is constantly replenished to perform the appropriate bone remodeling (17). To explain the remodeling activation due to mechanical requirements, Palumbo and coworkers (30), proposed a sequence of phases, through which osteocytes coordinate OCL and OB recruitment only when the micro-deformations induced by loading exceed the physiological range (i.e., fall above and below the lower and upper setpoint values, respectively) in mineralized matrix. The osteogenic cell system is organized in the Bone Basic Cellular System-BBCS (16), the functional syncytium formed by osteocytes, bone lining cells and stromal cells. The bone remodeling process is characterized by distinct phases. Under the above conditions, osteocytes drive steady ionic currents (45) outside the bone matrix to maintain the steady state. During unloading or when sensitivity to strain is altered by hormones, such as parathyroid hormone (PTH), estrogens etc, osteocytes stop producing a steady resting state ionic current and the bone lining cells, stromal cells and above all the osteocytes themselves (sensitive to loading changes) produce RANKL, as recently confirmed (27, 46–48) (1st Phase-Resorption). During the progression of erosive activity, the only cells which can inhibit OCLs are the surviving overstrained osteocytes that arrest OCL erosion when the local upper setpoint is exceeded. In this regard, it has been shown that an unexpected high number (about 60%) of osteocytes survive the end of OCL disruption (30). After this, the successive 2nd Phase-Reversion begins and the cells of the reversal phase (probably

of stromal-fibroblast origin) differentiate into OBs. The exact signals that couple bone resorption to subsequent bone formation are not yet fully understood. Various authors believe that the cells of the reversal phase could be involved in sending or receiving these signals (22, 49, 50). It has also been postulated that OCLs may be the source of coupling factors, either secreting cytokines or via regulatory receptors and their membrane bound ligands (51). Other signaling pathways may include matrix derived factors such as bone morphogenic protein (BMP)-2), transforming growth factor β and insulin-like growth factor (19, 26). In the last 3rd Phase-Deposition, bone is progressively rebuilt. When the local strains fall again within the physiological range, the osteocytes in the newly-laid-down bone matrix restore the steady ionic current returning the bone to the resting state, therefore halting OB activity.

Osteocytes play a key role in remodeling modulation via secretion of antagonists of the Wnt signaling pathway, such as Scl (18). Scl, encoded by the gene SOST is secreted by osteocytes and negatively regulates Wnt signaling by binding the co-receptors low-density lipoprotein receptor-related protein (LRP5/6). During the new resting phase, osteocyte expression of the Wnt inhibitors SOST, and DKK-1/2 prevents further bone formation in the quiescent bone, (52, 53). Thus, during the bone remodeling cycle, Scl osteocyte expression declines leading to an OB-mediated new bone formation after bone resorption. Finally, newly formed osteocytes become entombed within the bone matrix and re-express SOST, resulting in cessation of bone formation.

ROLE OF SCLEROSTIN IN THE REGULATION OF BONE REMODELING

Various molecular mechanisms, underlying the osteocyte's regulatory role in response to skeletal and mineral homeostasis, have been reported. As widely described by Sapir-Koren and Livshits (4), three categories of molecules are involved: (i) Scl, due to SOST promoter hypomethylation (54); (ii) the group of "mineralization-related genes," involved in regulating mineralization and phosphate metabolism: dentin matrix protein 1 (DMP1), matrix extracellular phosphor glycoprotein (MEPE), and fibroblast growth factor 23 (FGF23) (18, 55, 56); (iii) proteins encoded by RANKL and OPG genes. Scl is currently considered the major mediator of the molecular osteocyte mechanisms involved in the process of adaptive bone responses. It is a 22-kDa glycoprotein produced by the SOST gene and displays both autocrine and paracrine effects. The SOST gene is mainly expressed in bone cells, although it is also expressed during fetal development in several tissues including cartilage, bone marrow (BM), pancreas, heart, aorta, liver, and kidney. However, postnatal expression of Scl is mostly limited to osteocytes, chondrocytes and cementocytes (57). In the mature skeleton, Scl is mainly synthesized by differentiated mature osteocytes entrapped within the mineralized matrix, while immature osteocytes, embedded in osteoid, bone lining cells and OBs, express very low levels of Scl.

Scl has provided a pivotal step in the knowledge of bone remodeling regulation. This central role is achieved through interplay between two opposing mechanisms: (1) unloading-induced high Scl levels, which simultaneously antagonize canonical Wnt in osteocytes and OBs and promote noncanonical Wnt and/or other pathways in osteocytes and OCLs (20, 58, 59); (2) mechanical loading-induced low Scl levels, that activates Wnt-canonical signaling and bone formation.

Thus, adaptive bone remodeling occurring in different bone compartments is driven by altered Scl levels, which regulate the expression of the other osteocyte-specific proteins, such as RANKL, its decoy receptor osteoprotegerin (OPG), and proteins encoded by "mineralization-related genes" (DMP1, PHEX, and probably FGF23). For example, under specific condition, Scl regulates differential RANKL, and OPG production, and creates a dynamic RANKL/OPG ratio (60–62), leading to either bone formation or resorption. It also controls the expression of PHEX, DMP1, and most likely FGF23 (55), leading to either bone matrix mineralization or its inhibition. Such opposite up- or down-regulation of the remodeling phases allows osteocytes (i.e., the cells always present in bone tissue) to function as "the orchestrators" of OCLs and OBs (i.e., the transient operating cells) ensuring the transition from bone resorption to bone formation. The physiological role of osteocytes and Scl in unloading and loading conditions is summarized in **Figures 1A,B**.

The inhibition of Scl could represent a promising strategy to target bone remodeling and has been investigated since 2009 in mouse and rat bone density disorder models (osteoporosis, rheumatoid arthritis, genetic disorders). In these models the use of Scl-Ab significantly increased bone mineral density (BMD), bone mass and strength and also OB surface while decreasing OCL surface (63, 64). Scl-Ab mechanism of action has been the focus of different studies. Specifically, in nonhuman primate and rat models, the short-term use of anti-Scl therapy improved and prolonged the bone formation by activating bone lining cells, while simultaneously reducing bone resorption (65, 66). In cynos, single dose of Scl-Ab, mimicking intermittent Scl inhibition, induced a rapid increase in serum procollagen type 1 amino-terminal propeptide (P1NP) and osteocalcin which returned to baseline as soon as the antibody was cleared from circulation (65). No increase in the serum levels of bone resorption marker C-telopeptide (CTX) was found in the serum levels, suggesting the anabolic effect of single and short treatment with Scl-Ab.

Interestingly, longer-term treatment resulted in a robust and transient increase in bone formation during the early phase of treatment followed by a progressive reduction. On the contrary, the anti-resorptive effects remained detectable throughout the whole period (67).

Expression analysis performed by microarray and TaqMan analysis on isolated OBs, bone-lining cells, and osteocytes isolated from both short-term and long-term Scl-Ab treated ovariectomized rats revealed the mechanisms underlying the bone response to Scl inhibition. Short-term treatment resulted in upregulated expression of canonical Wnt target genes: *Wisp1,* a negative regulator of bone resorption, and *Twist1* an inhibitor of bone formation. In the same conditions, an increased

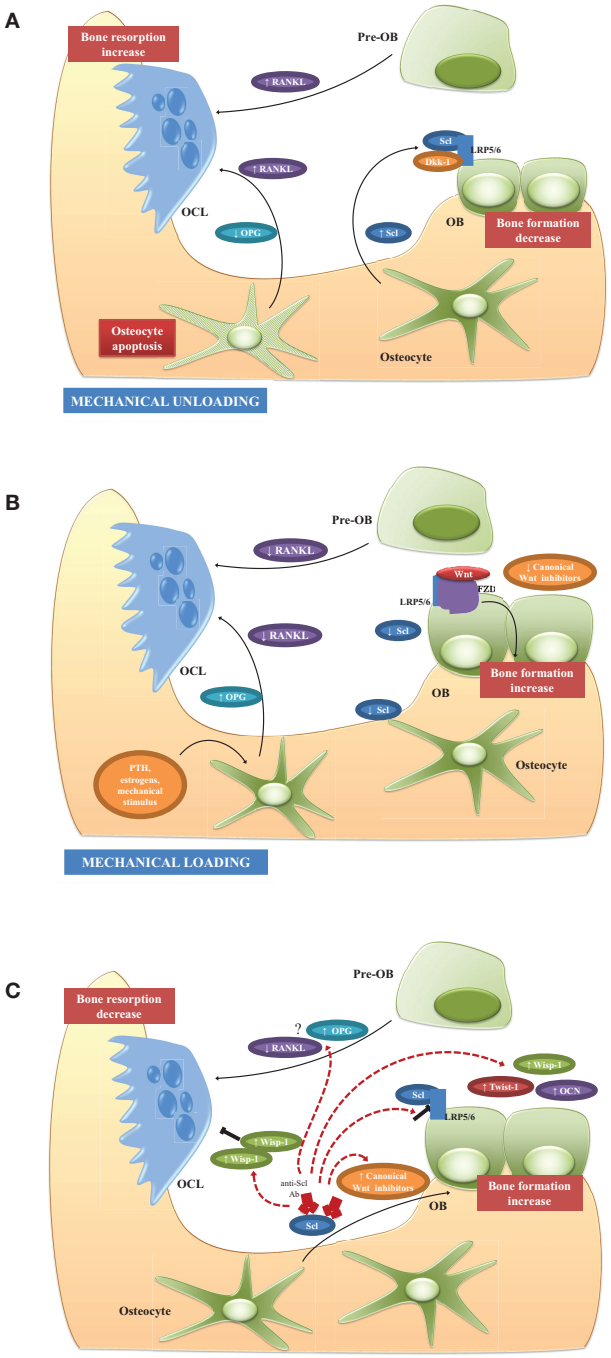

FIGURE 1 | Physiological role of osteocytes and Scl and potential mechanism of action of Scl-Ab in BM microenvironment. **(A)** Unloading conditions induce the production of high Scl levels which, in turn, promote the production of RANKL and decrease OPG with consequent increased RANKL/OPG ratio, osteoclastogenesis, and enhanced bone resorption. Another possible triggering event of RANKL release in BM microenvironment is the osteocyte apoptosis. Simultaneously, high Scl inhibits Wnt signaling and osteoblast formation. **(B)** Mechanical loading and other factors, such as PTH and estrogens, suppress Scl expression with the consequent induction of Wnt signaling and enhanced bone formation. The production of high levels of OPG and the reduction of RANKL production lead to the suppression of *(Continued)*

FIGURE 1 | resorption-associated activities. **(C)** Scl inhibition stimulates bone formation and reduces bone resorption by different mechanisms. Firstly, by blocking the binding between Scl and LRP5/6, Scl-Ab activates a set of Wnt target genes associated with bone formation and resorption (*Wisp* and *Twist*) and increased expression of extracellular matrix proteins, such as osteocalcin. The increased of Twist, an inhibitor of bone formation, limits the early response to Scl inhibition, whereas Wisp, a negative bone resorption, sustains the anti-osteoclastogenic activity. The feedback mechanisms following Scl inhibition, is associated with increased levels of Wnt antagonist to attenuate the bone-forming response and prevent excessive bone accrual. Although the anti-resorptive activity is demonstrated in animal studies and in clinical trials, the regulation of osteoclastogenic factors, such as RANKL and OPG, is not clearly and need to be elucidated in further studies. See text for details.

expression of all three osteogenic cell types of extracellular matrix and mineralization genes, such as *Bglap*, has also been observed within the first week of treatment (67, 68). Probably, the upregulation of *Twist* may limit the stimulatory response following Scl-Ab treatment. The progressive upregulation of matrix genes in lining cells supports the notion that Scl-Ab therapy differentiates lining cells into matrix-producing OBs on the quiescent surface without prior bone resorption (model-based bone formation) (68).

Interestingly, during extended treatment, at the time of peak bone formation rate, there was a decrease in the number of osteoprogenitor cells with a concomitant change in the global gene expression of osteocytes. In particular, *Twist1* returned to baseline levels while *Wisp1* remained increased suggesting a switch from anabolic to anti-anabolic expression profile in response to longer Scl-Ab treatment (67). The anti-resorptive activity of long-term treatment seemed to be accompanied by a reduction of *Csf1, a* gene encoding OCL regulator Macrophage Colony Stimulating Factor 1 (MCSF1), and an increased *Opg* expression (67). Further studies are needed to clarify how Scl-Ab modulates bone resorption since some authors reported the lack of modulation of RANKL, OPG, and other regulators of osteoclastogenesis during Scl-Ab treatment (68, 69).

Further pathways that inhibit canonical Wnt signaling such as Hippo, noncanonical Wnt (e.g., Wnat5b) and transforming growth factor (TGF)-β are significantly modulated by long-term treatment. These changes are likely driven by (i) increased p53, (ii) decreased c-Myc, and (iii) induction of Wnt inhibitors production dickkopf (Dkk)-1 and Scl, resulting in a self-regulated inhibition of bone formation to prevent excessive bone accrual (67, 70). The main effects on the BM microenvironment during treatment with Scl-Ab are illustrated in **Figure 1C**.

CLINICAL STUDIES WITH SCL-AB IN SKELETAL DISEASE

Given the numerous findings regarding the involvement of Scl in bone remodeling and bone disease, humanized Scl-Abs antibodies have been developed.

Romosozumab (AMG 785; Amgen, Thousand Oaks, CA, USA, and UCB, Belgium) is a humanized monoclonal IgG2 antibody with high specificity for human Scl. It has been

investigated as bone-forming drug among osteoporotic patients with increased risk of fractures. The first clinical study was a phase I randomized, double blind trial conducted in a cohort of healthy men and postmenopausal women (71) The subjects were randomized to receive subcutaneous or intravenous romosozumab or placebo. Administration of romosozumab was accompanied by an increase of serum levels of bone formation markers P1NP, bone-specific alkaline phosphatase (BSAP), osteocalcin, and decreased bone resorption CTX (71) compared with placebo. The study of romosozumab effects on trabecular and cortical bone was assessed in subject with low bone mass in phase I-II studies. The authors observed a significant improvement in vertebral trabecular and cortical bone maintained during the off-treatment follow-up period (72, 73). Moreover, romosozumab was superior to the bisphosphonate aldronate and teriparatide, in increasing bone formation and reducing bone resorption. Romosozumab administration, in phase a III trial, was associated with a lower risk of vertebral and clinical fractures as compared with placebo treatment. A more recent study compared the effectiveness of starting with romosozumab and transitioning to antiresorptive agent alendronate vs. alendronate alone in reducing the risk of fracture among postmenopausal women with osteoporosis (74). Treatment with romosozumab before aledronate reduced the risks of a new vertebral, clinical, nonvertebral, and hip fracture compared to alendronate alone associated with a rapid gain in BMD.

Blosozumab (Eli Lilly and Company, Indianapolis, IN, USA) is a humanized monoclonal IgG4 antibody targeted against Scl that displayed similar bone anabolic properties to romosozumab. Specifically, results of a randomized, placebo-controlled phase II clinical trial in postmenopausal women with low BMD demonstrated that blosozumab increased bone formation and spine and total hip BMD, while decreasing bone resorption (10).

The significant decrease in biochemical markers of bone resorption observed with both drugs may be related to a decreased RANKL and increased OPG levels, with a reduction in the RANKL/OPG ratio and in bone resorption.

BPS804 (Novartis, Basel, Switzeland) is a human IgG2 Scl-Ab being evaluated in clinical trials for osteogenesis imperfecta (OI) has demonstrated a stimulatory effect on bone formation and inhibitory effect on bone resorption (75).

Some limitation for the use of both drugs, came from the studies reporting a reduction of circulating bone formation and resorption markers to baseline levels within a year (10, 73). This effect may be partly due to a Scl-independent bone response: the reduced stresses and strains within the skeleton following the new bone formation, determines a reduction of positive signal for bone formation (76). In addition, Dkk-1, which is upregulated in Scl deficiency (77) might reduce bone formation as a compensatory mechanism in the absence of Scl. Moreover, these Scl-Abs showed immunogenic properties leading to the development of anti-drug antibody (ADA) even after short-term treatment (71, 75). However, this phenomenon does not affect the pharmacodynamics and pharmacokinetic properties and does not induce adverse effects. Both pre-clinical and clinical data showed that Scl-Ab administration increased the expression of SOST and the level of serum Scl that decreased after discontinuation. These effects might be due to either the formation of Ab-Scl complex or the presence of a feedback mechanism by which the blockade of Scl triggers its production (67, 78).

MYELOMA BONE DISEASE

Bone remodeling alteration is one of the hallmarks of MM (79). In this hematological malignancy, the plasma cell accumulation into the BM leads to bone destruction due to a severe unbalanced and uncoupled bone remodeling (80, 81) Indeed an increase of OCL enrollment and activity together with a deep OB suppression have been shown in MM patients (80, 81). MM bone disease occurs in about 80% of MM patients at diagnosis (82), resulting in pathological fractures, spinal cord compression and pain, significantly impacting their quality of life (80, 81). BPs, such as zoledronic acid and pamidronate, are the current treatments of choice for MM bone disease. BPs bind avidly to bone matrix and are incorporated into areas of active bone remodeling (83). During bone resorption OCLs incorporate BPs, leading to reduced OCL recruitment, maturation and activity (83).

Either soluble factors or the cell-to-cell contacts between MM and microenvironment cells are involved in bone alterations, resulting in the stimulation of OCL formation and activity, and inhibition of OB differentiation. These alterations of BM microenvironment and, consequently MM bone disease development, provide a permissive niche that promotes growth and survival of MM cells (80, 81). Several cytokines and chemokines contribute to the bone remodeling alterations in MM. These soluble factors are directly released by MM cells and/or produced by stromal and osteoprogenitor cells after interaction with MM cells. Indeed, the cell-to-cell interaction with MM cells, upregulates RANKL while downregulates OPG production in stromal cells, sustaining OCL recruitment and survival (80, 81). Furthermore chemokine (C-C motif) ligand (CCL)-3, interleukin (IL)-1, IL-3, IL-6, activin A, and tumor necrosis factor (TNF) α are known to be upregulated into the MM BM microenvironment and involved in OCL formation (80, 81, 84–86).

The interaction between MM cells and stromal cells also inhibits in stromal cells the activity of Runx2, the main pro-osteoblastogenic transcription factor, leading to the suppression of OB differentiation (87). Moreover, MM patients show high BM levels of cytokines such as IL-7 and HGF that contribute to the Runx2 inhibition and osteoblastogenesis decrease (88, 89). Together with their role in MM-induced enhanced osteoclastogenesis, IL-3 and Activin A also have a role in OB inhibition in MM patients (90, 91). Lastly, it has been shown that MM patients have high BM level of several Wnt signaling inhibitors such as Dkk-1, soluble frizzled related protein (sFRP)-2, and sFRP-3, that contribute to MM-induced OB suppression and MM bone disease (80, 88, 92–94).

OSTEOCYTE AND MYELOMA BONE DISEASE

As described above, bone destruction in MM relies upon the exchange of soluble factors as well as the interactions between MM cells and OCLs and OBs. Nevertheless, little is known about the interplay between MM cells and osteocytes and their role in MM bone disease. A preliminary paper by Eisenberger et al. (95) presented a transcriptome analysis of the *in vivo* effects of MM cells on osteocytes. The study clearly demonstrated that MM-induced stress generated specific gene expression footprints in osteocytes. More recently, a histological study performed on human bone biopsies, revealed that MM patients were characterized by increased osteocyte death and fewer viable osteocytes when compared with healthy controls (3). Moreover, the presence of osteolysis in MM patients correlated with the increased osteocyte death, probably due to increased osteocyte apoptosis. Interestingly, MM patients, when compared to healthy controls or monoclonal gammopathy of undetermined significance (MGUS) patients, showed a higher number of OCLs negatively correlating with the number of viable osteocytes. The same study showed that in a co-culture system, MM cells upregulated the production of pro-osteoclastogenic molecules such as IL-11, Matrix metalloproteinase-1 (MMP-1), and CCL3/macrophage inflammatory proteins (MIP)-1α by preosteocytes (3). Indeed, the conditioned media of these co-cultures increased the *in vitro* OCL formation that was inhibited by the presence of anti-CCL3 and anti-IL11 antibodies. The immunohistochemical analysis of bone biopsies showed that the osteocytic expression of IL-11 was higher in osteolytic MM patients when compared to non-osteolytic ones, even though there were no differences between MM and MGUS patients. Later, the same group demonstrated that MM cells induced autophagic cell death in co-cultured osteocytes, thus supporting the notion that other mechanisms, other than apoptosis, underlie the role of osteocytes in MM bone disease (2).

Osteocytes are in direct contact with MM cells in MM-bearing mice and so, these interactions increase apoptosis and the production of RANKL and Scl by osteocytes (5). *In vitro* experiments demonstrated that the activation of Notch signaling underlined the increased osteocytic apoptosis resulting in: (1) increased expression of RANKL and ability of osteocytes to recruit OCL precursors, and (2) increased production of Scl, which in turn inhibits Wnt signaling and OB differentiation. No less important, this physical interaction induces the reciprocal activation of Notch pathway in osteocytes and MM cells, supporting the growth, and proliferation of tumor cells (5). One of the possible MM-factors responsible for increased osteocyte death was TNF-α, as recombinant TNF-α increased osteocyte apoptosis and neutralizing anti-human TNFα antibody blocked the MM-induced reduction of osteocyte viability (5).

Together these data suggest that, in MM-colonized bone, osteocytes are responsible for the increased OCL recruitment as well as the inhibition of bone formation through cell-to-cell interactions and release of soluble factors. The complex interplay between MM cells and osteocytes is shown in **Figure 2**.

OSTEOCYTE AS THERAPEUTIC TARGETS IN MM

The recent improvements in the knowledge of osteocyte role in MM bone disease, have raised the possibility of targeting osteocytes as new therapeutic strategy to treat bone disease. Different studies sought to determine the effects of the main anti-MM drugs, such as proteasome inhibitors (PIs), as well as anti-resorptive agents BPs, and PTH on osteocytes. The first observation came from a study by Terpos et al. reporting a reduction of serum levels of Scl in MM patients receiving four cycles of bortezomib monotherapy. On the basis of this evidence, Toscani et al. investigated the effect of bortezomib therapy on osteocyte viability on BM biopsies taken from MM patients. Interestingly, patients treated with a bortezomib-based regimen showed a significant higher number of viable osteocytes compared with those treated without bortezomib. Additionally, bortezomib counterbalanced the negative effect of dexamethasone on osteocyte viability. A similar reduction of apoptotic osteocytes was also observed (2). In keeping with data described above reporting the ability of MM cells to induce autophagic cells death in cocultured osteocytes, *ex vivo* analysis showed that patients treated with bortezomib had a reduction of autophagic osteocytes compared with controls treated without bortezomib thus confirming the great impact of proteasome inhibition in preventing osteocyte death. *In vitro,* PIs were also able to block osteocyte death induced by MM cells and high doses of dexamethasone by inhibiting the activation of the autophagic pathway and the formation of autophagosome (2). Also, BPs are able to target osteocytes. It has been reported that osteoporotic patients treated with BPs had increased levels of serum Scl and reduced bone turnover markers (96). The mechanism by which BPs might modulate Scl levels remains unclear. It has been suggested that BPs induce the accumulation of Scl-secreting OCL precursors (96). Others linked the effect of BPs on Scl levels to the anabolic effects of intermittent PTH (97, 98).

PRECLINICAL STUDIES WITH ANTI-SCL ANTIBODY IN MM

Several clinical studies showed that patients with active MM were characterized by high levels of circulating Scl, which correlated with the presence of osteolytic fractures, disease stage and biochemical markers of bone remodeling (7, 99). There are controversial reports regarding the cellular origin of Scl in MM. Some authors showed that MM cells directly produced Scl (100) or were able to induce its production by osteocytes (5, 6). Nevertheless, Giuliani et al. did not find any significant difference in the expression of Scl in bone biopsies of MM patients (3).

More recently, Eda et al. identified spindle-shaped BM stromal cells and OBs as the main source of Scl in BM biopsy samples from MM patients (101), suggesting that, other than osteocytes, these cells are responsible for the increased levels of Scl in MM patients.

Delgado-Calle et al. generated a MM immunodeficient mouse model with a global deletion of SOST (Sost–/–) injected with

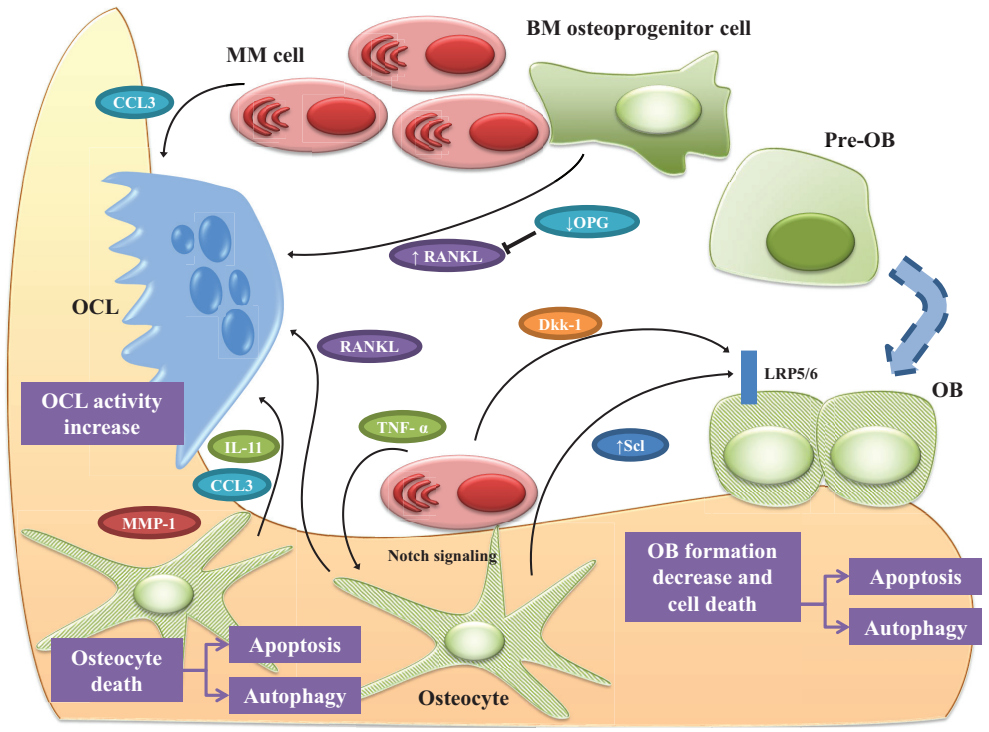

FIGURE 2 | Osteocyte role in MM bone disease. Bone destruction in MM rely up the exchange of soluble factors as well as the interactions between MM cells and OCLs and OBs. Osteocytes play a pivotal role in orchestrating this interplay. Cell-to cell interaction with MM cells, upregulates RANKL while downregulates OPG in osteoprogenitor cells, thus stimulating OCL survival. Under MM stimuli, osteocytes and OBs undergo apoptosis and autophagic cell death. In this scenario, osteocytes produce the pro-osteoclastogenic factors IL-11, CCL3, and MMP1 increasing OCL activity. The physical contact between MM cells and osteocytes induce the reciprocal activation of Notch pathway resulting in increased expression of RANKL, which stimulates OCL, and Scl, which suppress bone formation by osteocytes as well as MM cells growth and osteocyte apoptosis. TNF-α produced by MM cells exacerbated these effects. The effects of MM cells on osteocytic expression of Scl is controversial since some authors reported that osteocytes isolated from tumor-bearing mice expressed lower Scl than non-tumor bearing mice. Moreover, MM cells induce the expression of Scl in OBs via secretion of Dkk-1. See text for details.

MM cells. Interestingly, the mice displayed decreased osteolysis and improved bone loss compared with wild type mice, without affecting MM growth (8). Moreover, whereas MM-injected wt mice displayed reduced bone surface and OB number, MM-injected Sost–/– mice did not display a reduction in the number or function of OBs suggesting that Scl is involved in the OB suppression induced by MM cells.

For further insight into the cellular effects of Scl inhibition, the authors treated an established MM immune-competent mouse model with Scl-Ab.

Mice receiving Scl-Ab showed reduced osteolysis and increased bone formation compared with mice receiving control IgG, no differences in MM growth and with a modest effect on OCLs (8). Furthermore, the increased bone volume was present in mice with both low and high tumor burden suggesting that the anabolic effect is independent of tumor cells presence.

By using a human MM xenograft mouse model, Eda et al. showed that, compared to controls, MM-bearing mice presented high levels of mouse Scl, together with the inhibition of activated β-catenin expression in bone (101).

The treatment with Scl-Ab determined an increase of bone volume and bone formation markers osteocalcin and P1NP as well as the increase of β-catenin staining in xenograft mouse bones. Interestingly, the combination therapy with carfilzomib increased bone formation together with important reduction of tumor burden when compared with mice treated with carfilzomib alone. Moreover, MM cells induced the expression of SOST in cocultured mature human OBs, via secretion of Dkk-1, and the treatment with neutralizing Scl-Ab blocked MM-induced OB suppression. Importantly, neutralizing Dkk-1 antibody blocked SOST upregulation induced by MM while recombinant Dkk-1 increased SOST expression in immature and mature OBs (101). RNA-seq analysis performed on osteocytes isolated from non-tumor bearing mice revealed that these cells expressed *Sost*, *Dkk1* and other Wnt antagonist such as *Sfrp1*, *Sfrp2* and frizzled-b (*Frzb*) (6). In contrast with previous results, the expression of *Sost* and *Dkk1* decreased in osteocytes isolated from tumor–bearing mice compared to naive non-tumor–bearing mice. This suggests that osteocytes respond differently in presence of MM cells although further studies are needed to clarify this aspect.

Given the data demonstrating that Dkk-1 is a direct transcriptional target of β-catenin (102), Florio et el. measured Dkk-1 expression in whole-bone lysate in SOST knockout mice

and mice treated with Scl-Ab. Dkk-1 was found significantly upregulated after Scl-Ab treatment probably due to a negative feedback regulation to prevent excessive bone accrual (70).

A bispecific antibody against Scl and Dkk-1 has been developed recently. In rat, mice and primates, the treatment increased bone mass and bone strength, and improved fracture repair while decreasing bone resorption. These effects were associated with a consistent upregulation of osteoblastic genes *Dkk1, Bglap, Opg,* and *Runx2* and osteocyte activity markers *SOST* and *MEPE* (70). Furthermore, treatment with a bispecific antibody induced a compensatory increase in other secreted Wnt antagonists such as WIF1 and SFRP4, thus suggesting a feedback regulation.

In view of a more realistic therapeutic strategy combining Scl-Ab and available anti-MM drugs, several groups are spending resources in this field. The *in vitro* treatment of MM cells with Scl-Ab in combination with anti-MM drugs, such as bortezomib and dexamethasone, did not affect their anti-MM activity thus promoting the use of combination therapy to improve bone disease and inhibit tumor progression (101). Lastly, a combination therapy of Scl-Ab and zoledronic acid and other anti-MM drugs has been tested. Delgado-Calle et al. demonstrated that Scl-Ab therapy did not impact negatively the anti-MM efficacy of Bortezomib and Dexamethasone *in vitro* (8), while others reported a superior effect of Scl-Ab combined with Zoledronic Acid in increasing bone volume and resistance to fracture *in vivo* (6). This data emphasizes (i) the importance of targeting Scl to improve bone disease in patients with skeletal disorders, (ii) the efficacy of therapies combining Scl-Ab and anti-MM drugs and antiresorptive agents, (iii) the feasibility of evaluating combinatory treatment in clinical studies in patients with MM.

CONCLUSIONS

MM patients'quality of life is strongly affected by the high incidence of bone pain, fractures and other skeletal-related events. Currently, few therapies are approved for the treatment of MM bone disease strongly encouraging the identification of new therapeutic approaches. Together with the physiological role of osteocytes in bone remodeling, recent studies highlight the involvement of osteocyte-MM cell interaction in the pathogenesis of MM bone disease. Numerous reports demonstrated that Scl, an inhibitor of canonical Wnt pathway, is a negative regulator of bone formation and plays a pivotal role in MM bone alterations thus supporting the use of anti-Scl therapy for the treatment of skeletal disease. Scl-Abs have been recently developed showing a good bone anabolic response in osteoporotic patients. Nevertheless, this anabolic effect is transient and followed by anti-catabolic effect with a net increase in bone mass. So far, there are no clinical trials in MM patients but numerous preclinical models of MM demonstrated that the use of Scl-Ab stimulated bone formation. Some concerns came from the controversial observations on the modulation of osteoclastogenic factors as well as increased levels of other Wnt antagonists that counterbalance the inhibition of Scl.

Moreover, since Scl-Ab induced strong bone anabolic responses, it is possible that, prolonged stimulation of bone formation, might cause bony overgrowth and skeletal complications.

Since the levels of Scl change in different diseases and with age, an antibody dose titration might be required. Moreover, the relevance of the increased levels of Scl after Scl-Ab treatment need to be clarified especially considering that other cell types, beyond osteocytes, produce Scl. It is conceivable that, upon inhibition of Scl, other cells are stimulated to produce Scl as a feedback mechanism. Lastly, the effects of ADA on the efficacy of drugs in patients treated with Scl-Ab therapy should be considered. To conclude, the immunotherapy approach targeting Scl appears to be promising also for the treatment of MM bone disease.

AUTHOR CONTRIBUTIONS

DT, MF, CP wrote the manuscript. NG and MB organized and edited the manuscript.

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REFERENCES

1. Roodman GD. Pathogenesis of myeloma bone disease. *J Cell Biochem.* (2010) 109:283–91. doi: 10.1002/jcb.22403
2. Toscani D, Palumbo C, Dalla Palma B, Ferretti M, Bolzoni M, Marchica V, et al. The Proteasome inhibitor Bortezomib maintains Osteocyte viability in multiple myeloma patients by reducing both apoptosis and autophagy: a new function for proteasome inhibitors. *J Bone Miner Res.* (2016) 31:815–27. doi: 10.1002/jbmr.2741
3. Giuliani N, Ferretti M, Bolzoni M, Storti P, Lazzaretti M, Dalla Palma B, et al. Increased osteocyte death in multiple myeloma patients: role in myeloma-induced osteoclast formation. *Leukemia* (2012) 26:1391–401. doi: 10.1038/leu.2011.381
4. Sapir-Koren R, Livshits G. Osteocyte control of bone remodeling: is sclerostin a key molecular coordinator of the balanced bone resorption-formation cycles? *Osteoporos Int.* (2014) 25:2685–700. doi: 10.1007/s00198-014-2808-0

5. Delgado-Calle J, Anderson J, Cregor MD, Hiasa M, Chirgwin JM, Carlesso N, et al. Bidirectional notch signaling and Osteocyte-derived factors in the bone marrow microenvironment promote tumor cell proliferation and bone destruction in multiple myeloma. *Cancer Res.* (2016) 76:1089–100. doi: 10.1158/0008-5472.CAN-15-1703
6. McDonald MM, Reagan MR, Youlten SE, Mohanty ST, Seckinger A, Terry RL, et al. Inhibiting the osteocyte-specific protein sclerostin increases bone mass and fracture resistance in multiple myeloma. *Blood* (2017) 129:3452–64. doi: 10.1182/blood-2017-03-773341
7. Terpos E, Christoulas D, Katodritou E, Bratengeier C, Gkotzamanidou M, Michalis E, et al. Elevated circulating sclerostin correlates with advanced disease features and abnormal bone remodeling in symptomatic myeloma: reduction post-bortezomib monotherapy. *Int J Cancer* (2012) 131:1466–71. doi: 10.1002/ijc.27342
8. Delgado-Calle J, Anderson J, Cregor MD, Condon KW, Kuhstoss SA, Plotkin LI, et al. Genetic deletion of Sost or pharmacological inhibition of sclerostin prevent multiple myeloma-induced bone disease without affecting tumor growth. *Leukemia* (2017) 31:2686–94. doi: 10.1038/leu.2017.152
9. Ominsky MS, Boyce RW, Li X, Ke HZ. Effects of sclerostin antibodies in animal models of osteoporosis. *Bone* (2017) 96:63–75. doi: 10.1016/j.bone.2016.10.019
10. Recker RR, Benson CT, Matsumoto T, Bolognese MA, Robins DA, Alam J, et al. A randomized, double-blind phase 2 clinical trial of blosozumab, a sclerostin antibody, in postmenopausal women with low bone mineral density. *J Bone Miner Res.* (2015) 30:216–24. doi: 10.1002/jbmr.2351
11. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, et al. Romosozumab treatment in Postmenopausal women with osteoporosis. *N Engl J Med.* (2016) 375:1532–43. doi: 10.1056/NEJMoa1607948
12. Palumbo C, Palazzini S, Zaffe D, Marotti G. Osteocyte differentiation in the tibia of newborn rabbit: an ultrastructural study of the formation of cytoplasmic processes. *Acta Anat.* (1990) 137:350–8. doi: 10.1159/000146907
13. Buenzli PR, Sims NA. Quantifying the osteocyte network in the human skeleton. *Bone* (2015) 75:144–50. doi: 10.1016/j.bone.2015.02.016
14. Palumbo C, Palazzini S, Marotti G. Morphological study of intercellular junctions during osteocyte differentiation. *Bone* (1990) 11:401–6. doi: 10.1016/8756-3282(90)90134-K
15. Palazzini S, Palumbo C, Ferretti M, Marotti G. Stromal cell structure and relationships in perimedullary spaces of chick embryo shaft bones. *Anat Embryol.* (1998) 197:349–57. doi: 10.1007/s004290050145
16. Marotti G. The osteocyte as a wiring transmission system. *J Musculoskelet Neuronal Interact.* (2000) 1:133–6.
17. Kenkre JS, Bassett J. The bone remodelling cycle. *Ann Clin Biochem.* (2018) 55:308–27. doi: 10.1177/0004563218759371
18. Bonewald LF. The amazing osteocyte. *J Bone Miner Res.* (2011) 26:229–38. doi: 10.1002/jbmr.320
19. Matsuo K, Otaki N. Bone cell interactions through Eph/ephrin: bone modeling, remodeling and associated diseases. *Cell Adh Migr.* (2012) 6:148–56. doi: 10.4161/cam.20888
20. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* (2013) 19:179–92. doi: 10.1038/nm.3074
21. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell… and more. *Endocr Revi.* (2013) 34:658–690. doi: 10.1210/er.2012-1026
22. Delaisse JM. The reversal phase of the bone-remodeling cycle: cellular prerequisites for coupling resorption and formation. *Bonekey Rep.* (2014) 3:561. doi: 10.1038/bonekey.2014.56
23. Lanske B, Densmore MJ, Erben RG. Vitamin D endocrine system and osteocytes. *Bonekey Rep.* (2014) 3:494. doi: 10.1038/bonekey.2013.228
24. Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. *Bonekey Rep.* (2014) 3:481. doi: 10.1038/bonekey.2013.215
25. Goldring SR. The osteocyte: key player in regulating bone turnover. *RMD Open* (2015) 1(Suppl. 1):e000049. doi: 10.1136/rmdopen-2015-000049
26. Sims NA, Martin TJ. Coupling signals between the Osteoclast and Osteoblast: how are messages transmitted between these temporary visitors to the bone surface? *Front. Endocrinol.* (2015) 6:41. doi: 10.3389/fendo.2015.00041
27. Xiong J, Piemontese M, Onal M, Campbell J, Goellner JJ, Dusevich V, et al. Osteocytes, not Osteoblasts or lining cells, are the main source of the RANKL required for Osteoclast formation in remodeling bone. *PLoS ONE* (2015) 10:e0138189. doi: 10.1371/journal.pone.0138189
28. Bassett JH, Williams GR. Role of Thyroid hormones in skeletal development and bone maintenance. *Endocr Rev.* (2016) 37:135–87. doi: 10.1210/er.2015-1106
29. Prideaux M, Findlay DM, Atkins GJ. Osteocytes: the master cells in bone remodelling. *Curr Opin Pharmacol.* (2016) 28:24–30. doi: 10.1016/j.coph.2016.02.003
30. Palumbo C, Ferretti M, Ardizzoni A, Zaffe D, Marotti G. Osteocyte-osteoclast morphological relationships and the putative role of osteocytes in bone remodeling. *J Musculoskelet Neuronal Interact.* (2001) 1:327–32.
31. Burr DB. Targeted and nontargeted remodeling. *Bone* (2002) 30:2–4. doi: 10.1016/S8756-3282(01)00619-6
32. Parfitt AM. Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. *Bone* (2002) 30:5–7. doi: 10.1016/S8756-3282(01)00642-1
33. Atkins GJ, Findlay DM. Osteocyte regulation of bone mineral: a little give and take. *Osteoporos Int.* (2012) 23:2067–79. doi: 10.1007/s00198-012-1915-z
34. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.* (2007) 5:464–75. doi: 10.1016/j.cmet.2007.05.001
35. Chen H, Senda T, Kubo KY. The osteocyte plays multiple roles in bone remodeling and mineral homeostasis. *Med Mol Morphol.* (2015) 48:61–8. doi: 10.1007/s00795-015-0099-y
36. Noble BS, Peet N, Stevens HY, Brabbs A, Mosley JR, Reilly GC, et al. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol.* (2003) 284:C934–43. doi: 10.1152/ajpcell.00234.2002
37. Kogianni G, Mann V, Noble BS. Apoptotic bodies convey activity capable of initiating osteoclastogenesis and localized bone destruction. *J Bone Miner Res.* (2008) 23:915–27. doi: 10.1359/jbmr.080207
38. Capulli M, Paone R, Rucci N. Osteoblast and osteocyte: games without frontiers. *Arch Biochem Biophys.* (2014) 561:3–12. doi: 10.1016/j.abb.2014.05.003
39. Bonewald L. Osteocytes as multifunctional cells. *J Musculoskelet Neuronal Interact.* (2006) 6:331–3.
40. Bonewald LF. Mechanosensation and transduction in osteocytes. *Bonekey Osteovision* (2006) 3:7–15. doi: 10.1138/20060233
41. *Bone*wald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. *Bone* (2008) 42:606–15. doi: 10.1016/j.bone.2007.12.224
42. Lau E, Al-Dujaili S, Guenther A, Liu D, Wang L, You L. Effect of low-magnitude, high-frequency vibration on osteocytes in the regulation of osteoclasts. *Bone* (2010) 46:1508–15. doi: 10.1016/j.bone.2010.02.031
43. Rochefort GY, Pallu S, Benhamou CL. Osteocyte: the unrecognized side of bone tissue. *Osteoporos Int.* (2010) 21:1457–69. doi: 10.1007/s00198-010-1194-5
44. Iolascon G, Resmini G, Tarantino U. Mechanobiology of bone. *Aging Clin Exp Res.* (2013) 25(Suppl. 1):S3–7. doi: 10.1007/s40520-013-0101-2
45. Rubinacci A, Villa I, Dondi Benelli F, Borgo E, Ferretti M, Palumbo C, et al. Osteocyte-bone lining cell system at the origin of steady ionic current in damaged amphibian bone. *Calcif Tissue Int.* (1998) 63:331–9. doi: 10.1007/s002239900536
46. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med.* (2011) 17:1231–4. doi: 10.1038/nm.2452
47. Amarasekara DS, Yun H, Kim S, Lee N, Kim H, Rho J. Regulation of Osteoclast differentiation by cytokine networks. *Immune Netw.* (2018) 18:e8. doi: 10.4110/in.2018.18.e8
48. Luo G, Li F, Li X, Wang ZG, Zhang B. TNFalpha and RANKL promote osteoclastogenesis by upregulating RANK via the NFkappaB pathway. *Mol Med Rep.* (2018) 17:6605–11. doi: 10.3892/mmr.2018.8698
49. Everts V, Delaisse JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J Bone Miner Res.* (2002) 17:77–90. doi: 10.1359/jbmr.2002.17.1.77
50. Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. *J Biol Chem.* (2010) 285:25103–8. doi: 10.1074/jbc.R109.041087

51. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab.* (2006) 4:111–21. doi: 10.1016/j.cmet.2006.05.012
52. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* (2012) 149:1192–205. doi: 10.1016/j.cell.2012.05.012
53. Williams BO. Insights into the mechanisms of sclerostin action in regulating bone mass accrual. *J Bone Miner Res.* (2014) 29:24–8. doi: 10.1002/jbmr.2154
54. Delgado-Calle J, Sanudo C, Bolado A, Fernandez AF, Arozamena J, Pascual-Carra MA, et al. DNA methylation contributes to the regulation of sclerostin expression in human osteocytes. *J Bone Miner Res.* (2012) 27:926–37. doi: 10.1002/jbmr.1491
55. Feng JQ, Ward LM, Liu SG, Lu YB, Xie YX, Yuan BZ, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet.* (2006) 38:1310–5. doi: 10.1038/ng1905
56. Sapir-Koren R, Livshits G. Bone mineralization and regulation of phosphate homeostasis. *IBMS BoneKEy* (2011) 8:286–300. doi: 10.1138/20110516
57. van Bezooijen RL, Bronckers AL, Gortzak RA, Hogendoorn PC, van der Wee-Pals L, Balemans W, et al. Sclerostin in mineralized matrices and van Buchem disease. *J Dent Res.* (2009) 88:569–74. doi: 10.1177/0022034509338340
58. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, et al. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem.* (2005) 280:19883–7. doi: 10.1074/jbc.M413274200
59. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest.* (2006) 116:1202–9. doi: 10.1172/JCI28551
60. You L, Temiyasathit S, Lee P, Kim CH, Tummala P, Yao W, et al. Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. *Bone* (2008) 42:172–9. doi: 10.1016/j.bone.2007.09.047
61. Wijenayaka AR, Kogawa M, Lim HP, Bonewald LF, Findlay DM, Atkins GJ. Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway. *PLoS ONE* (2011) 6:e25900. doi: 10.1371/journal.pone.0025900
62. Honma M, Ikebuchi Y, Kariya Y, Hayashi M, Hayashi N, Aoki S, et al. RANKL subcellular trafficking and regulatory mechanisms in osteocytes. *J Bone Miner Res.* (2013) 28:1936–49. doi: 10.1002/jbmr.1941
63. Li X, Ominsky MS, Warmington KS, Morony S, Gong J, Cao J, et al. Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. *J Bone Miner Res.* (2009) 24:578–88. doi: 10.1359/jbmr.081206
64. Veverka V, Henry AJ, Slocombe PM, Ventom A, Mulloy B, Muskett FW, et al. Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation. *J Biol Chem.* (2009) 284:10890–900. doi: 10.1074/jbc.M807994200
65. Ominsky MS, Vlasseros F, Jolette J, Smith SY, Stouch B, Doellgast G, et al. Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength. *J Bone Miner Res.* (2010) 25:948–59. doi: 10.1002/jbmr.14
66. Ominsky MS, Niu QT, Li C, Li X, Ke HZ. Tissue-level mechanisms responsible for the increase in bone formation and bone volume by sclerostin antibody. *J Bone Miner Res.* (2014) 29:1424–30. doi: 10.1002/jbmr.2152
67. Taylor S, Ominsky MS, Hu R, Pacheco E, He YD, Brown DL, et al. Time-dependent cellular and transcriptional changes in the osteoblast lineage associated with sclerostin antibody treatment in ovariectomized rats. *Bone* (2016) 84:148–59. doi: 10.1016/j.bone.2015.12.013
68. Nioi P, Scott T, Rong H, Efrain P, D HY, Hisham H, et al. Transcriptional profiling of laser capture microdissected subpopulations of the osteoblast lineage provides insight into the early response to Sclerostin antibody in rats. *J Bone Miner Res.* (2015) 30:1457–67. doi: 10.1002/jbmr.2482
69. Li X, Niu QT, Warmington KS, Asuncion FJ, Dwyer D, Grisanti M, et al. Progressive increases in bone mass and bone strength in an ovariectomized rat model of osteoporosis after 26 weeks of treatment with a sclerostin antibody. *Endocrinology* (2014) 155:4785–97. doi: 10.1210/en.2013-1905
70. Florio M, Gunasekaran K, Stolina M, Li X, Liu L, Tipton B, et al. A bispecific antibody targeting sclerostin and DKK-1 promotes bone mass accrual and fracture repair. *Nat Commun.* (2016) 7:11505. doi: 10.1038/ncomms11505
71. Padhi D, Jang G, Stouch B, Fang L, Posvar E. Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. *J Bone Miner Res.* (2011) 26:19–26. doi: 10.1002/jbmr.173
72. Graeff C, Campbell GM, Pena J, Borggrefe J, Padhi D, Kaufman A, et al. Administration of romosozumab improves vertebral trabecular and cortical bone as assessed with quantitative computed tomography and finite element analysis. *Bone* (2015) 81:364–9. doi: 10.1016/j.bone.2015.07.036
73. McClung MR, Grauer A, Boonen S, Bolognese MA, Brown JP, Diez-Perez A, et al. Romosozumab in postmenopausal women with low bone mineral density. *N Engl J Med.* (2014) 370:412–20. doi: 10.1056/NEJMoa1305224
74. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, et al. Romosozumab or Alendronate for fracture prevention in women with osteoporosis. *N Engl J Med.* (2017) 377:1417–27. doi: 10.1056/NEJMoa1708322
75. Glorieux FH, Devogelaer JP, Durigova M, Goemaere S, Hemsley S, Jakob F, et al. BPS804 anti-sclerostin antibody in adults with moderate osteogenesis imperfecta: results of a randomized phase 2a trial. *J Bone Miner Res.* (2017) 32:1496–504. doi: 10.1002/jbmr.3143
76. Morse A, McDonald MM, Kelly NH, Melville KM, Schindeler A, Kramer I, et al. Mechanical load increases in bone formation via a sclerostin-independent pathway. *J Bone Miner Res.* (2014) 29:2456–67. doi: 10.1002/jbmr.2278
77. van Lierop AH, Moester MJ, Hamdy NA, Papapoulos SE. Serum Dickkopf 1 levels in sclerostin deficiency. *J Clin Endocrinol Metab.* (2014) 99:E252–256. doi: 10.1210/jc.2013-3278
78. Recknor CP, Recker RR, Benson CT, Robins DA, Chiang AY, Alam J, et al. The effect of discontinuing treatment with blosozumab: follow-up results of a phase 2 randomized clinical trial in postmenopausal women with low bone mineral density. *J Bone Miner Res.* (2015) 30:1717–25. doi: 10.1002/jbmr.2489
79. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
80. Roodman GD. Pathogenesis of myeloma bone disease. *Leukemia* (2009) 23:435–41. doi: 10.1038/leu.2008.336
81. Yaccoby S. Advances in the understanding of myeloma bone disease and tumour growth. *Br J Haematol.* (2010) 149:311–21. doi: 10.1111/j.1365-2141.2010.08141.x
82. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc.* (2003) 78:21–33. doi: 10.4065/78.1.21
83. Lee OL, Horvath N, Lee C, Joshua D, Ho J, Szer J, et al. Bisphosphonate guidelines for treatment and prevention of myeloma bone disease. *Intern Med J.* (2017) 47:938–51. doi: 10.1111/imj.13502
84. Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD, et al. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood* (2000) 96:671–5.
85. Kitaura H, Sands MS, Aya K, Zhou P, Hirayama T, Uthgenannt B, et al. Marrow stromal cells and osteoclast precursors differentially contribute to TNF-alpha-induced osteoclastogenesis *in vivo*. *J Immunol.* (2004) 173:4838–46. doi: 10.4049/jimmunol.173.8.4838
86. Lee JW, Chung HY, Ehrlich LA, Jelinek DF, Callander NS, Roodman GD, et al. IL-3 expression by myeloma cells increases both osteoclast formation and growth of myeloma cells. *Blood* (2004) 103:2308–15. doi: 10.1182/blood-2003-06-1992
87. Giuliani N, Colla S, Morandi F, Lazzaretti M, Sala R, Bonomini S, et al. Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation. *Blood* (2005) 106:2472–83. doi: 10.1182/blood-2004-12-4986
88. Giuliani N, Rizzoli V, Roodman GD. Multiple myeloma bone disease: pathophysiology of osteoblast inhibition. *Blood* (2006) 108:3992–6. doi: 10.1182/blood-2006-05-026112
89. Toscani D, Bolzoni M, Accardi F, Aversa F, Giuliani N. The osteoblastic niche in the context of multiple myeloma. *Ann N Y Acad Sci.* (2015) 1335:45–62. doi: 10.1111/nyas.12578
90. Ehrlich LA, Chung HY, Ghobrial I, Choi SJ, Morandi F, Colla S, et al. IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. *Blood* (2005) 106:1407–14. doi: 10.1182/blood-2005-03-1080
91. Vallet S, Mukherjee S, Vaghela N, Hideshima T, Fulciniti M, Pozzi S, et al. Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease. *Proc Natl Acad Sci USA.* (2010) 107:5124–9. doi: 10.1073/pnas.0911929107

92. Oshima T, Abe M, Asano J, Hara T, Kitazoe K, Sekimoto E, et al. Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. *Blood* (2005) 106:3160–5. doi: 10.1182/blood-2004-12-4940
93. Giuliani N, Morandi F, Tagliaferri S, Lazzaretti M, Donofrio G, Bonomini S, et al. Production of Wnt inhibitors by myeloma cells: potential effects on canonical Wnt pathway in the bone microenvironment. *Cancer Res.* (2007) 67:7665–74. doi: 10.1158/0008-5472.CAN-06-4666
94. Palma BD, Guasco D, Pedrazzoni M, Bolzoni M, Accardi F, Costa F, et al. Osteolytic lesions, cytogenetic features and bone marrow levels of cytokines and chemokines in multiple myeloma patients: role of chemokine (C-C motif) ligand 20. *Leukemia* (2016) 30:409–16. doi: 10.1038/leu.2015.259
95. Eisenberger S, Ackermann K, Voggenreiter G, Sultmann H, Kasperk C, Pyerin W. Metastases and multiple myeloma generate distinct transcriptional footprints in osteocytes *in vivo*. *J Pathol.* (2008) 214:617–26. doi: 10.1002/path.2322
96. Gatti D, Viapiana O, Adami S, Idolazzi L, Fracassi E, Rossini M. Bisphosphonate treatment of postmenopausal osteoporosis is associated with a dose dependent increase in serum sclerostin. *Bone* (2012) 50:739–42. doi: 10.1016/j.bone.2011.11.028
97. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone* (2005) 37:148–58. doi: 10.1016/j.bone.2005.03.018
98. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, et al. Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS ONE* (2008) 3:e2942. doi: 10.1371/journal.pone.0002942
99. Wang XT, He YC, Zhou SY, Jiang JZ, Huang YM, Liang YZ, et al. Bone marrow plasma macrophage inflammatory protein protein-1 alpha(MIP-1 alpha) and sclerostin in multiple myeloma: relationship with bone disease and clinical characteristics. *Leuk Res.* (2014) 38:525–31. doi: 10.1016/j.leukres.2014.02.010
100. Colucci S, Brunetti G, Oranger A, Mori G, Sardone F, Specchia G, et al. Myeloma cells suppress osteoblasts through sclerostin secretion. *Blood Cancer J.* (2011) 1:e27. doi: 10.1038/bcj.2011.22
101. Eda H, Santo L, Wein MN, Hu DZ, Cirstea DD, Nemani N, et al. Regulation of Sclerostin expression in multiple myeloma by Dkk-1: a potential therapeutic strategy for myeloma bone disease. *J Bone Miner Res.* (2016) 31:1225–34. doi: 10.1002/jbmr.2789
102. Chamorro MN, Schwartz DR, Vonica A, Brivanlou AH, Cho KR, Varmus HE. FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development. *EMBO J.* (2005) 24:73–84. doi: 10.1038/sj.emboj.7600460

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REVIEW
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Mechanisms of NK Cell Activation and Clinical Activity of the Therapeutic SLAMF7 Antibody, Elotuzumab in Multiple Myeloma

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Multiple myeloma (MM) is a bone marrow plasma cell neoplasm and is the second most-common hematologic malignancy. Despite advances in therapy, MM remains largely incurable. Elotuzumab is a humanized IgG1 monoclonal antibody targeting SLAMF7, which is highly expressed on myeloma cells, and the antibody is approved for the treatment of relapsed and/or refractory (RR) MM in combination with lenalidomide and dexamethasone. Elotuzumab can stimulate robust antibody-dependent cellular cytotoxicity (ADCC) through engaging with FcγRIIIA (CD16) on NK cells and antibody-dependent cellular phagocytosis (ADCP) by macrophages. Interestingly, SLAMF7 is also expressed on cytolytic NK cells, which also express the requisite adaptor protein, EAT-2, to mediate activation signaling. Accumulating evidence indicates that antibody crosslinking of SLAMF7 on human and mouse NK cells can stimulate EAT-2-dependent activation of PLCγ, ERK, and intracellular calcium mobilization. The binding of SLAMF7 by elotuzumab can directly induce signal transduction in human NK cells, including co-stimulation of the calcium signaling triggered through other surface receptors, such as NKp46 and NKG2D. In RRMM patients, elotuzumab monotherapy did not produce objective responses, but did enhance the activity of approved standard of care therapies, including lenalidomide or bortezomib, which are known to enhance anti-tumor responses by NK cells. Taken together, these preclinical results and accumulating experience in the clinic provide compelling evidence that the mechanism of action of elotuzumab in MM patients involves the activation of NK cells through both CD16-mediated ADCC and direct co-stimulation via engagement with SLAMF7, as well as promoting ADCP by macrophages. We review the current understanding of how elotuzumab utilizes multiple mechanisms to facilitate immune-mediated attack of myeloma cells, as well as outline goals for future research.

Keywords: multiple myeloma, SLAMF7, elotuzumab, NK cells, ADCC, ADCP, macrophage

INTRODUCTION

Multiple myeloma (MM) is a deadly hematopoietic cancer characterized by the expansion of monotypic plasma cells in the bone marrow, accumulation of monoclonal immunoglobulin in the serum, and end-organ damage such as anemia, lytic bone lesions, and renal dysfunction (1). It is estimated that almost 31,000 cases of MM will be diagnosed in the U.S. in 2018 and almost 13,000 will die of the disease. Incidence increases with age, which accounts for a steadily rising prevalence of MM overall (2). Rates of median survival are improving, with overall 5-year survival of about 50%, although survival is better in younger patients (2). Nonetheless, MM is still a largely incurable disease, highlighting the need for improved therapeutic options, which may include new agents with novel mechanisms of action and innovative combination therapies.

A variety of recently-developed therapies have contributed to the extended survival of MM patients, including proteasome inhibitors (bortezomib, carfilzomib, and ixazomib), immunomodulatory imide drugs (IMiDs; namely thalidomide, lenalidomide and pomalidomide), and monoclonal antibodies (daratumumab and elotuzumab). Clinical results with these therapies have been previously summarized in a variety of quality reviews (3–6) and will not be further discussed here. Importantly, however, optimal long-term control of MM requires combinations of two or even three different classes of drugs (7). Furthermore, in contrast to older MM therapies such as steroids or cytotoxic chemotherapies, these newer therapies can mediate their anti-myeloma activity not just by acting directly on the myeloma cell, but also through modulation of the patient's immune system (8). Thus, gaining a greater understanding of the mechanisms of action of these new therapies, and particularly how they impact host innate and adaptive immunity, will be critical to further developing optimal combinations for treatment.

Here, we will review current understanding of the mechanisms by which elotuzumab promotes immune responses toward MM, especially through facilitating NK cell-mediated anti-tumor activity. We further summarize clinical results from the use of elotuzumab in combination immunotherapies and discuss how the immune potentiating mechanisms may be contributing to anti-tumor responses in patients. While the CD38 targeting antibody, daratumumab, shares some mechanistic attributes with elotuzumab, we will only touch upon some aspects of the effects of daratumumab, in view of recently published reviews on the topic (9, 10).

NK CELLS AND MULTIPLE MYELOMA

NK cells are believed to play important roles in immune surveillance of cancer, limiting neoplastic progression, and effectors of anti-tumor therapies (11, 12). Their stimulation is triggered upon recognition of certain ligands on tumor cells by cell surface activating receptors [including NKG2D, CD16, 2B4, NKp80, and DNAM-1, and natural cytotoxicity receptors (NCR: NKp30, NKp44, and NKp46)] (13). NK cell stimulation is, however, tightly regulated by their expression of major histocompatibility class I (MHC-I)-binding inhibitory receptors [killer cell Ig-like receptors (KIR; CD158), CD94/NKG2A heterodimers, and ILT2/LIR1/CD85j], which efficiently block NK cell activation toward MHC-I-expressing normal cells (12). Therefore, when an NK cell conjugates with an abnormal tumor cell lacking MHC-I and expressing ligands for activating receptors, the inhibitory receptors are not engaged, and unsuppressed activating signals trigger targeted attack of the conjugated cell.

The importance of NK cells in mediating anti-myeloma activity has been demonstrated in several ways. A graft-vs.-myeloma effect has been shown by the differences in post-allogeneic stem cell transplant relapse rates based on the inherited repertoire of *KIR* genes expressed by donor NK cells (14, 15), indicating a role for NK cell-mediated suppression of relapse. NK cells can clearly mediate direct cytotoxicity and ADCC against myeloma cells *in vitro* and *in vivo* (16–19). This response depends on the expression of activating receptors, such as NKG2D, DNAM-1, and the NCRs, on the NK cells, along with their respective ligands on the myeloma cells (16, 17, 20). Several studies have now shown that the balance of activating and inhibitory NK cell receptors and ligands is significantly altered in MM patients, especially in advanced disease (16, 21–26). For example, myeloma cells derived from a patient late in disease course (from a pleural effusion) expressed much higher levels of MHC-I (an inhibitory ligand) and lower levels of MICA (a ligand for the NK cell activating receptor, NKG2D) and were much more resistant to NK cell-mediated lysis than myeloma cells derived earlier from the bone marrow of the same patient (16). In addition, MICA can be shed off the myeloma cell surface and reportedly down-regulate or block engagement of the activating NKG2D receptor on NK and T cells (27, 28). This mutual "immuno-editing" of receptor and ligand expression on the surface of NK and myeloma cells, respectively, implies a strong selective pressure of NK cells on the tumor, and suggests that strategies augmenting NK cell activity may overcome this immune evasion and eliminate MM. Finally, data that currently-used therapies (e.g., melphalan, bortezomib, lenalidomide) can augment NK cell-mediated cytotoxicity against MM (3, 20, 24, 26, 29–34) provide strong support for exploring combinations of NK cell-targeted therapies with these active anti-myeloma agents.

SLAMF7 AS A PROMINENT BIOMARKER AND POTENTIAL THERAPEUTIC TARGET ON MYELOMA CELLS

Signaling Lymphocyte Activation Marker Family member 7 (SLAMF7) was found highly expressed on human plasma cells and corresponding myeloma cells (18, 19). While the physiological function of SLAMF7 on plasma cells is still unknown, the high expression on myeloma cells raised interest as a therapeutic antibody target. Hsi and colleagues detected high levels of SLAMF7 mRNA in $CD138^{+}$ plasma cells from healthy donors, patients with MGUS, smoldering myeloma and newly diagnosed patients, whereas NK cells expressed

a substantially lower level of SLAMF7 mRNA (18). High expression on myeloma cells was also found in MM patients, regardless of cytogenetic abnormalities. Examination of SLAMF7 protein expression on MM, other plasma cell tumors, and normal tissues was consistent with mRNA expression patterns, where strong surface staining was found on plasmacytomas (18), most myeloma cells from bone marrow biopsies, neoplastic plasma cells from most lymphoplasmacytic lymphoma, and some peripheral T cell lymphomas. Importantly, SLAMF7 expression was preserved on myeloma cells at significant levels upon relapse in most patients (18). Tai et al. further confirmed that SLAMF7 mRNA is expressed in $CD138^+$ tumor cells from more than 97% of MM patient analyzed and surface SLAMF7 protein was detected on several myeloma cell lines and 12 representative MM tumor samples (19). The same study also detected soluble SLAMF7 in 32 of 54 serum samples from MM patients, but not healthy donors, which they suggest could serve as a biomarker of active disease (19). It was also shown that myeloma cells with t(4;14) translocations (found in about 15% of MM patients) express higher levels of SLAMF7 mRNA and surface protein, which appears to be due to overexpression of MMSET (35). Interestingly, shRNA-mediated knockdown of SLAMF7 expression in t(4;14) myeloma cells reduced colony formation and induced G1 arrest and apoptosis, indicating that maintaining high SLAMF7 expression promotes growth of these myeloma cells (35). A recent analysis of gene expression data in hematopoietic malignancies confirmed high SLAMF7 expression on myeloma tumors, but also identified high SLAMF7 expression on tumors in patients with myelodysplastic syndrome, chronic lymphocytic leukemia, and diffuse large B cell lymphoma (36). This result suggests that SLAMF7 may also be a useful diagnostic marker and therapeutic target in other hematopoietic cancers. However, the biological role of SLAMF7 on the pathogenesis of these tumors types has not been thoroughly evaluated.

ELOTUZUMAB AS A NEW THERAPEUTIC TO TARGET MULTIPLE MYELOMA

Preclinical Studies: ADCC by NK Cells as a Major Mechanism of Action

Elotuzumab (Elo; formerly HuLuc63) is a humanized IgG1 monoclonal antibody that was developed to target SLAMF7. HuLuc63 was originally engineered by PDL BioPharma as a humanized version of the SLAMF7 monoclonal antibody, MuLuc63, which was originally generated in BALB/c mice (18, 19). HuLuc63 binds to the carboxy-terminal Ig-like constant 2 (C2) domain of SLAMF7, which encompasses amino acids 170-227 (U.S. patent 7842293B2). It is important to note that HuLuc63 does not cross-react with other SLAM family proteins and did not activate complement-dependent lysis or direct cytotoxicity of myeloma cells (19, 37).

From the earliest studies, Elo was found to promote antibody-dependent cellular cytotoxicity (ADCC) of myeloma cells by NK cells both *in vitro* and *in vivo* (18, 19, 38). Hsi et al. found that HuLuc63 induced specific myeloma cell lysis in multiple assays using PBMCs or purified NK cells from healthy allogeneic donors or autologous NK cells toward myeloma cells or myeloma cell lines (18). HuLuc63 was also shown to induce similar lysis of patient myeloma cells by NK cells from allogeneic healthy donors as compared to NK cells from the same MM patient, even in patients who were resistant to conventional therapy (18). Furthermore, HuLuc63 was significantly more effective in inducing ADCC responses by NK cells than another chimerized SLAMF7 antibody (human IgG1-human Fc/mouse variable regions) named ChLuc90 (18, 19, 39). Tai et al. also demonstrated that HuLuc63 stimulated ADCC responses by NK cells from healthy donors to a variety of myeloma cell lines, and they further showed strong ADCC of autologous myeloma cells by NK cells from MM patients, even if patients were resistant to conventional therapies (19).

Preclinical *in vitro* studies also found minimal loss of NK cells in PBMC treated with Elo, indicating that the antibody does not induce significant NK cell fratricide upon binding to SLAMF7 on NK cells themselves. Treatment of whole blood overnight with 100 or 200 μg/ml HuLuc63 resulted in a loss of only 20% of NK cells (18). Another study found that in cultures of PBMC overnight with up to 100 μg/ml Elo, NK cell viability was retained at >95% (39). Fratricide was also not observed when purified NK cells were exposed to 100 μg/ml Elo for 72 h, possibly due to upregulation of MHC class I on the NK cell surface as a ligand for inhibitory signaling (37). Similarly, Elo therapy results in only a transient loss of NK cells in peripheral blood of patients within hours after the initial dose that recovers over time (40). A parallel loss of T and B cells also occurred, which the authors attributed to an early increase in serum levels of the chemokine IP-10 (CXCL10), which induces migration of lymphocytes and myeloid cells. A similar transient early loss and recovery of NK cells in peripheral blood was also noted in another clinical trial (41). Thus, it appears that NK cell fratricide is minimal in patients treated with Elo. In contrast, patients treated with daratumumab exhibit significant loss of NK cells in peripheral blood, due to ADCC-mediated fratricide (42), although daratumumab-treated patients alternatively benefit from depletion of immunosuppressive regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells to boost anti-myeloma immune responses (9).

Preclinical Studies of Combination Therapies With Elotuzumab

The early study by Tai et al. showed enhanced NK cell-mediated ADCC responses by HuLuc63 if the myeloma cell lines were pretreated with several drugs, including bortezomib, dexamethasone, and lenalidomide (19). These experiments provided the first preclinical evidence for the use of Elo in combination with other therapies to treat MM patients.

Van Rhee at al. subsequently tested the effect of Elo in combination with the 26S proteasome inhibitor, bortezomib, in a severe combined immunodeficiency (SCID)-human mouse xenograft model engrafted with primary myeloma cells (38). Treatment of mice with MuLuc63 (the parental mouse mAb from which Elo was derived) alone promoted significant reductions of tumor volume and human IgG in serum, with equivalent

responses to high- or low-risk myeloma samples (38). Treating a MM target cell line or autologous myeloma cells with bortezomib was found to enhance *in vitro* susceptibility to Elo-mediated ADCC by NK cells (38). This increased susceptibility is consistent with reports that bortezomib treatment reduces expression of the NK cell inhibitory receptor ligand, MHC class I, and increases expression of ligands for the activating receptor, NKG2D (3, 30, 43). Van Rhee et al. also showed that mice treated with the combination of Elo plus bortezomib had significantly more efficient anti-tumor response in an OPM2 myeloma cell line xenograft mouse model, compared to treatment with either agent alone (38). The group additionally showed that SLAMF7 expression on myeloma cells was not affected in patients treated with bortezomib (38).

Balasa et al. investigated *in vitro* and *in vivo* effect of Elo in combination with lenalidomide on NK cell activation, cytokine production and myeloma cell death (44). Lenalidomide is a member of the IMiD family, which also includes thalidomide and pomalidomide, that can augment function of T and NK cells, suppress angiogenesis, and directly restrain myeloma cell growth (3). Treatment of the OPM2 xenograft mouse model with Elo plus lenalidomide resulted in significantly greater reduction in tumor volume and increased infiltration of NK cells into the tumor microenvironment (44). The combination of Elo with lenalidomide in co-cultures of peripheral blood mononuclear cells (PBMC) and myeloma cells also increased upregulation of NK cell activation marker, adhesion molecules, and cytokine production (IFN-γ, TNF-α, IL-2), as compared to either agent alone (44). These effects required the Fc domain of Elo, indicating the primary role of NK cell-mediated ADCC, and were enhanced by IL-2 production by $CD56^+$ T cells within the PBMC. In addition TNF-α production contributed significantly to NK cell activation and myeloma cell cytotoxicity (44). Therefore, Elo in combination with lenalidomide was highly effective and appeared to primarily benefit from NK cell activation in response to IL-2 produced by T cells and TNF-α production by monocytes and NK cells.

Bezman et al. studied the *in vivo* impact of a murine IgG2a-modified version of Elo (Elo-g2a) in xenograft mouse models using immunocompetent mice and syngeneic mouse tumors expressing human SLAMF7 (25). Treatment with Elo-g2a significantly reduced tumor volume and the effect was reversed if NK cells were depleted from the mice or mice were instead treated with a Fc mutant form of Elo-g2a that cannot bind Fcγ receptors (25). In these xenograft mouse models, PD-1 expression was found to be increased on tumor-infiltrating T cells and the tumors expressed PD-L1. Consistent with this observation, the combined treatment with Elo-g2a and PD-1 antibody resulted in significantly reduced tumor volume and increased survival compared to either agent alone (25). The combination therapy resulted in increased expression of IFN-γ, TNF-α, CD69, and CD107a degranulation marker on tumor-infiltrating NK cells, as compared to treatment with either antibody alone. Furthermore, long-term surviving mice from one of these mouse models were protected from subsequent challenge with the same tumor, indicating that immunological memory had been established in response to the combination therapy (25). The results suggest that the combination of Elo with PD-1/PD-L1 blocking antibody therapy may also be an effective strategy in treating MM patients.

Taken together, these combination therapy preclinical studies provided further evidence that NK cells play an important role in the mechanism by which Elo effectively reduces MM tumor burden in mice. In particular, both bortezomib and lenalidomide are known to boost NK cell function, which could contribute to better ADCC responsiveness (3). The potentiation of Elo effectiveness by PD-1 blockade also corresponded to enhanced NK cell responsiveness, likely due to overriding this important immune checkpoint on T cells and perhaps NK cells (23, 45, 46).

Clinical Trials With Elotuzumab to Treat Multiple Myeloma (Summarized in Table 1)

Zonder et al. performed the first-in-human phase I study to evaluate safety, tolerability, pharmacokinetics and pharmacodynamics of Elo (40). Thirty-five relapsed/refractory (RR) MM patients were treated with 0.5–20 mg/kg of Elo every 2 weeks. Elo was generally well-tolerated, even at the highest dose, and saturation of over 95% of SLAMF7 receptors on bone marrow plasma cells was achieved at 10 and 20 mg/kg (40). However, no objective anti-myeloma response was observed in the MM patients treated with Elo as a single agent in this highly pre-treated population, despite reaching high SLAMF7 saturation with Elo (40).

Jakubowiak et al. performed the first phase I trial of Elo in combination with bortezomib in RRMM (41). The combination was safe and showed promising activity with an objective response in 48% of 27 patients, including partial responses in patients refractory to previous bortezomib therapy. Serum concentrations of Elo in these patients were >100 ug/ml at doses of 10 or 20 mg/kg with 80 or 95% median saturation of SLAMF7 on $CD38^+$ myeloma cells in bone marrow, respectively (41). The most frequent adverse events were lymphopenia and fatigue.

A subsequent phase II study comparing the effects of Elo plus bortezomib/dexamethasone (Bor/Dex; 75 patients) vs. Bor/Dex

TABLE 1 | Comparisons of response rates (RR) progression free survival (PFS) in elotuzumab clinical trials.

Phase	Regimen*	N*	Overall RR	Median PFS	References
I	Elo	35	0%	N/A	(40)
I	Elo/Bor	27	48%	9.46 months	(41)
II	Elo/Bor/Dex vs. Bor/Dex	150	66 vs. 63%	9.7 vs. 6.9 months	(47)
I	Elo/Len/Dex	28	82%	N/A	(48)
Ib/II	Elo/Len/Dex	73	84%	28.6 months	(49)
III	Elo/Len/Dex vs. Len/Dex	646	79 vs. 66%	19.4 vs. 14.9 months	(50)
II	Elo/Td/Dex	40	38%	3.9 months	(51)
II	Elo/Pom/Dex vs. Pom/Dex	117	53 vs. 26%	10.3 vs. 4.7 months	(52)

**Elo, elotuzumab; Bor, bortezomib; Dex, dexamethasone; Len, lenalidomide; Td, thalidomide; Pom, pomalidomide; n, number of evaluable patients.*

alone (75 patients) for RRMM was also reported by Jakubowiak et al. (47). At 1 year, progression free survival rate was 39% for the Elo-treated group compared to 33% for Bor/Dex alone, while the rate was 18 vs. 11%, respectively at 2 years (47). Strikingly, patients homozygous for the high affinity polymorphic variant of FcγRIIIa (CD16 158V/V) in the Elo/Bor/Dex group had a median progression free survival of 22.3 months compared to only 9.8 months for patients homozygous for the lower affinity variant (CD16 158 F/F) (47). This result suggests an important role for CD16 in the mechanism of Elo activity in MM patients, analogous to enhanced ADCC and clinical response to rituximab in follicular lymphoma patients having CD16 158/V/V genotype (53, 54). No significant increase in toxicity was noted when Elo was added to Bor/Dex therapy, with the most common side effects being infection, diarrhea, and thrombocytopenia (47).

The initial phase I trial of the combination of Elo/Lenalidomide/Dexamethasone (Elo/Len/Dex) was carried out by Lonial et al. (48). In that study, 29 previously-treated advanced MM patients were treated with 5, 10, and 20 mg/kg of Elo. Neutropenia and thrombocytopenia were the most frequent adverse events. Outcomes were encouraging, with 82% of patients achieving an objective response, including patients who had received prior thalidomide, bortezomib or lenalidomide therapy (48). Treatment with 10 or 20 mg/ml Elo resulted in full saturation of more than 80% of SLAMF7 binding sites on $CD38^+$ myeloma cells and consistently achieved Elo serum concentration of more than 70 ug/ml in patients, with peak concentrations of up to 1 mg/ml in serum (48).

Richardson et al. subsequently reported on the phase II portion of this trial (49). In this phase, 73 patients were randomized to either 10 or 20 mg/kg of Elo, in combination with Len/Dex. Objective responses were observed in 84% of patients with a better response in the low-dose group, and no dose-limiting toxicities observed. Median progression free survival was 29 months (10 mg/kg, 32 months; 20 mg/kg, 25 months) (49). Retrospective analysis of bone marrow samples obtained from this trial revealed increased infiltration of $CD56^{dim}CD16^+$ NK cells exhibiting higher expression of the adhesion molecule, CD54 (ICAM-1), and concomitant reduction in $CD45^{dim}CD138^+$ myeloma cells at cycle 1 day 22, as compared to baseline (25).

Lonial et al. performed a phase III Elo/Len/Dex trial (ELOQUENT-2) comparing treatment of 321 RRMM patients with 10 mg/kg Elo/Len/Dex and a control arm of 325 patients treated with Len/Dex alone (50). At 1 year, progression free survival in the Elo-treated patients was 68% compared to 57% in the Len/Dex control group, and 41 and 27%, respectively at 2 years. Follow up analysis at 3 years found 26% progression free survival for the Elo-treated patients vs. 18% for the control group (55). Overall response rate was 79% for the Elo-treated patients vs. 66% in the control group (50). Common adverse events were lymphocytopenia, anemia, thrombocytopenia, neutropenia, fatigue and diarrhea, but there was no evidence of autoimmunity in Elo-treated patients. The significant reduction in disease progression and death in the Lonial et al. study (50) was instrumental in subsequent U.S. FDA approval in November 2015 for the use of the Elo/Len/Dex combination to treat RRMM patients that have received one to three previous lines of therapy.

In addition to the combined use of Elo with Lenalidomide and dexamethasone, clinical trials are underway combining Elo with related IMiD drugs, thalidomide and pomalidomide. The combination of Elo with thalidomide/dexamethasone was shown to be safe and effective in a phase II study by Mateos et al. (51). Furthermore, results from the randomized phase II ELOQUENT-3 trial comparing Elo/pomalidomide/dexamethasone with pomalidomide/dexamethasone in RRMM patients were recently presented at the 2018 European Hematology Association meeting by Dimopoulos et al. (52). Overall response rate of the Elo-treated group was 53% compared to 26% for the control group, and median progression free survival was 10.3 vs. 4.7 months, respectively. Therefore, combination therapies of Elo, particularly with IMiDs have demonstrated significant clinical activity in RRMM disease and additional combination studies, including in newly-diagnosed patients, are underway.

SLAMF7 EXPRESSION AND SIGNALING IN LEUKOCYTES (SUMMARIZED IN TABLE 2)

Expression and Structure of SLAMF7

SLAMF7 (CD319) was originally discovered as a CD2-related receptor by the labs of Marco Colonna, who called it CRACC (CD2-like receptor activating cytotoxic cells) (57), and Porunelloor Mathew, who named it CS1 (CD2 subset 1) (56). The gene encoding SLAMF7 is found on chromosome 1 in humans in a locus at 1q23-24 that contains most of the other SLAM (signaling lymphocyte activation molecule) family receptors (56, 57, 70). In addition to plasma cells and myeloma cells, SLAMF7 is expressed in healthy donors on essentially all $CD56^{dim}$ NK cells, the majority of $CD56^{bright}$ NK cells, many $CD56^+$ T cells, mature dendritic cells, and small subsets of $CD4^+$ T cells and B cells (18, 39, 57). IL-12 produced by dendritic cells has been shown to increase expression of SLAMF7 on NK cells (71). B cells have also been reported to increase SLAMF7 expression upon activation with various stimuli (57, 70, 72). While early studies did not detect SLAMF7 on $CD14^+$ monocytes (18, 57), a more recent report found significant expression on most non-classical ($CD14^{low}CD16^+$) and a fraction of intermediate ($CD14^+CD16^+$) monocytes (39). In addition, SLAMF7 is preferentially expressed at higher levels on M1 macrophages, as compared to M2 macrophages in humans (73).

Mature SLAMF7 is expressed as a 66 kDa glycoprotein (57). Similar to most SLAM family members, the extracellular domain contains an amino terminal Ig-like variable (V) domain and a carboxy-terminal Ig-like constant 2 (C2) domain and can interact with other SLAMF7 extracellular domains via the V domains as self-ligands (56–58). The cytoplasmic domain of SLAMF7 contains four tyrosine residues, one of which ($TVY_{304}STV$) is within an immunoreceptor tyrosine-based switch motifs (ITSM; T-V/I-Y-x-x-V/I) and another that is embedded in a similar ITSM-like sequence ($TEY_{284}DTI$). ITSM sequences are also found in most SLAM family receptors, and have

TABLE 2 | Main biological functions of SLAMF7.

Function	References
SLAMF7 is a self-ligand	(56–58)
SLAMF7-S isoform lacking ITSM-like sequence	(59)
NK cells activation upon SLAMF7 engagement with mAbs	(57–62)
SLAMF7 preferentially recruits EAT-2 over SAP	(57, 61–65)
EAT-2 stimulates calcium mobilization and ERK activation	(63, 66)
SLAMF7 mediates inhibitory function in the absence of EAT-2	(37, 61, 67)
Memory-like NK cells lack EAT-2	(68, 69)
SLAMF7 signals through association with Mac-1 in macrophages	(36)

capacity to switch between activating and inhibitory signaling (64). Tyrosine phosphorylation is mediated by Src family kinases, including Fyn, Lyn, and Src (67, 74). Upon tyrosine phosphorylation, an ITSM can recruit either SLAM adaptor protein (SAP, encoded by *SH2D1A*) or EWS-FLI1 activated transcript 2 (EAT-2, encoded by *SH2D1B*) (65) to mediate activation signaling in human leukocytes (mice also express ERT, which is not found in humans). Interestingly, in addition to this ITSM-containing form of SLAMF7 with a long cytoplasmic domain (SLAMF7-L), a mRNA splice variant encodes a receptor with a shorter cytoplasmic domain (SLAMF7-S) lacking the two ITSM-like sequences and possessing an alternative ITSM-like motif (SKYGLL) (59). NK cells predominantly express the SLAMF7-L variant, which exclusively exhibits activation signaling properties (59). It is currently unclear if a subset of NK cells can predominantly express SLAMF7-S or if some individuals preferentially express this truncated isoform.

SLAMF7 Signaling and the Importance of EAT-2

From early studies, SLAMF7 was found to activate NK cells upon engagement with monoclonal antibodies (mAb), SLAMF7-Ig fusion protein, or exposure to SLAMF7$^+$ target cells (57, 58, 60, 61). Crosslinking SLAMF7 with mAb induced the serine phosphorylation of Akt and ERK and tyrosine phosphorylation of phospholipase C (PLC)-γ1, PLC-γ2, c-Cbl, Vav1, and SHIP-1 (57, 62). Engagement of SLAMF7 with biotinylated antibody + streptavidin crosslinking was shown to stimulate intracellular calcium mobilization in mouse NK cells that required EAT-2 (61), and engaging human SLAMF7 in a rat NK cell line with the 1G10 mAb + secondary crosslinking antibody induced a strong calcium signal (59). In contrast, Pazina did not observe any elevation of intracellular calcium when primary NK cells from healthy donors were treated with biotinylated Elo ± streptavidin (39), indicating that calcium signaling through engaging SLAMF7 does not occur in normal human NK cells or Elo binds to an epitope on SLAMF7 that is incapable of properly engaging the receptor to directly mediate calcium signaling.

Tyrosine phosphorylated SLAMF7 was found to preferentially recruit EAT-2, but not SAP, at tyrosine(Y)-304 within the ITSM (61, 62). Although SAP association with SLAMF7 has also been reported (59, 62), EAT-2 is recruited to tyrosine phosphorylated SLAMF7 at >100-fold higher affinity than SAP (63). In addition, antibody engagement of SLAMF7 stimulated comparable levels of cytotoxicity by NK cells from both SAP-deficient X-linked lymphoproliferative disease patients and healthy donors, further indicating that SLAMF7 activation signaling is independent of SAP (57). EAT-2 expression was shown to promote the tyrosine phosphorylation of SLAMF7 by Src family kinases in one study (62), but this was not confirmed in another study (61). Importantly, EAT-2 is strongly expressed in NK cells, somewhat in γδ T cells, but not in B, CD4$^+$ T, CD8$^+$ T, or dendritic cells (62, 75). Interestingly, CD16$^+$ non-classical monocytes express both SLAMF7 and EAT-2 (39, 76), indicating that SLAMF7 is likely signaling competent in this population of monocytes that is also capable of mediating antibody-dependent cellular phagocytosis (ADCP) through CD16 (77).

EAT-2 is a small cytosolic adaptor protein that consists of an SH2 domain, which binds to Y304 on SLAMF7, and a short C-terminal sequence including a tyrosine at position 127 (Y127), which is required for SLAMF7 activating function (61). When EAT-2 is tyrosine phosphorylated on Y127, it can subsequently recruit PLC-γ1 and PLC-γ2 to stimulate downstream intracellular calcium mobilization and ERK activation (63, 66). Consistent with these findings, expression of EAT-2 in the EAT-2-deficient human NK cell line, YT-S, enhanced tyrosine phosphorylation of PLC-γ1 and Cbl and serine phosphorylation of ERK in response to crosslinking the SLAM family receptor, 2B4 (66). Functionally, EAT-2 signaling in NK cells can enhance polarization of cytolytic granules and the microtubular organizing center (MTOC) toward target cells and degranulation responses, but does not promote conjugate formation with target cells (66). The known SLAMF7 signaling mechanisms are outlined in **Figure 1**.

SLAMF7 has also been observed to exhibit some inhibitory function in NK cells from EAT-2-deficient mice or the human NK cell line, YT-S, in the absence of EAT-2 (61). Therefore, available data indicate that EAT-2 is required for activating function of SLAMF7, but the receptor can mediate inhibitory function in the absence of EAT-2. Early work was unable to demonstrate co-immunoprecipitation of the inhibitory phosphatases, SH2 domain-containing 5′-inositol phosphatase (SHIP)-1, SH2 domain-containing tyrosine phosphatase (SHP)-1, or SHP-2 with tyrosine phosphorylated SLAMF7 in the EAT-2-expressing human NK cell line, NK-92, treated with pervanadate (57, 59), but a subsequent study by Guo et al. demonstrated recruitment of SHIP-1 to mouse SLAMF7 when expressed in the EAT-2-deficient human NK cell line, YT-S, and engaged with a SLAMF7 mAb (67). The stimulation of these YT-S cells with SLAMF7 mAb also induced tyrosine phosphorylation of SHIP-1, but this did not occur if Y261 (analogous to human Y284) on the mouse SLAMF7 was mutated to phenylalanine, even though direct binding to Y261 was not demonstrated (67). There is also no evidence that EAT-2 and SHIP-1 compete for binding to SLAMF7. These results indicate that the inhibitory function of mouse SLAMF7 in the absence of EAT-2 is mediated by SHIP-1 and requires Y261 (human Y284) on SLAMF7. Interestingly, "adaptive" or "memory-like" NK cells have been reported to lack expression of EAT-2 (68, 69). It is currently unclear whether the adaptive/memory-like cells, which exist as a subset of the

NK cell repertoire in many human cytomegalovirus seropositive individuals, are inhibited toward SLAMF7$^+$ target cells or have unique responsiveness to Elo compared to conventional NK cells.

Importantly, plasma cells and myeloma cells express high levels of SLAMF7, but lack expression of EAT-2, thereby compromising their activation signaling capacity through SLAMF7 (37, 67). This lack of EAT-2 suggested that SLAMF7 may function as an inhibitory receptor in myeloma cells. However, while treatment with SLAMF7 antibody (162) and secondary crosslinker can induce varying levels of tyrosine phosphorylation of the receptor in different myeloma cell lines, SHIP-1 was only minimally tyrosine phosphorylated, if at all (67). The defective tyrosine phosphorylation of SHIP-1 was attributed to lack of CD45 expression in myeloma cells, since CD45 is a tyrosine phosphatase required for maintaining activity of Src family kinases that mediate the tyrosine phosphorylation of SHIP-1 (67). Thus, SLAMF7 does not have activation or inhibitory signaling function in myeloma cells, due to the lack of EAT-2 to mediate activation and the lack of CD45 to maintain Src family kinases in an active state that is required to phosphorylate SLAMF7 and the inhibitory SHIP-1 phosphatase. Consistent with these findings, Elo was found to be incapable of inducing proliferation or apoptosis of myeloma cell lines, even in plate bound form (25, 67).

Recent intriguing work by Chen et al. established a novel mechanism for SLAMF7 signaling through physical association with the integrin receptor CD11b/CD18 (Mac-1) in the plasma membrane on the surface of macrophages (36). This group found that SLAMF7 serves as a key receptor promoting the phagocytosis of SLAMF7-expressing hematopoietic tumor cells by macrophages when the inhibitory SIRP-α receptor on macrophages is blocked by antibodies from detecting its ligand, CD47, on the same tumor target cells (36). The SLAMF7 activation signaling was independent of the cytoplasmic tyrosines, but instead relied upon signaling through Mac-1 association with DAP12 and FcR-γ, as well as their operative protein tyrosine kinases, Syk and Btk (36).

Fc-DEPENDENT NK CELL ACTIVATION BY ELOTUZUMAB THROUGH FCγRIIIA (CD16)

Elo is an IgG1 mAb and thereby possesses an Fc domain that is capable of efficiently binding to CD16 on the surface of leukocytes. FcγRIII is expressed in two distinct forms that exhibit nearly identical extracellular amino acid sequence: (1) as a transmembrane receptor, designated FcγRIIIA, or (2) as a glycophosphatidylinositol-linked surface receptor, called FcγRIIIB (78, 79). FcγRIIIA is expressed on the surface of the cytolytic CD56^{dim} subset of NK cells, as well as intermediate and non-classical monocytes and macrophages, and can trigger potent intracellular signaling, including tyrosine phosphorylation and calcium mobilization, through physical association with the transmembrane adaptor proteins TCR-ζ and FcR-γ, which contain immunoreceptor tyrosine-based activation motifs (ITAM) (79). In contrast, FcγRIIIB is expressed on the surface of neutrophils, and signals through interactions with FcγRIIA (CD32A) (80).

Elo was found to trigger robust ADCC responses by NK cells through Fc-dependent interaction with FcγRIIIA. In the early preclinical studies by Hsi et al., HuLuc63-mediated lysis of myeloma cell lines in cultures with PBMC or in SCID mice was significantly impaired by blocking CD16 with antibodies, using an Fc mutant form of HuLuc63 with reduced CD16 binding capacity, or depleting NK cells (18). These results strongly implied that the *in vitro* and *in vivo* anti-tumor activity of Elo is mediated primarily by NK cells in a CD16-dependent manner. *In vitro* Elo begins to stimulate ADCC responses at concentration around 0.1 ng/ml, with peak responses in the range of 100 ng/ml (19, 39). However, early preclinical studies in mice found that serum concentrations that generated the most effective responses to myeloma cell lines was 70–430 μg/ml, whereas no biological activity was observed with serum concentrations of <1 μg/ml (19).

ALTERNATIVE MECHANISMS OF ACTION

In addition to boosting ADCC responses by NK cells, several alternative immune-promoting mechanisms of action may contribute to the anti-myeloma responses of Elo in patients, as detailed in this section. These alternative mechanisms include promoting SLAMF7-SLAMF7 interactions between NK cells and myeloma cells, co-stimulating calcium signaling by other activating receptors in NK cells, and promoting ADCP of myeloma cells by macrophages (**Figure 2**).

Alternative Mechanisms Involving NK Cells

Collins et al. were the first to provide *in vitro* evidence that Elo can induce NK cell activation through direct binding to SLAMF7 on NK cells (37). The bulk of their studies used purified primary NK cells from healthy donors treated for 24 h with 100 μg/ml Elo prior to addition to assays. The addition of either Elo or F(ab')$_2$ Elo to purified NK cells was found to induce expression of the activation marker CD69 in an Fc-independent manner, whereas granzyme B release (degranulation) required the Fc domain on Elo, characteristic of ADCC responses (37). In contrast, when MM target cells were added, granzyme B was released by NK cells that had been pretreated with Elo or a mutant form of Elo (G2M3) with reduced CD16-binding capacity. It should be noted that the actual mutations in Elo-G2M3 were not described and lack of affinity toward CD16 was not demonstrated (37). Nonetheless, this Fc-independent activation of NK cells suggested a mechanism involving direct engagement with SLAMF7 by the Fab domains of Elo. In accordance with these results, the CD16$^-$ SLAMF7$^+$ NK-92 cell line was stimulated by Elo to kill SLAMF7$^+$ target cells (37). This CD16-independent cytotoxicity did not occur toward SLAMF7$^-$ target cells, suggesting that Elo was "stabilizing" the SLAMF7 between NK and target cell to promote NK cell-mediated cytotoxicity. Furthermore, the effect was unique to Elo, since another SLAMF7 antibody that inhibits SLAMF7-SLAMF7 homotypic interactions instead inhibited cytotoxicity of SLAMF7$^+$ target

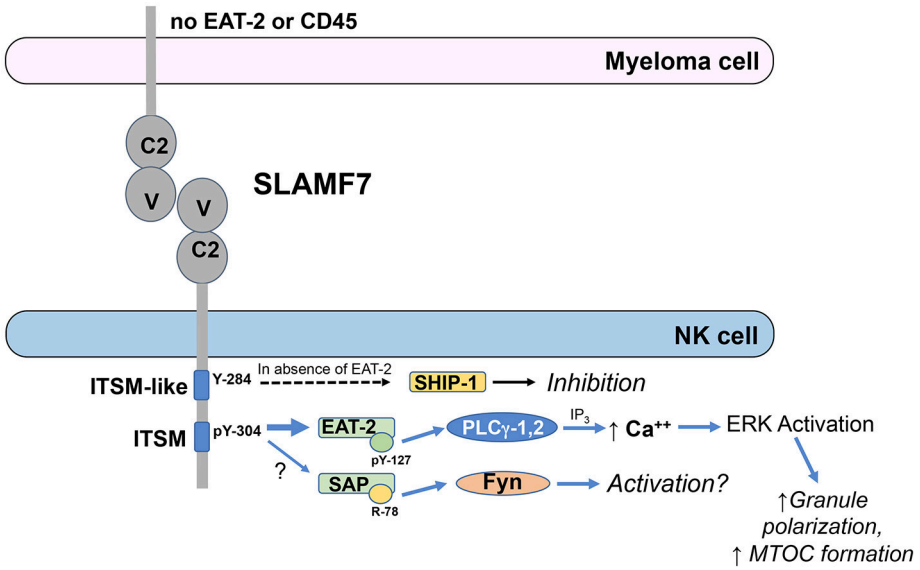

FIGURE 1 | ITSM-mediated signaling by SLAMF7 in NK cells. EAT-2 is predominantly recruited to phosphorylated tyrosine (pY)-304 within the ITSM of SLAMF7 (TVYSTV; numbering follows NCBI reference sequence NP_067004.3 and UniProt Q9NQ25-1) and PLCγ-1 and PLCγ-2 are recruited to pY-127 on EAT-2. Activated PLCγ generates inositol trisphosphate (IP_3), which induces release of calcium from the endoplasmic reticulum into the cytosol, causing activation of ERK and downstream co-stimulation of polarization of the microtubular organizing center and cytolytic granules toward the tumor cell to enhance cytotoxicity. SAP has also been reportedly recruited to pY-304 at lower affinity and can recruit Fyn to arginine (R)-78, resulting in downstream activation, although the functional relevance of SAP recruitment is unclear. SHIP-1 can also be recruited to SLAMF7 to mediate inhibitory signaling in cells lacking EAT-2 expression and this recruitment requires Y-284 in the ITSM-like sequence on SLAMF7, but direct binding has not been demonstrated to that site to date. These signaling pathways are abrogated in myeloma cells, due to their lack of EAT-2 and CD45 expression.

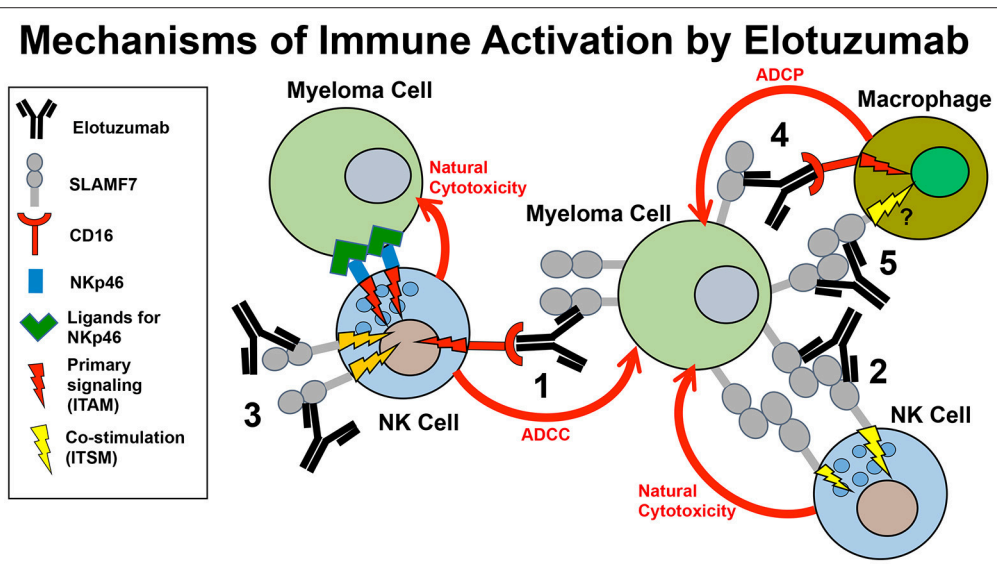

FIGURE 2 | Model of the mechanisms of innate immune activation by elotuzumab. Elotuzumab promotes numerous innate immune mechanisms to enhance attack of myeloma tumor cells by NK cells and macrophages. The known mechanisms are: (1) Facilitating NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of myeloma cells through Fc-dependent interactions with FcγRIIIA (CD16). (2) Promoting SLAMF7-SLAMF7 interactions to enhance ITSM-mediated co-stimulatory signaling in NK cells, thereby potentiating natural cytotoxicity of myeloma cells. This mechanism likely requires simultaneous engagement of ITAM-linked activating receptors on NK cells with ligands on myeloma cells. (3) Triggering ITSM-mediated co-stimulatory signaling in NK cells to enhance calcium signaling originating from ITAM-linked activating receptors (such as NKp46 or CD16) engaging with ligands on myeloma cells. (4) Promoting macrophage-mediated antibody-dependent cellular phagocytosis (ADCP) of myeloma cells through Fc-dependent interactions with Fcγ receptors. The operative Fcγ receptors in macrophages that can promote ADCP are FcγRIIIA (CD16), FcγRIIA (CD32), and FcγRI (CD64). (5) Although not yet established, it is possible that elotuzumab may also be able to promote SLAMF-SLAMF7 interactions and co-stimulatory signaling to enhance ADCP in macrophages expressing both SLAMF7 and EAT-2.

cells by NK-92 cells (37). Although further work is necessary to convincingly prove this mechanism, the evidence from Collins et al. suggest that Elo can also facilitate SLAMF7-SLAMF7 interactions between NK cells and myeloma cells to promote natural cytotoxicity, in addition to its capacity to promote ADCC responses. Although currently unpublished, Pazina et al. recently presented further evidence that Elo has unique properties among several SLAMF7 antibodies in facilitating SLAMF7-SLAMF7 interactions between NK cells and myeloma cells at the 2018 European Hematology Association meeting (81).

Pazina et al. also tested for CD16-independent effects of Elo on activation of NK cells within healthy donor peripheral blood mononuclear cells (PBMC) *in vitro* (39). Elo strongly promoted degranulation of NK cells that required integrity of the Fc domain and correlated with SLAMF7 expression level on the myeloma cells, thereby confirming that the main mechanism of action of Elo is NK cell-mediated ADCC (39). Pazina saw CD69 induction on NK in PBMC, but it was Fc-dependent, so mediated by opsonization of other SLAMF7$^+$ immune cells and engaging CD16 on the NK cells. In stark contrast, no increase in CD69 expression (overnight assay) or degranulation (2 h assay) was detected on NK cells when PBMC were incubated with F(ab')$_2$ Elo or an Fc mutant form of Elo that they showed to be incapable of binding to recombinant CD16 (39). Given these results, it is unclear why Collins et al. observed Fc-independent CD69 induction on purified NK cells after 24 h treatment with F(ab')2 or Fc mutant (G2M3) Elo, but perhaps due to their use of purified NK cells (39). Pazina et al. also showed that a non-fucosylated form of Elo that exhibited higher affinity toward CD16 induced more potent degranulation and CD69 expression in the presence or absence of myeloma cells, thereby providing further support for the role of the Fc domain interacting with FcγRIIIA. Whereas, Lee et al. demonstrated that the 1G2 mAb could trigger intracellular calcium mobilization upon engaging SLAMF7 expressed in a rat NK cell line (59), Pazina et al. found that crosslinking SLAMF7 with Elo alone had no impact on intracellular calcium concentrations in primary NK cells (39).

Despite defining the clear importance of the ADCC response, Pazina et al. also detected a novel co-stimulatory signaling effect that resulted when Elo engaged with SLAMF7 on the NK cell surface (39). They found that Elo binding to SLAMF7 can significantly enhance the intensity of intracellular calcium responses triggered by the ITAM-linked NK cell activating receptor, NKp46, and this co-stimulatory effect was independent of the Fc domain of Elo (39). Interestingly, while the calcium signaling required multimeric crosslinking of biotinylated NKp46 antibody with streptavidin, the Elo was not biotinylated, so was not forcibly co-aggregated with the NKp46 receptors (39). This is important, since it suggests that Elo binding to SLAMF7 on the surface of NK cells (as would occur in treated patients) can co-stimulate calcium signaling responses triggered by NKp46 and other ITAM-coupled receptors engaging with ligands on the surface of myeloma cells. In addition, non-biotinylated Elo could even further boost calcium signaling beyond levels achieved with the combination of biotinylated antibodies to NKp46 and the co-stimulatory NKG2D receptor (39). In this way, Elo demonstrates unique co-stimulatory signaling capacity, presumably resulting from SLAMF7 recruiting EAT2, which recruits PLC-γ to initiate intracellular calcium mobilization (66). It is also important that this co-stimulation effect by Elo has the potential to enhance specific tumor target cell recognition through other activating receptors, but would not universally activate NK cells in a tumor non-specific and potentially autoimmune manner. Interestingly, a similar boost in calcium signaling was previously observed in mouse NK cells stimulated with antibodies toward 2B4 + CD16, as compared to CD16 alone, and the effect was nearly lost in NK cells from EAT-2-deficient mice (66). On the other hand, biotinylated Elo plus streptavidin was unable to stimulate calcium mobilization in human NK cells by Pazina et al., whereas previous work with other SLAMF7 antibodies and secondary crosslinkers stimulated strong calcium mobilization in mouse and human NK cells (59, 61), indicating that Elo has unique properties, presumably through binding a distinct epitope within the C2 domain of SLAMF7.

Alternative Mechanisms Involving Other Immune Cells

Recently, Kurdi et al. found that Elo can also stimulate ADCP of tumor cells by tumor-associated macrophages (TAMs) in an Fcγ receptor-dependent manner (77). Elo significantly reduced tumor burden and prolonged survival in a xenograft mouse model using SCID-beige mice implanted with a myeloma cell line. SCID-beige mice lack T and B cells and have compromised NK cell cytolytic function (82), leaving monocytes/macrophages as the primary anti-tumor effector cells. The effects were abrogated using a form of Elo with the Fc domain mutated to prevent interactions with Fcγ receptors or if macrophage function was compromised by using NOD SCID gamma (NSG) immunodeficient mice (77). Elo also enhanced infiltration of TAMs, which displayed higher expression of activation markers. Finally, TAMs that had been polarized to the M1 phenotype in culture demonstrated enhanced *in vitro* ADCP capacity toward myeloma cells in the presence of Elo (77).

Although the Kurdi et al. study relies on the interactions of mouse Fcγ receptors with the humanized Elo antibody, the results open a new chapter of understanding by showing that TAMs may be an additional innate immune effector cell contributing to the mechanism of Elo anti-tumor activity in human patients (77). ADCP by monocytes and macrophages can be triggered through their surface expression of FcγRIIIA (CD16), FcγRIIA (CD32), or FcγRI (CD64) and can contribute significantly to anti-tumor effects of IgG antibodies, such as rituximab (83–86). Of note, the Fc domain of Elo has been shown to bind with approximately 5,000-fold higher affinity to CD64 than to the high affinity isoform of CD16 (39), exemplifying the potential biological relevance of this mechanism. It should be further noted that depletion of NK cells in an immunocompetent xenograft mouse model by Bezman et al. significantly reduced the anti-tumor effects of Elo, but activity was not completely

lost (25). This result indicates that NK cells play a major role in Elo function, but other immune cells are also involved. Furthermore, Kurdi et al. found that either depletion of NK cells or macrophages resulted in essentially identical loss of the anti-tumor benefits of Elo in immunocompetent xenograft mice (77), although it is unclear if these results in a mouse model phenocopy the roles of these innate effector cells in humans. In addition, Bezman et al. found significantly enhanced tumor growth if $CD8^+$ T cells were depleted in combination with Elo, thereby further implicating a cooperative role for cytotoxic T cells in the anti-tumor function of Elo (25). Taken together, these mouse studies demonstrate that NK cells are key effectors in mediating the biological anti-myeloma effects of Elo through ADCC and direct engagement of SLAMF7, but significant contributions are likely also derived from ADCP by TAMs, as well as supporting adaptive immune responses involving cytotoxic T cells, at least in these mouse models.

FUTURE RESEARCH AND CLINICAL TRIALS

In summary, accumulating published evidence demonstrates that Elo mediates strong ADCC by NK cells, enhanced SLAMF7-SLAMF7 interactions, co-stimulatory signaling in NK cells, and ADCP by macrophages (**Figure 2**), but additional questions remain to fully elucidate the mechanism of action by which Elo boosts immune function toward MM in patients. In addition, new preclinical studies and clinical trials are needed to develop additional effective combination therapies, to establish roles of other immune cells in Elo function, and to find biomarkers that identify patients that will best respond to Elo therapy.

A variety of mechanistic questions also remain to fully understand the mechanism of Elo activation of NK cells. For instance, accumulating data suggest that Elo binds to a unique epitope on SLAMF7 to mediate co-stimulation or facilitate SLAMF7-SLAMF7 interactions. Improved understanding of this binding site and how binding influences SLAMF7 structure, orientation, etc. are of high interest. In addition, while elegant SLAMF7 signaling function studies have been performed on NK cells in mice, particularly knockout models, more mechanistic studies in human NK cells are needed.

As with other immunotherapies, certain MM patients respond substantially better to Elo therapy, and further work is necessary to identify molecular characteristcs that are unique to high-responding vs. low-responding patients. Such findings could result in the identification of biomarkers that stratify the patients most likely to respond and tailor their therapy accordingly. For example, little is known about the expression of SLAMF7-L vs. SLAMF7-S alternative splice variants in NK cell subsets and whether these expression patterns change in subsets of MM patients or different stages of disease. Predominant expression of SLAMF7-S could render a subset of NK cells resistant to co-stimulatory signaling or perhaps inhibitory, and therefore differentially responsive to Elo. Of note, a subset of HCMV seropositive individuals exhibit "adaptive" or "memory-like" NK cell subsets lacking expression of EAT-2, and these NK cells are likely incapable of co-stimulatory signaling through SLAMF7 in HCMV seropositive individuals (68, 69) or may demonstrate inhibitory signaling through the receptor.

Since Elo is only therapeutically effective when used in combination with IMiDs or bortezomib and dexamethasone, the biological basis for these synergies require further resolution and additional combination therapies should be tested. Importantly, the mechanistic basis by which IMiDs enhance NK cell function to benefit therapeutic efficacy of Elo are largely unexplored. It was previously shown that lenalidomide can lower the threshold of NK cell activation to promote cytotoxicity and IFN-γ responses (87), but this has not yet been studied in combination with Elo. Also, early clinical trial results suggest unique synergies when pomalidomide is combined with Elo (52), raising questions of whether immune cells in addition to NK cells are contributing or if supplementation with additional immune stimulating drugs could further improve responses. Furthermore, it may be worthwhile to combine or alternate Elo therapy with the other currently available antibody that promotes NK cell-mediated ADCC in MM, daratumumab. The availability of two antibodies targeting distinct myeloma cell surface markers (SLAMF7 and CD38, respectively) provides an advantage that could be exploited in treating MM patients. In addition, expression of SLAMF7 has also been identified on tumor cells in a subset of patients with chronic lymphocytic leukemia, myelodysplastic syndrome, diffuse large B cell lymphoma, and peripheral T cell lymphoma. Therefore, other hematopoietic malignancies may be amenable to treatment with Elo.

The pre-clinical finding that non-fucosylated Elo is more potent in stimulating NK cell activation than conventional Elo (39) suggests that this enhanced form of the mAb could have improved efficacy in the clinic. While the potential toxicity of hyperactive ADCC responses by NK cells or ADCP responses by macrophages must be considered, non-fucosylated Elo may, in fact, have efficacy as a single agent or may have even better efficacy in combination therapies.

Further research is also clearly warranted to improve our minimal understanding of the impacts of Elo on anti-myeloma responses by SLAMF7-expressing monocytes, macrophages, and DC. The discoveries that SLAMF7 is highly expressed on M1 macrophages (73) and Elo promotes ADCP by inflammatory M1 macrophages (77) provide an exciting new direction that has only been minimally studied to date. Furthermore, it is interesting that non-classical "patrolling" monocytes express CD16, SLAMF7 and EAT-2 (39, 76), which are the same molecular components necessary for NK cell activation by Elo and likely contribute to ADCP and possibly co-stimulatory signaling through direct SLAMF7 engagement (**Figure 2**). Finally, studies are needed to test whether Elo activity is affected by SLAMF7 expression on other cells, such as subsets of B cells and $CD8^+$ T cells.

Taken together, Elo has proven to be safe and effective when used in combination therapy to treat MM. Although our understanding is rapidly expanding, this unique

antibody offers a wide array of additional opportunities for performing further research and for conducting new combination clinical trials to improve efficacy in treating MM and potentially other hematopoietic cancers.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
2. Turesson I, Bjorkholm M, Blimark CH, Kristinsson S, Velez R, Landgren O. Rapidly changing myeloma epidemiology in the general population: increased incidence, older patients, and longer survival. *Eur J Haematol.* (2018) 101:237–44. doi: 10.1111/ejh.13083
3. Mentlik James A, Cohen AD, Campbell KS. Combination immune therapies to enhance anti-tumor responses by NK cells. *Front Immunol.* (2013) 4:481. doi: 10.3389/fimmu.2013.00481
4. Vandross A. Proteasome inhibitor-based therapy for treatment of newly diagnosed multiple myeloma. *Semin Oncol.* (2017) 44:381–4. doi: 10.1053/j.seminoncol.2018.01.002
5. Chim CS, Kumar SK, Orlowski RZ, Cook G, Richardson PG, Gertz MA, et al. Management of relapsed and refractory multiple myeloma: novel agents, antibodies, immunotherapies and beyond. *Leukemia* (2018) 32:252–62. doi: 10.1038/leu.2017.329
6. Varga C, Laubach JP, Anderson KC, Richardson PG. Investigational agents in immunotherapy: a new horizon for the treatment of multiple myeloma. *Br J Haematol.* (2018) 181:433–46. doi: 10.1111/bjh.15116
7. Moreau P. How I treat myeloma with new agents. *Blood* (2017) 130:1507–13. doi: 10.1182/blood-2017-05-743203
8. Neri P, Bahlis NJ, Lonial S. New strategies in multiple myeloma: immunotherapy as a novel approach to treat patients with multiple myeloma. *Clin Cancer Res.* (2016) 22:5959–65. doi: 10.1158/1078-0432.CCR-16-0184
9. van de Donk NWCJ, Usmani SZ. CD38 antibodies in multiple myeloma: mechanisms of action and modes of resistance. *Front Immunol.* (2018) 9:2134. doi: 10.3389/fimmu.2018.02134
10. Plesner T, Krejcik J. Daratumumab for the treatment of multiple myeloma. *Front Immunol.* (2018) 9:1228. doi: 10.3389/fimmu.2018.01228
11. Binyamin L, Alpaugh RK, Hughes TL, Lutz CT, Campbell KS, Weiner LM. Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J Immunol.* (2008) 180:6392–401. doi: 10.4049/jimmunol.180.9.6392
12. Purdy AK, Campbell KS. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer Biol Ther.* (2009) 8:2209–18. doi: 10.4161/cbt.8.23.10455
13. Lanier LL. NK cell recognition. *Annu Rev Immunol.* (2005) 23:225–74. doi: 10.1146/annurev.immunol.23.021704.115526
14. Kroger N, Shaw B, Iacobelli S, Zabelina T, Peggs K, Shimoni A, et al. Comparison between antithymocyte globulin and alemtuzumab and the possible impact of KIR-ligand mismatch after dose-reduced conditioning and unrelated stem cell transplantation in patients with multiple myeloma. *Br J Haematol.* (2005) 129:631–43. doi: 10.1111/j.1365-2141.2005.05513.x
15. Gabriel IH, Sergeant R, Szydlo R, Apperley JF, DeLavallade H, Alsuliman A, et al. Interaction between KIR3DS1 and HLA-Bw4 predicts for progression-free survival after autologous stem cell transplantation in patients with multiple myeloma. *Blood* (2010) 116:2033–9. doi: 10.1182/blood-2010-03-273706
16. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* (2005) 105:251–8. doi: 10.1182/blood-2004-04-1422
17. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, et al. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res.* (2007) 67:8444–9. doi: 10.1158/0008-5472.CAN-06-4230
18. Hsi ED, Steinle R, Balasa B, Szmania S, Draksharapu A, Shum BP, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. *Clin Cancer Res.* (2008) 14:2775–84. doi: 10.1158/1078-0432.CCR-07-4246
19. Tai YT, Dillon M, Song W, Leiba M, Li XF, Burger P, et al. Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood* (2008) 112:1329–37. doi: 10.1182/blood-2007-08-107292
20. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* (2009) 113:3503–11. doi: 10.1182/blood-2008-08-173914
21. Fauriat C, Mallet F, Olive D, Costello RT. Impaired activating receptor expression pattern in natural killer cells from patients with multiple myeloma. *Leukemia* (2006) 20:732–3. doi: 10.1038/sj.leu.2404096
22. Bernal M, Garrido P, Jimenez P, Carretero R, Almagro M, Lopez P, et al. Changes in activatory and inhibitory natural killer (NK) receptors may induce progression to multiple myeloma: implications for tumor evasion of T and NK cells. *Hum Immunol.* (2009) 70:854–7. doi: 10.1016/j.humimm.2009.07.004
23. Benson DMJr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1 / PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel, monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
24. Guillerey C, Nakamura K, Vuckovic S, Hill GR, Smyth MJ. Immune responses in multiple myeloma: role of the natural immune surveillance and potential of immunotherapies. *Cell Mol Life Sci.* (2016) 73:1569–89. doi: 10.1007/s00018-016-2135-z
25. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K, et al. PD-1 blockade enhances elotuzumab efficacy in mouse tumor models. *Blood Adv.* (2017) 1:753–65. doi: 10.1182/bloodadvances.2017004382
26. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, et al. Restoring natural killer cell immunity against multiple myeloma in the era of new drugs. *Front Immunol.* (2017) 8:1444. doi: 10.3389/fimmu.2017.01444
27. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* (2002) 419:734–8. doi: 10.1038/nature01112
28. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc Natl Acad Sci USA.* (2008) 105:1285–90. doi: 10.1073/pnas.0711293105
29. Davies FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* (2001) 98:210–6. doi: 10.1182/blood.V98.1.210
30. Shi J, Tricot GJ, Garg TK, Malaviarachchi PA, Szmania SM, Kellum RE, et al. Bortezomib down-regulates the cell-surface expression of HLA class

I and enhances natural killer cell-mediated lysis of myeloma. *Blood* (2008) 111:1309–17. doi: 10.1182/blood-2007-03-078535
31. Fionda C, Soriani A, Malgarini G, Iannitto ML, Santoni A, Cippitelli M. Heat shock protein-90 inhibitors increase MHC class I-related chain A and B ligand expression on multiple myeloma cells and their ability to trigger NK cell degranulation. *J Immunol.* (2009) 183:4385–94. doi: 10.4049/jimmunol.0901797
32. Fionda C, Abruzzese MP, Zingoni A, Cecere F, Vulpis E, Peruzzi G, et al. The IMiDs targets IKZF-1/3 and IRF4 as novel negative regulators of NK cell-activating ligands expression in multiple myeloma. *Oncotarget* (2015) 6:23609–30. doi: 10.18632/oncotarget.4603
33. Antonangeli F, Soriani A, Ricci B, Ponzetta A, Benigni G, Morrone S, et al. Natural killer cell recognition of *in vivo* drug-induced senescent multiple myeloma cells. *Oncoimmunology* (2016) 5:e1218105. doi: 10.1080/2162402X.2016.1218105
34. Borrelli C, Ricci B, Vulpis E, Fionda C, Ricciardi MR, Petrucci MT, et al. Drug-Induced senescent multiple myeloma cells elicit NK cell proliferation by direct or exosome-mediated IL15 trans-presentation. *Cancer Immunol Res.* (2018) 6:860–9. doi: 10.1158/2326-6066.CIR-17-0604
35. Xie Z, Gunaratne J, Cheong LL, Liu SC, Koh TL, Huang G, et al. Plasma membrane proteomics identifies biomarkers associated with MMSET overexpression in T(4;14) multiple myeloma. *Oncotarget* (2013) 4:1008–18. doi: 10.18632/oncotarget.1049
36. Chen J, Zhong MC, Guo H, Davidson D, Mishel S, Lu Y, et al. SLAMF7 is critical for phagocytosis of haematopoietic tumour cells via Mac-1 integrin. *Nature* (2017) 544:493–7. doi: 10.1038/nature22076
37. Collins SM, Bakan CE, Swartzel GD, Hofmeister CC, Efebera YA, Kwon H, et al. Elotuzumab directly enhances NK cell cytotoxicity against myeloma via CS1 ligation: evidence for augmented NK cell function complementing ADCC. *Cancer Immunol Immunother.* (2013) 62:1841–9. doi: 10.1007/s00262-013-1493-8
38. van Rhee F, Szmania SM, Dillon M, van Abbema AM, Li X, Stone MK, et al. Combinatorial efficacy of anti-CS1 monoclonal antibody elotuzumab (HuLuc63) and bortezomib against multiple myeloma. *Mol Cancer Ther.* (2009) 8:2616–24. doi: 10.1158/1535-7163.MCT-09-0483
39. Pazina T, James AM, MacFarlane AW, Bezman NA, Henning KA, Bee C, et al. The anti-SLAMF7 antibody elotuzumab mediates NK cell activation through both CD16-dependent and -independent mechanisms. *Oncoimmunology* (2017) 6:e1339853. doi: 10.1080/2162402X.2017.1339853
40. Zonder JA, Mohrbacher AF, Singhal S, van Rhee F, Bensinger WI, Ding H, et al. A phase 1, multicenter, open-label, dose escalation study of elotuzumab in patients with advanced multiple myeloma. *Blood* (2012) 120:552–9. doi: 10.1182/blood-2011-06-360552
41. Jakubowiak AJ, Benson DM, Bensinger W, Siegel DS, Zimmerman TM, Mohrbacher A, et al. Phase I trial of anti-CS1 monoclonal antibody elotuzumab in combination with bortezomib in the treatment of relapsed/refractory multiple myeloma. *J Clin Oncol.* (2012) 30:1960–5. doi: 10.1200/JCO.2011.37.7069
42. Wang Y, Zhang Y, Hughes T, Zhang J, Caligiuri MA, Benson DM, et al. Fratricide of NK cells in daratumumab therapy for multiple myeloma overcome by *ex vivo*-expanded autologous NK cells. *Clin Cancer Res.* (2018) 24:4006–17. doi: 10.1158/1078-0432.CCR-17-3117
43. Armeanu S, Krusch M, Baltz KM, Weiss TS, Smirnow I, Steinle A, et al. Direct and natural killer cell-mediated antitumor effects of low-dose bortezomib in hepatocellular carcinoma. *Clin Cancer Res.* (2008) 14:3520–8. doi: 10.1158/1078-0432.CCR-07-4744
44. Balasa B, Yun R, Belmar NA, Fox M, Chao DT, Robbins MD, et al. Elotuzumab enhances natural killer cell activation and myeloma cell killing through interleukin-2 and TNF-alpha pathways. *Cancer Immunol Immunother.* (2015) 64:61–73. doi: 10.1007/s00262-014-1610-3
45. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29:1441–4. doi: 10.1038/leu.2015.11
46. Della Chiesa M, Pesce S, Muccio L, Carlomagno S, Sivori S, Moretta A, et al. Features of memory-like and PD-1^{+} human NK cell subsets. *Front Immunol.* (2016) 7:351. doi: 10.3389/fimmu.2016.00351
47. Jakubowiak A, Offidani M, Pegourie B, De La Rubia J, Garderet L, Laribi K, et al. Randomized phase 2 study: elotuzumab plus bortezomib/dexamethasone vs bortezomib/dexamethasone for relapsed/refractory MM. *Blood* (2016) 127:2833–40. doi: 10.1182/blood-2016-01-694604
48. Lonial S, Vij R, Harousseau JL, Facon T, Moreau P, Mazumder A, et al. Elotuzumab in combination with lenalidomide and low-dose dexamethasone in relapsed or refractory multiple myeloma. *J Clin Oncol.* (2012) 30:1953–9. doi: 10.1200/JCO.2011.37.2649
49. Richardson PG, Jagannath S, Moreau P, Jakubowiak AJ, Raab MS, Facon T, et al. Elotuzumab in combination with lenalidomide and dexamethasone in patients with relapsed multiple myeloma: final phase 2 results from the randomised, open-label, phase 1b-2 dose-escalation study. *Lancet Haematol.* (2015) 2:e516–27. doi: 10.1016/S2352-3026(15)00197-0
50. Lonial S, Dimopoulos M, Palumbo A, White D, Grosicki S, Spicka I, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Med.* (2015) 373:621–31. doi: 10.1056/NEJMoa1505654
51. Mateos MV, Granell M, Oriol A, Martinez-Lopez J, Blade J, Hernandez MT, et al. Elotuzumab in combination with thalidomide and low-dose dexamethasone: a phase 2 single-arm safety study in patients with relapsed/refractory multiple myeloma. *Br J Haematol.* (2016) 175:448–56. doi: 10.1111/bjh.14263
52. Dimopoulos MA, Dytfeld D, Grosicki S, Moreau P, Takezako N, Hori M, et al. *Elotuzumab Plus Pomalidomide/Dexamethasone (EPD) vs. PD for Treatment of Relapsed/Refractory Multiple Myeloma (RRMM): Results from the Phase 2, Randomized Open-Label ELOQUENT-3 Study*. Stockholm: EHA Congress (2018). Abstract LB2606, June 14–17, 2018.
53. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* (2002) 99:754–8. doi: 10.1182/blood.V99.3.754
54. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol.* (2003) 21:3940–7. doi: 10.1200/JCO.2003.05.013
55. Dimopoulos MA, Lonial S, White D, Moreau P, Palumbo A, San-Miguel J, et al. Elotuzumab plus lenalidomide/dexamethasone for relapsed or refractory multiple myeloma: ELOQUENT-2 follow-up and post-hoc analyses on progression-free survival and tumour growth. *Br J Haematol.* (2017) 178:896–905. doi: 10.1111/bjh.14787
56. Boles KS, Nakajima H, Colonna M, Chuang SS, Stepp SE, Bemmett M, et al. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* (1999) 54:27–34. doi: 10.1034/j.1399-0039.1999.540103.x
57. Bouchon A, Cella M, Grierson HL, Cohen JI, Colonna M. Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J Immunol.* (2001) 167:5517–21. doi: 10.4049/jimmunol.167.10.5517
58. Kumaresan PR, Lai WC, Chuang SS, Bennett M, Mathew PA. CS1, a novel member of the CD2 family, is homophilic and regulates NK cell function. *Mol Immunol.* (2002) 39:1–8. doi: 10.1016/S0161-5890(02)00094-9
59. Lee JK, Boles KS, Mathew PA. Molecular and functional characterization of a CS1 (CRACC) splice variant expressed in human NK cells that does not contain immunoreceptor tyrosine-based switch motifs. *Eur J Immunol.* (2004) 34:2791–9. doi: 10.1002/eji.200424917
60. Stark S, Watzl C. 2B4 (CD244), NTB-A and CRACC (CS1) stimulate cytotoxicity but no proliferation in human NK cells. *Int Immunol.* (2006) 18:241–7. doi: 10.1093/intimm/dxh358
61. Cruz-Munoz ME, Dong Z, Shi X, Zhang S, Veillette A. Influence of CRACC, a SLAM family receptor coupled to the adaptor EAT-2, on natural killer cell function. *Nat Immunol.* (2009) 10:297–305. doi: 10.1038/ni.1693
62. Tassi I, Colonna M. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J Immunol.* (2005) 175:7996–8002. doi: 10.4049/jimmunol.175.12.7996
63. Wilson TJ, Garner LI, Metcalfe C, King E, Margraf S, Brown MH. Fine specificity and molecular competition in SLAM family receptor signalling. *PLoS ONE* (2014) 9:e92184. doi: 10.1371/journal.pone.0092184

64. Veillette A, Dong Z, Perez-Quintero LA, Zhong MC, Cruz-Munoz ME. Importance and mechanism of 'switch' function of SAP family adapters. *Immunol Rev.* (2009) 232:229–39. doi: 10.1111/j.1600-065X.2009.00824.x
65. Thompson AD, Braun BS, Arvand A, Stewart SD, May WA, Chen E, et al. EAT-2 is a novel SH2 domain containing protein that is up regulated by Ewing's sarcoma EWS/FLI1 fusion gene. *Oncogene* (1996) 13:2649–58.
66. Perez-Quintero LA, Roncagalli R, Guo H, Latour S, Davidson D, Veillette A. EAT-2, a SAP-like adaptor, controls NK cell activation through phospholipase Cγ, Ca^{++}, and Erk, leading to granule polarization. *J Exp Med.* (2014) 211:727–42. doi: 10.1084/jem.20132038
67. Guo H, Cruz-Munoz ME, Wu N, Robbins M, Veillette A. Immune cell inhibition by SLAMF7 is mediated by a mechanism requiring src kinases, CD45, and SHIP-1 that is defective in multiple myeloma cells. *Mol Cell Biol.* (2015) 35:41–51. doi: 10.1128/MCB.01107-14
68. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity* (2015) 42:431–42. doi: 10.1016/j.immuni.2015.02.013
69. Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* (2015) 42:443–56. doi: 10.1016/j.immuni.2015.02.008
70. Murphy JJ, Hobby P, Vilarino-Varela J, Bishop B, Iordanidou P, Sutton BJ, et al. A novel immunoglobulin superfamily receptor (19A) related to CD2 is expressed on activated lymphocytes and promotes homotypic B-cell adhesion. *Biochem J.* (2002) 361:431–6. doi: 10.1042/bj3610431
71. Kramer B, Kebschull M, Nowak M, Demmer RT, Haupt M, Korner C, et al. Role of the NK cell-activating receptor CRACC in periodontitis. *Infect Immun.* (2013) 81:690–6. doi: 10.1128/IAI.00895-12
72. Lee JK, Mathew SO, Vaidya SV, Kumaresan PR, Mathew PA. CS1 (CRACC, CD319) induces proliferation and autocrine cytokine expression on human B lymphocytes. *J Immunol.* (2007) 179:4672–8. doi: 10.4049/jimmunol.179.7.4672
73. Beyer M, Mallmann MR, Xue J, Staratschek-Jox A, Vorholt D, Krebs W, et al. High-resolution transcriptome of human macrophages. *PLoS ONE* (2012) 7:e45466. doi: 10.1371/journal.pone.0045466
74. Tassi I, Presti R, Kim S, Yokoyama WM, Gilfillan S, Colonna M. Phospholipase C-gamma 2 is a critical signaling mediator for murine NK cell activating receptors. *J Immunol.* (2005) 175:749–54. doi: 10.4049/jimmunol.175.2.749
75. Veillette A. NK cell regulation by SLAM family receptors and SAP-related adapters. *Immunol Rev.* (2006) 214:22–34. doi: 10.1111/j.1600-065X.2006.00453.x
76. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* (2011) 118:e16–31. doi: 10.1182/blood-2010-12-326355
77. Kurdi AT, Glavey SV, Bezman NA, Jhatakia A, Guerriero JL, Manier S, et al. Antibody-dependent cellular phagocytosis by macrophages is a novel mechanism of action of elotuzumab. *Mol Cancer Ther.* (2018) 17:1454–63. doi: 10.1158/1535-7163.MCT-17-0998
78. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J Exp Med.* (1989) 170:481–97.
79. Trinchieri G, Valiante N. Receptors for the Fc fragment of IgG on natural killer cells. *Nat Immun.* (1993) 12:218–34.
80. Selvaraj P, Fifadara N, Nagarajan S, Cimino A, Wang G. Functional regulation of human neutrophil Fc gamma receptors. *Immunol Res.* (2004) 29:219–30. doi: 10.1385/IR:29:1-3:219
81. Pazina T, James AM, Jhatakia A, Graziano RF, Bezman NA, Robbins MD, et al. *Elotuzumab Promotes Self-Engagement of SLAMF7 Between Natural Killer and Multiple Myeloma Cells to Enhance Cytotoxicity*. Stockholm: EHA Congress (2018). Abstract PS1277, June 14–17, 2018.
82. Roder J, Duwe A. The beige mutation in the mouse selectively impairs natural killer cell function. *Nature* (1979) 278:451–3. doi: 10.1038/278451a0
83. Richards JO, Karki S, Lazar GA, Chen H, Dang W, Desjarlais JR. Optimization of antibody binding to FcgammaRIIa enhances macrophage phagocytosis of tumor cells. *Mol Cancer Ther.* (2008) 7:2517–27. doi: 10.1158/1535-7163.MCT-08-0201
84. Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells *in vitro*. *J Immunol.* (2009) 182:4415–22. doi: 10.4049/jimmunol.0713732
85. Gordan S, Biburger M, Nimmerjahn F. bIgG time for large eaters: monocytes and macrophages as effector and target cells of antibody-mediated immune activation and repression. *Immunol Rev.* (2015) 268:52–65. doi: 10.1111/imr.12347
86. Weiskopf K, Weissman IL. Macrophages are critical effectors of antibody therapies for cancer. *MAbs* (2015) 7:303–10. doi: 10.1080/19420862.2015.1011450
87. Lagrue K, Carisey A, Morgan DJ, Chopra R, Davis DM. Lenalidomide augments actin remodeling and lowers NK-cell activation thresholds. *Blood* (2015) 126:50–60. doi: 10.1182/blood-2015-01-625004

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MINI REVIEW
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Perspectives for the Development of CD38-Specific Heavy Chain Antibodies as Therapeutics for Multiple Myeloma

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The NAD^+-metabolizing ectoenzyme CD38 is an established therapeutic target in multiple myeloma. The CD38-specific monoclonal antibodies daratumumab and isatuximab show promising results in the clinic. Nanobodies correspond to the single variable domains (VHH) derived from heavy chain antibodies that naturally occur in camelids. VHHs display high solubility and excellent tissue penetration *in vivo*. We recently generated a panel of CD38-specific nanobodies, some of which block or enhance the enzymatic activity of CD38. Fusion of such a nanobody to the hinge, CH2, and CH3 domains of human IgG1 generates a chimeric llama/human hcAb of about half the size of a conventional moAb (75 vs. 150 kDa). Similarly, a fully human CD38-specific hcAb can be generated using a CD38-specific human VH3 instead of a CD38-specific camelid nanobody. Here we discuss the advantages and disadvantages of CD38-specific hcAbs vs. conventional moAbs and provide an outlook for the potential use of CD38-specific hcAbs as novel therapeutics for multiple myeloma.

Keywords: antibody engineering, CD38, heavy chain antibody, monoclonal antibody, multiple myeloma, nanobody

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INTRODUCTION

CD38 is a cell surface ectoenzyme that metabolizes NAD^+ released from damaged cells in inflammation (1). In concert with the ecto-enzymes CD203 and CD73, CD38 contributes to the conversion of NAD^+ to immunosuppressive extracellular adenosine. In the tumor microenvironment, CD38 may promote tumor growth by suppressing effector T cell responses (1, 2). Since CD38 is overexpressed by multiple myeloma cells and other hematological tumors, it has attracted interest as a target for therapeutic antibodies (3–5).

Nanobodies are single domain antibody fragments derived from the heavy chain IgG antibodies naturally occurring in llamas and other camelids (6–8). In these animals, the IgG2 and IgG3 isotypes lack the CH1 domain and do not bind to light chains. Nanobodies correspond to the variable domain (VHH) of these heavy chain antibodies. VHHs carry characteristic residues in the framework region 2 (FR2) that render them highly soluble in the absence of a paired light chain (8–10). VHHs often have a long complementarity determining region 3 (CDR3) that can mediate binding to the catalytic cavity of an enzyme and other hidden epitopes that are not accessible for conventional antibodies (11–13). Their robust, soluble single domain format renders nanobodies amenable for genetic fusion to the hinge and Fc domains of other antibody isotypes (14, 15). Owing to their high solubility, it is much easier to link two or more VHHs into bi- or multivalent formats than the corresponding VH+VL domains of conventional antibodies.

CD38-SPECIFIC THERAPEUTIC CHIMERIC MOUSE/HUMAN AND FULLY HUMAN CONVENTIONAL ANTIBODIES

The conventional CD38-specific moAbs daratumumab and isatuximab have proven therapeutic efficacy in multiple myeloma (5, 16). Both antibodies were derived from mice immunized with human CD38. While daratumumab was generated from CD38-immunized transgenic mice that carry genomic loci encoding human IgH and IgL (17), isatuximab was generated from CD38-immunized wild type mice (18). The VH and VL domains of the murine moAb were genetically fused to the CH1-hinge-CH2-CH3 domains of human IgG1 and to the constant domain of the kappa light chain (Cκ), respectively, generating a classic mouse/human chimeric antibody (**Figure 1A**). The crystal structure of isatuximab in complex with CD38 indicates that its capacity to inhibit the enzymatic activity of CD38 is by an allosteric mechanism (18). Recently, the VH and VL domains of daratumumab were used to construct a single-chain human anti-CD38 cytokine-antibody fusion protein termed IL2-αCD38-αCD38-scTRAIL (19). The bivalent tandem scFv of daratumumab mediated specific binding to CD38 expressing myeloma cells, while the engineered homotrimeric format of TRAIL induced apoptosis of these cells, presumably by binding to cognate death receptors.

CD38-SPECIFIC CHIMERIC LLAMA/HUMAN AND FULLY HUMAN HEAVY CHAIN ANTIBODIES

Recently, CD38-specific nanobodies were generated from CD38-immunized llamas (20, 21). Some of these nanobodies inhibited or enhanced CD38 enzymatic activity in a dose dependent manner and effectively targeted CD38 on human tumor cells in a mouse Xenograft model (20). Several nanobodies bind independently of daratumumab. Such nanobodies have proven useful for detecting cell surface CD38 in patients treated with daratumumab (22). Owing to their high solubility, the nanobodies can readily be fused to other protein domains, including the hinge and Fc domains of human IgG1 (**Figure 1B**). Such chimeric llama/human heavy chain antibodies acquire the capacity to induce classic Fc-mediated effector functions, including ADCC and CDC (23).

In contrast to the hydrophylic nature of camelid VHH domains, human VH domains display a natural "stickiness" and tendency to aggregate in the absence of a light chain (24–26). This "stickiness" is attributed to the hydrophobic interface that helps to properly orient VH and VL domains for joint interaction with the target antigen (27). "Camelization" of human VH domains by substituting hydrophobic amino acid residues in FR2 with hydrophilic residues can greatly enhance the solubility of human VH domains (28–30). In order to express human heavy chain antibodies in transgenic mice or rats it is therefore advisable to inactivate not only the endogenous rodent heavy chain locus, but also the kappa and lambda light chain loci. Interestingly, during the ensuing immune response, somatic hypermutation and selection drive the expansion of VH variants that increase the solubility of heavy chain antibodies (31, 32). A similar mechanism was observed when human VH domains were affinity matured *in vitro* (33). Recently, CD38-specific human

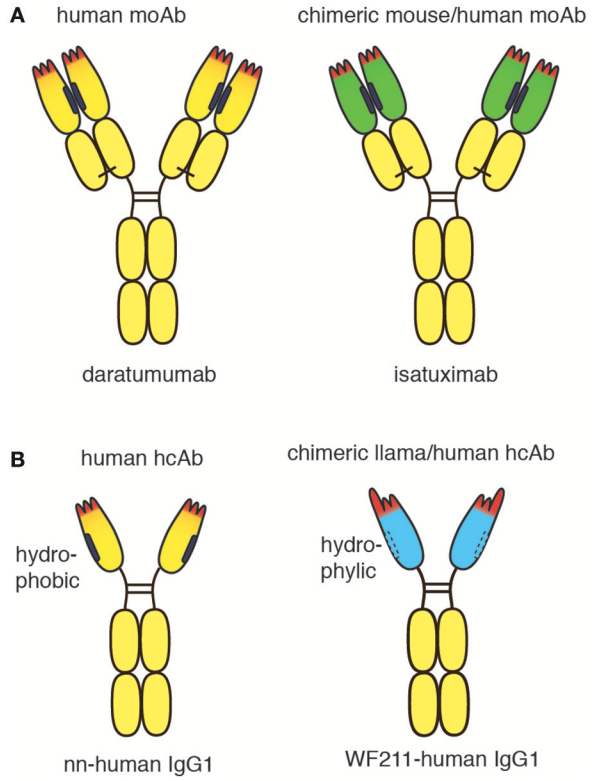

FIGURE 1 | Schematic diagram of conventional and heavy chain CD38-specific antibodies. **(A)** Schematic diagrams of therapeutic CD38-specific conventional antibodies. Daratumumab is derived from a CD38-immunized human-antibody transgenic mouse. Isatuximab is derived from a CD38-immunized wildtype mouse. The chimeric antibody was generated by genetic fusion of the VH and VL domains of the mouse monoclonal antibody to the constant domains of human IgG1 and kappa, respectively. **(B)** Schematic diagrams of CD38-specific heavy chain antibodies. A fully human CD38-specific heavy chain antibody has been derived from a CD38-immunized human heavy chain transgenic rat. The chimeric WF211-human IgG1 heavy chain antibody was generated by genetic fusion of the VHH domain of a llama heavy chain antibody to the hinge, CH2 and CH3 domains of human IgG1. Human heavy chain antibodies display a natural "stickiness," i.e., tendency to bind light chains via the hydrophobic interface that helps to stabilize the orientation of the VH-VL pair in conventional antibodies. In contrast, chimeric llama-human heavy chain antibodies do not show any natural "stickiness" or tendency to bind light chains. VHH domains have been shaped by 50 Mio years of evolution for high solubility in the absence of a light chain.

Abbreviations: ADCC, antibody-dependent cytotoxicity; CDC, complement-dependent cytotoxicity; CDR, compelementarity determining region; Fc, crystallizing fragment; FR, framework region; Ig, immunoglobulin; kD, kilodalton; NAD, nicotinamide adenine dinucleotide; moAb, monoclonal antibody; Nb, nanobody; Nb-hcAb, nanobody-based (human) heavy chain antibody; scFv, single chain variable fragment; VH, variable domain of a conventional heavy chain; VHH, variable domain of a camelid heavy chain antibody.

heavy chain antibodies were generated successfully from CD38-immunized human heavy chain-only transgenic rats (32).

ADVANTAGES AND DISADVANTAGES OF CD38-SPECIFIC HEAVY CHAIN ANTIBODIES VS. CONVENTIONAL MOABS

Tissue Penetration and *in vivo* Half Life

Heavy chain antibodies are only half the size of conventional moAbs (75 vs. 150 kDa). HcAbs may therefore penetrate more effectively into CD38-expressing tumors than full size moAbs, particularly when the tumors promote increased interstitial pressure. Better tissue penetration has indeed been demonstrated for nanobodies vs. conventional antibodies in solid tumors and subcutaneous tumors (34, 35). Tissue penetration is a highly relevant issue, in particular when considering that multiple myeloma resides in the bone marrow and is surrounded by a dense immune suppressive microenvironment (1). It will be important to determine whether nanobody-based hcAbs do reach myeloma cells in the bone marrow more efficiently than conventional antibodies.

The half life of therapeutic antibodies is influenced by several factors, including size, glycosylation, and affinity to the neonatal Fc receptor. While a smaller size may facilitate tissue penetration, a small size may also facilitate renal filtration and thereby shorten the persistence of the therapeutic *in vivo*. Several strategies have been employed successfully to prolong the *in vivo* half life of nanobodies, including conjugation to polyethylene glycol polymers (36), genetic fusion to an albumin-specific nanobody (34, 37). In case of nanobody based hcAbs, Fc engineering could be used to introduce mutations that enhance binding to the neonatal Fc receptor and thereby prolong persistence *in vivo* (38, 39).

Developability of Bispecific Therapeutics

The soluble nature of the nanobody VHH domain, facilitates the construction and production of bispecific antibodies. For example, a bispecific nanobody-based heavy chain antibody can readily be generated simply by fusing a second nanobody to the N-terminus of a nanobody-based hcAb. Importantly, nanobody-based bispecific hcAbs are composed of two identical polypeptide chains, i.e., their production does not require any "knob in hole" technology or adjusting the of expression levels of two or more vectors (40, 41). This simplifies the production and developability of bispecific hcAbs, although the moderate increase in size of a bispecific vs. a mono-specific hcAb (from ∼75 to ∼100 kDa) may compromise tissue penetration. By tandem fusion of two nanobodies that recognize independent epitopes of CD38 to the Fc domain of human IgG, we recently generated tetravalent biparatopic hcAbs that exhibit a markedly enhanced capacity to induce CDC of CD38-expressing myeloma cells.

Modulation of Enzyme Activity

Owing to the inherent capacity of nanobodies to extend into and block active site crevices (11, 12), a heavy chain antibody containing a CD38-antagonistic nanobody may provide an additional therapeutic benefit by inhibiting the production of immunosuppressive adenosine (1, 2). Conceivably, the potency of enzyme inhibition may be enhanced by fusion of an enzyme-inhibiting nanobody to a nanobody recognizing a distinct epitope of CD38, e.g., in a biparatopic activity blocking hcAb.

Immunogenicity

The potential immunogenicity of antibody therapeutics is a relevant concern (5, 42, 43). The development of neutralizing antibodies against the therapeutic antibody by the patient usually renders the patient resistant to the therapeutic. This risk for developing such antibodies is larger for chimeric antibodies that contain murine VH and VL domains such as rituximab and isatuximab than for fully human antibodies such as dartumumab which is composed only of human domains. However, it is impossible to fully humanize the idiotype of an antibody without losing specificity of effectivity since the unique CDR loops of the VH and VL domains are required for specificity. Hence, the potential development of antibodies directed against the unique CDR loops remains a concern for any therapeutic antibody. Drug antibodies have not yet been detected in any daratumumab-treated patients (5). However, it is uncertain to what extent this is due to the lack of a sensitive assay for such antibodies.

The human germline encodes ∼50 distinct VH domains and 4 distinct IgG isotypes (**Figure 2A**) (44, 45). V-D-J recombination during B-cell development generates millions of distinct idiotypes (antigen binding paratopes). Subsequent to antigen encounter, somatic hypermutation generates many more variant VH domains. During pregnancy, maternal IgG is translocated from the maternal blood through placental trophoblasts into the blood stream of the fetus, leading to tolerization of the new born immune system against millions of VH variants, but only 4 distinct IgG isotypes. In germline configuration, llama VHH domains show ∼80–90% amino acid sequence identity to human VH3 domains, i.e., the predominant VH subset found in human immunoglobulins (46). As a result of somatic hypermutation, two matured human VH domains often differ more from one another than a germline human VH3 domain from a llama VHH3 domain. A few hydrophilic amino acid residues in framework region 2 and the long CDR3 that can partially fold back onto the former interface to the VL domain largely account for the dramatically improved solubility of camelid VHH domains vs. human VH3 domains. These residues cannot be fully humanized without compromising solubility. Notwithstanding, the idiotype (CDR regions 1, 2, and 3) cover a much larger space (both, in the literal sense and in terms of potential immunogenicity) than these hydrophilic amino acids in the former VL interface.

Although the immunogenicity of a therapeutic antibody can be reduced by humanization, the residual risk remains for any therapeutic antibody that the patient develops antibodies directed against the idiotype (**Figure 2B**). Such anti-drug antibodies usually render the therapeutic useless for the patient. If more than one therapeutic antibody is available for a particular target, an option in such cases is to switch to a different biologic targeting the same molecule (e.g., from daratumumab to isatuximab or vice versa). It is conceivable that in the future, the risk of developing anti-drug antibodies can be reduced further

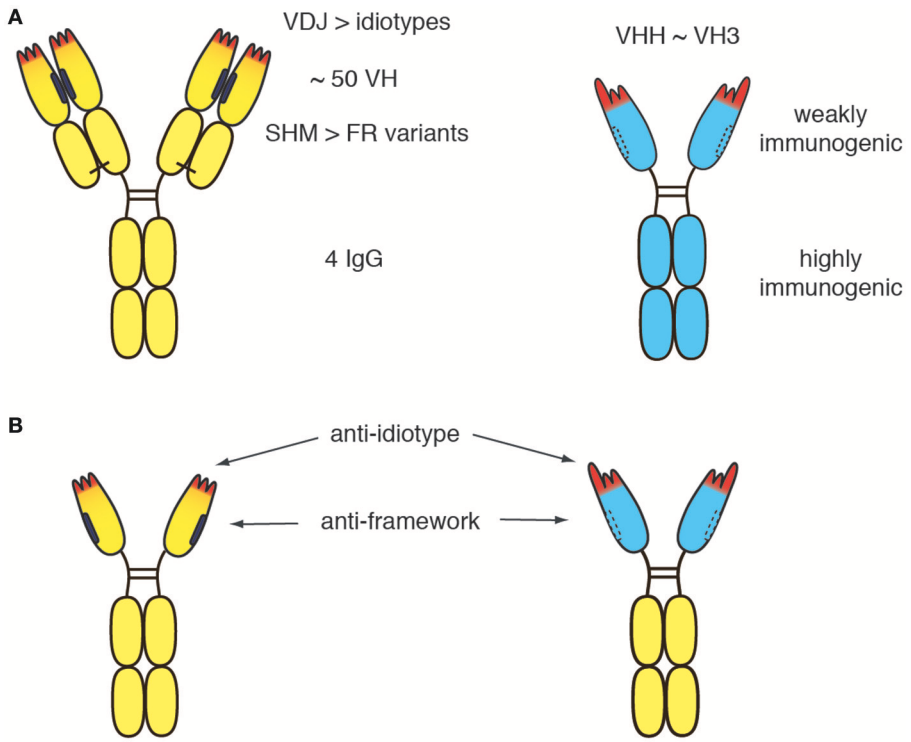

FIGURE 2 | Potential immunogenicity of heavy chain antibodies. **(A)** The human germline encodes ~50 distinct VH domains and 4 distinct IgG isotpyes. V-D-J recombination during B-cell development generates millions of distinct idiotypes (antigen binding paratopes, CDR regions 1, 2 indicated in red). Subsequent to antigen encounter, somatic hypermutation generates many more variant VH domains. During pregnancy, maternal IgGs are translocated through the placental trophoblasts to the fetus, leading to tolerization of the new born human immune system against millions of VH variants, but only 4 distinct IgG isotypes. **(B)** In germline configuration, llama VHH domains show ~80-90% amino acid sequence identitiy to human VH3 domains. A few amino acid substitutions in the VL face (mainly framework region 2, indicated by dashed lines) and a long CDR3 that can partially fold back onto this face largely account for the dramatically improved solubility of camelid VHH domains *vs.* human VH3 domains. The solubility of human VH can be improved by "camelization," i.e., by replacing hydrophobic residues at the interface of the VL domain (indicated in black) with hydrophylic residues resembling those found in VHH domains. Conversely, camelid VHH domains can be "humanized," i.e., by replacing amino acid residues in the framework with residues corresponding to germlin human VH domains. However, the idiotype of a therapeutic moAb or hcAb cannot be fully humanized without compromising binding to the target antigen. Similarly, the VL face cannot be fully humanized without compromising solubility. Therefore, small risks remain, that the patient will develop antibodies against the idiotype and/or against the (much smaller) hydrophilic VL face.

by tolerization strategies that will become available and permit tolerization of the patient to the therapeutic antibody before treatment is initiated.

CONCLUSIONS AND OUTLOOK

Later this year, Caplacizumab, a dimeric nanobody directed against the van Willebrand factor, is expected to receive FDA approval as the first nanobody in the clinic (47, 48). Nanobodies that antagonize CD38 provide proof of concept for the notion that these small biologics represent attractive alternatives to small molecule inhibitors for inhibiting the production of immunosuppressive adenosine. Nanobody-based heavy chain antibodies retain all effector functions of full sized moAbs, at half the size. This size advantage will likely facilitate targeting of tumor cells *in vivo*, even under conditions of increased interstitial pressure within tumors. Owing to their excellent solubility, it is much easier to link different nanobodies in a single therapeutic than the combined VH+VL domains of conventional moAbs. The high solubility of CD38-specific heavy chain antibodies may come at the price of a slightly higher risk for inducing anti-drug antibodies compared to conventional human CD38-specific moAbs. In addition to the complementarity determining regions, the VL face of heavy chain antibodies may provide a second, albeit much smaller, vulnerability than the idiotype. It will be interesting to see whether "humanized" nanobody heavy chain antibodies or "camelized" human heavy chain antibodies will hold the leading nose in the race to the clinic.

AUTHOR CONTRIBUTIONS

PB and FK-N conceived the project. FK-N wrote the manuscript. Both authors reviewed and approved the manuscript.

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REFERENCES

1. Horenstein AL, Chillemi A, Quarona V, Zito A, Roato I, Morandi F, et al. NAD(+)-Metabolizing ectoenzymes in remodeling tumor-host interactions: the human myeloma model. *Cells* (2015) 4:520–37. doi: 10.3390/cells4030520
2. Chini EN, Chini CCS, Espindola Netto JM, de Oliveira GC, van Schooten W. The pharmacology of CD38/NADase: an emerging target in cancer and diseases of aging. *Trends Pharmacol Sci.* (2018) 39:424–36. doi: 10.1016/j.tips.2018.02.001
3. Afifi S, Michael A, Lesokhin A. Immunotherapy: A new approach to treating multiple myeloma with daratumumab and elotuzumab. *Ann Pharmacother.* (2016) 50:555–68. doi: 10.1177/1060028016642786
4. Rajan AM, Kumar S. New investigational drugs with single-agent activity in multiple myeloma. *Blood Cancer J.* (2016) 6:e451. doi: 10.1038/bcj.2016.53
5. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood* (2017) 131:13–29. doi: 10.1182/blood-2017-06-740944
6. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. *Nature* (1993) 363:446–8. doi: 10.1038/363446a0
7. Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem.* (2013) 82:775–97. doi: 10.1146/annurev-biochem-063011-092449
8. Ingram JR, Schmidt FI, Ploegh HL. Exploiting Nanobodies' Singular Traits. *Annu Rev Immunol.* (2018) 36:695–715. doi: 10.1146/annurev-immunol-042617-053327
9. Goldman ER, Liu JL, Zabetakis D, Anderson GP. Enhancing stability of camelid and shark single domain antibodies: an overview. *Front Immunol.* (2017) 8:865. doi: 10.3389/fimmu.2017.00865
10. Konning D, Zielonka S, Grzeschik J, Empting M, Valldorf B, Krah S, et al. Camelid and shark single domain antibodies: structural features and therapeutic potential. *Curr Opin Struct Biol.* (2017) 45:10–6. doi: 10.1016/j.sbi.2016.10.019
11. Lauwereys M, Arbabi Ghahroudi M, Desmyter A, Kinne J, Holzer W, De Genst E, et al. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J.* (1998) 17:3512–20. doi: 10.1093/emboj/17.13.3512
12. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, Kinne J, et al. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci USA.* (2006) 103:4586–91. doi: 10.1073/pnas.0505379103
13. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol.* (2009) 198:157–74. doi: 10.1007/s00430-009-0116-7
14. Bannas P, Hambach J, Koch-Nolte F. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. *Front Immunol.* (2017) 8:1603. doi: 10.3389/fimmu.2017.01603
15. Ingram JR, Blomberg OS, Rashidian M, Ali L, Garforth S, Fedorov E, et al. Anti-CTLA-4 therapy requires an Fc domain for efficacy. *Proc Natl Acad Sci USA.* (2018) 115:3912–7. doi: 10.1073/pnas.1801524115
16. Martin T, Baz R, Benson DM, Lendvai N, Wolf J, Munster P, et al. A phase 1b study of isatuximab plus lenalidomide and dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 129:3294–303. doi: 10.1182/blood-2016-09-740787
17. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol.* (2011) 186:1840–8. doi: 10.4049/jimmunol.1003032
18. Deckert J, Wetzel MC, Bartle LM, Skaletskaya A, Goldmacher VS, Vallee F, et al. SAR650984, a novel humanized CD38-targeting antibody, demonstrates potent antitumor activity in models of multiple myeloma and other CD38+ hematologic malignancies. *Clin Cancer Res.* (2014) 20:4574–83. doi: 10.1158/1078-0432.CCR-14-0695
19. De Luca R, Kachel P, Kropivsek K, Snijder B, Manz MG, Neri D. A novel dual-cytokine-antibody fusion protein for the treatment of CD38-positive malignancies. *Protein Eng Des Sel.* (2018) 31:173–9. doi: 10.1093/protein/gzy015
20. Fumey W, Koenigsdorf J, Kunick V, Menzel S, Schutze K, Unger M, et al. Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of CD38(+) tumors in mouse models *in vivo*. *Sci Rep.* (2017) 7:14289. doi: 10.1038/s41598-017-14112-6
21. Li T, Qi S, Unger M, Hou YN, Deng QW, Liu J, et al. Immuno-targeting the multifunctional CD38 using nanobody. *Sci Rep.* (2016) 6:27055. doi: 10.1038/srep27055
22. Oberle A, Brandt A, Alawi M, Langebrake C, Janjetovic S, Wolschke C, et al. Long-term CD38 saturation by daratumumab interferes with diagnostic myeloma cell detection. *Haematologica* (2017) 102:e368–370. doi: 10.3324/haematol.2017.169235
23. Nolte F, Bannas P, Schütze K, Fumey F, Schriewer L. *Antigen-Binding Polypeptides directed against CD38*. WO2017081211A2 (2017).
24. Barthelemy PA, Raab H, Appleton BA, Bond CJ, Wu P, Wiesmann C, et al. Comprehensive analysis of the factors contributing to the stability and solubility of autonomous human VH domains. *J Biol Chem.* (2008) 283:3639–54. doi: 10.1074/jbc.M708536200
25. Ewert S, Cambillau C, Conrath K, Pluckthun A. Biophysical properties of camelid V(HH) domains compared to those of human V(H)3 domains. *Biochemistry* (2002) 41:3628–36. doi: 10.1021/bi011239a
26. Feige MJ, Buchner J. Principles and engineering of antibody folding and assembly. *Biochim Biophys Acta* (2014) 1844:2024–31. doi: 10.1016/j.bbapap.2014.06.004
27. Teplyakov A, Obmolova G, Malia TJ, Luo J, Muzammil S, Sweet R, et al. Structural diversity in a human antibody germline library. *MAbs* (2016) 8:1045–63. doi: 10.1080/19420862.2016.1190060
28. Davies J, Riechmann L. 'Camelising' human antibody fragments: NMR studies on VH domains. *FEBS Lett.* (1994) 339:285–90.
29. Dudgeon K, Rouet R, Kokmeijer I, Schofield P, Stolp J, Langley D, et al. General strategy for the generation of human antibody variable domains with increased aggregation resistance. *Proc Natl Acad Sci USA.* (2012) 109:10879–84. doi: 10.1073/pnas.1202866109
30. Kim DY, Hussack G, Kandalaft H, Tanha J. Mutational approaches to improve the biophysical properties of human single-domain antibodies. *Biochim Biophys Acta* (2014) 1844:1983–2001. doi: 10.1016/j.bbapap.2014.07.008
31. Nilvebrant J, Tessier PM, Sidhu SS. Engineered Autonomous Human Variable Domains. *Curr Pharm Des.* (2016) 22:6527–37. doi: 10.2174/1381612822666160921143011
32. Aldred SF, Bourdreau A, Buelow B, Clarke S, Dank K, Davison L, et al. Multispecific antibodies targeting CD38 show potent tumor-specific cytotoxicity. (Abstract). *J Clin Oncol.* (2018) 36(5Suppl.):57. doi: 10.1200/JCO.2018.36.5_suppl.57
33. Julian MC, Li L, Garde S, Wilen R, Tessier PM. Efficient affinity maturation of antibody variable domains requires co-selection of compensatory mutations to maintain thermodynamic stability. *Sci Rep.* (2017) 7:45259. doi: 10.1038/srep45259
34. Tijink BM, Laeremans T, Budde M, Stigter-van Walsum M, Dreier T, de Haard HJ, et al. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. *Mol Cancer Ther.* (2008) 7:2288–97. doi: 10.1158/1535-7163.MCT-07-2384
35. Bannas P, Well L, Lenz A, Rissiek B, Haag F, Schmid J, et al. *In vivo* near-infrared fluorescence targeting of T cells: comparison of nanobodies and conventional monoclonal antibodies. *Contrast Media Mol Imaging* (2014) 9:135–42. doi: 10.1002/cmmi.1548
36. Rashidian M, Keliher EJ, Bilate AM, Duarte JN, Wojtkiewicz GR, Jacobsen JT, et al. Noninvasive imaging of immune responses. *Proc Natl Acad Sci USA.* (2015) 112:6146–51. doi: 10.1073/pnas.1502609112
37. Danquah W, Meyer-Schwesinger C, Rissiek B, Pinto C, Serracant-Prat A, Amadi M, et al. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. *Sci Transl Med.* (2016) 8:366ra162. doi: 10.1126/scitranslmed.aaf8463
38. Ghetie V, Popov S, Borvak J, Radu C, Matesoi D, Medesan C, et al. Increasing the serum persistence of an IgG fragment by random mutagenesis. *Nat Biotechnol.* (1997) 15:637–40. doi: 10.1038/nbt0797-637
39. Scheuplein F, Rissiek B, Driver JP, Chen YG, Koch-Nolte F, Serreze DV. A recombinant heavy chain antibody approach blocks ART2 mediated deletion of an iNKT cell population that upon activation inhibits autoimmune diabetes. *J Autoimmun.* (2010) 34:145–54. doi: 10.1016/j.jaut.2009.08.012

40. Ridgway JB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng.* (1996) 9:617–21.
41. De Nardis C, Hendriks LJA, Poirier E, Arvinte T, Gros P, Bakker ABH, et al. A new approach for generating bispecific antibodies based on a common light chain format and the stable architecture of human immunoglobulin G1. *J Biol Chem.* (2017) 292:14706–17. doi: 10.1074/jbc.M117.793497
42. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol.* (2009) 157:220–33. doi: 10.1111/j.1476-5381.2009.00190.x
43. van Schie KA, Wolbink GJ, Rispens T. Cross-reactive and pre-existing antibodies to therapeutic antibodies–Effects on treatment and immunogenicity. *MAbs* (2015) 7:662–71. doi: 10.1080/19420862.2015.1048411
44. Chothia C, Lesk AM, Gherardi E, Tomlinson IM, Walter G, Marks JD, et al. Structural repertoire of the human VH segments. *J Mol Biol.* (1992) 227:799–817.
45. de Bono B, Madera M, Chothia C. VH gene segments in the mouse and human genomes. *J Mol Biol.* (2004) 342:131–43. doi: 10.1016/j.jmb.2004.06.055
46. Klarenbeek A, El Mazouari K, Desmyter A, Blanchetot C, Hultberg A, de Jonge N, et al. Camelid Ig V genes reveal significant human homology not seen in therapeutic target genes, providing for a powerful therapeutic antibody platform. *MAbs* (2015) 7:693–706. doi: 10.1080/19420862.2015.1046648
47. Kaplon H, Reichert JM. Antibodies to watch in 2018. *MAbs* (2018) 10:183–203. doi: 10.1080/19420862.2018.1415671
48. Peyvandi F, Scully M, Kremer Hovinga JA, Cataland S, Knobl P, Wu H, et al. Caplacizumab for acquired thrombotic thrombocytopenic purpura. *N Engl J Med.* (2016) 374:511–22. doi: 10.1056/NEJMoa1505533

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MINI REVIEW
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Vγ9Vδ2 T Cells as Strategic Weapons to Improve the Potency of Immune Checkpoint Blockade and Immune Interventions in Human Myeloma

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The advent of immune checkpoint (ICP) blockade has introduced an unprecedented paradigm shift in the treatment of cancer. Though very promising, there is still a substantial proportion of patients who do not respond or develop resistance to ICP blockade. *In vitro* and *in vivo* models are eagerly needed to identify mechanisms to maximize the immune potency of ICP blockade and overcome primary and acquired resistance to ICP blockade. Vγ9Vδ2 T cells isolated from the bone marrow (BM) from multiple myeloma (MM) are excellent tools to investigate the mechanisms of resistance to PD-1 blockade and to decipher the network of mutual interactions between PD-1 and the immune suppressive tumor microenvironment (TME). Vγ9Vδ2 T cells can easily be interrogated to dissect the progressive immune competence impairment generated in the TME by the long-lasting exposure to myeloma cellss. BM MM Vγ9Vδ2 T cells are PD-1$^+$ and anergic to phosphoantigen (pAg) stimulation; notably, single agent PD-1 blockade is insufficient to fully recover their anti-tumor activity *in vitro* indicating that additional players are involved in the anergy of Vγ9Vδ2 T cells. In this mini-review we will discuss the value of Vγ9Vδ2 T cells as investigational tools to improve the potency of ICP blockade and immune interventions in MM.

Keywords: Vγ9Vδ2 T cells, immune checkpoint blockade, immunotherapy, tumor vaccination, multiple myeloma

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INTRODUCTION

Multiple myeloma (MM) is a disease characterized by the malignant growth of clonal plasma cells (hereafter referred to as myeloma cells) driven by intrinsic and extrinsic mechanisms. MM is uniformly preceded by a premalignant phase, termed monoclonal gammopathy of undetermined significance (MGUS). The risk of progression from MGUS to MM varies from 1 to 5% per year (1). Interestingly, myeloma cells isolated from the BM of MGUS already harbor many of the genetic and epigenetic abnormalities of myeloma cells isolated from patients with overt disease. Interestingly, long-term follow up has shown that almost 50% of high-risk MGUS never progresses to overt MM (2). These clinical data strongly support the concept that other factors, in addition to intrinsic myeloma cell features, are important to determine the fate and aggressiveness of myeloma cells.

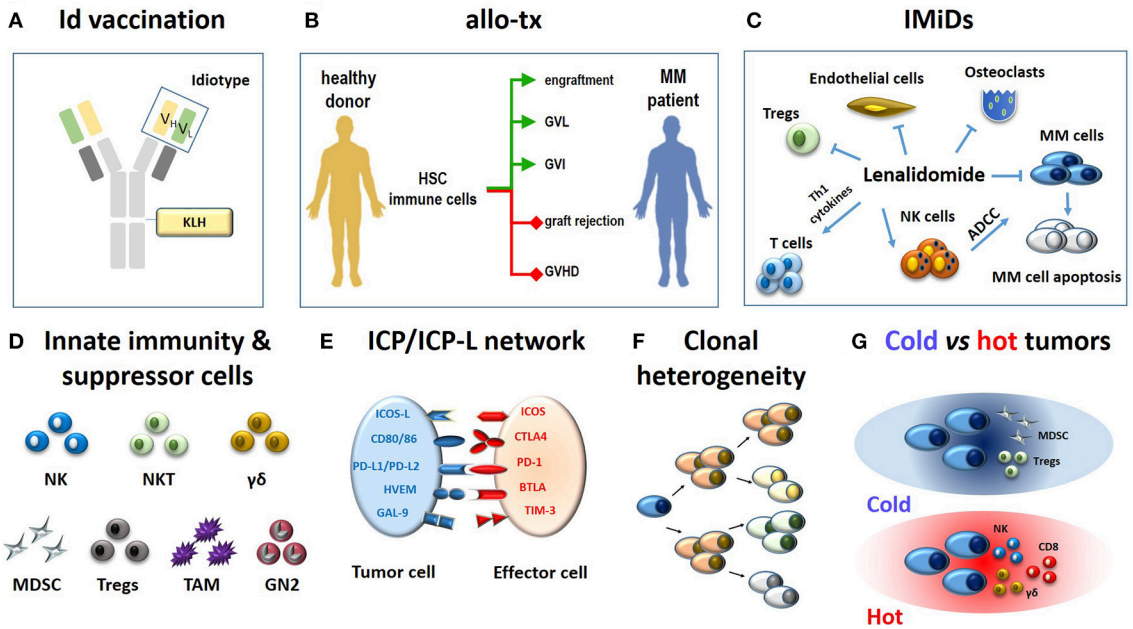

FIGURE 1 | Immune-based approaches in MM patients **(A–C)** and major hurdles to their definitive clinical success **(D-G). (A)** The monoclonal immunoglobulin produced by myeloma cells is a very specific TAA. The antigenic determinants localized in the complementary determining regions of monoclonal heavy and light chains (yellow and green rectangles) are termed idiotypes (Id) and are tumor-specific. Id specifities have been used to address tumor-specific immune responses. A vaccine formulation consisting of Id-specific proteins conjugated with KLH as immunogenic carrier has been shown to generate very specific and long-lasting anti-myeloma immune responses (6). **(B)** The ultimate goals of allogeneic transplantation (allo-tx) are to ensure a rapid blood engraftment mediated by donor HSC and concurrently address donor immune effector cells to eliminate residual malignant cells (GVL) and to control post-transplant infections (GVI; green lines). Ideally, these goals are achieved in the absence of graft rejection and/or GVHD (red lines) (72). So far, it has not been possible to clearly separate GVL from GVHD in the clinical practice. **(C)** Immunomodulatory drugs like lenalidomide fine-tune multiple immune functions in MM patients: (i) they enhance and potentiate the cytototoxic and ADCC activity of T cells and NK cells, respectively; (ii) they inhibit myeloma cell growth and induce apoptosis; (iii) they inhibit osteoclasts, ECs, and Tregs suppressor functions. **(D)** The role of innate effector cells such as NK cells, NKT cells and γδ T cells has been neglected when initial immunotherapy approaches have been developed; the need to overcome or neutralize the suppressor role of MDSCs, Tregs, TAMs, and suppressive neutrophils type II (GN2) was unkown and not addressed. **(E)** Another major hurdle is represented by the ICP/ICP-L immune suppressive circuitry. The interactions between ICP expressed by effector cells (ICOS, CTLA-4, PD-, BTLA, TIM-3) and ICP-L expressed by myeloma cells and bystander cells in the TME (ICOS-L, CD80/CD86, PDL-1/PDL-2, HVEM, GAL-9) impair anti-myeloma immunity. **(F)** MM is characterized by clonal and subclonal diversity which is shaped over time by repeated treatments, responses, and relapses. This clonal heterogeneity facilitates the immune escape of myeloma cells. **(G)** The TME immune infiltration discriminates between cold and hot tumors. The former are characterized by the local recruitment and/or activation of immune suppressor cells like Tregs and MDSC; the latter are characterized by the presence of cytotoxic cells (NK, CD8, γδ T cells). Clonal diversity, mutational load, and treatments are key factors to drive the immune infiltration of cold vs. hot tumors. Hot tumors are more sensitive to immunotherapy than cold tumors. The MM TME is closer to cold than hot tumors. Id, idiotype; KLH, keyhole limpet hemocyanin; allo-tx, allogeneic transplantation; HSC, hematopoietic stem cells; MM, multiple myeloma; graft-vs.-leukemia, GVL; graft-vs.-infections, GVI; graft-vs. host desease (GVHD); IMIDs, immunomodulatory drugs; Tregs, regulatory T cells; MDSC, myeloid derived suppressor cell; tumor-associated macrophages (TAM); GN, granulocyte neutrofils; NK, natural killer; ADCC, antibody-dependent-cellular-cytotoxicity; NKT, natural killer T cells; ICP, immune checkpoint; ICP-L, immune checkpoint ligands; TAA: tumor associated antigen.

The nature and relevance of the tumor microenvironment (TME) in MM have comprehensively been described elsewhere, including the role of immune cells (3, 4). We have anticipated these insights in the mid '80s, when we have shown a defective CD73 expression in $CD8^+$ cells which was correlated with the proliferative activity of BM PC in both MGUS and MM (5). These initial findings have been corroborated by many other preclinical studies leading to the pioneeristic development of active specific immunotherapy approaches. The unique expression of idiotype (Id) by clonal B cells encouraged the generation of a variety of Id-specific vaccines (from protein- to DNA-based vaccines) which were able to induce long-lasting and tumor-specific immune responses (6).

Clinical results in allo-transplanted MM patients have strengthened the perception that the only chance to permanently eliminate residual myeloma cells [including those surviving high dose melphalan and autologous stem cell transplantation (ASCT)] is the recognition and elimination by allogeneic immune effector cells (7). The development of immunomodulatory imide drugs (IMiDs) and the clinical results obtained with lenalidomide (including maintenance treatment after ASCT) have brought further evidences that immune cells in the TME are key targets to interrupt the myeloma cell prosurvival network (8).

These approaches have significantly impacted on the clinical outcome, but none of them has generated such an impressive cure rate to definitely change the natural history of the disease (**Figure 1**).

RECONSIDERING THE IMMUNE COMPETENCE OF MGUS AND MM PATIENTS

The unsatisfactory results of immune-based approaches in MM should not generate a pessimistic view. The reasons are rooted in the increased knowledge about the pathogenesis of the disease, the pathophysiology of immune responses, and the innovative technologies available to monitor the disease, assess clinical responses, and develop novel strategies of immune interventions. Additional progresses have been made by shedding some misconceptions like the wisdom that MGUS are immunologically blessed conditions in which myeloma cells are hold in check by very effective immune responses. This misconception was based on mouse models and preclinical results obtained in humans when much less was known about the mechanisms of immune surveillance and immune escape (9). Only recently, this misconception has been breached by us and others revealing that multiple immune dysfunctions are already present in MGUS (10–13).

Another misconception to be abandoned is that the remission state after ASCT represents a unique opportunity for immune interventions since it is possible to achieve a minimal residual disease (MRD) condition in this setting. We have shown more than 10 years ago that the T-cell receptor (TCR) repertoire is highly disrupted in patients in remission after ASCT (14). These results have been confirmed and consolidated (15) explaining why Id vaccination could not fulfil clinical expectations and why lenalidomide maintenance, even nowadays, significantly extends progression free survival (PFS), but does not definitely protect MM patients from late or very late relapse (8).

The time is ripe to apply more informative assays to investigate the immune competence of MGUS and MM. The aim of this minireview is to recapitulate how interrogating the immune competence of BM Vγ9Vδ2T cells has deepen our knowledge about the immune derangement occurring in MGUS and MM patients and how these informations can be applied to design more effective immune interventions in MM.

Vγ9Vδ2 T CELLS AS ULTRASENSITIVE TOOLS TO ASSESS THE IMMUNE SUPPRESSIVE TME COMMITMENT IN MGUS AND MM

Vγ9Vδ2 T-cells are non-conventional T cells half-way between adaptive and innate immunity with a natural inclination to react against malignant B cells, including malignant myeloma cells (16). These cells are able to sense supra-physiological concentrations of phosphorylated metabolites (pAgs) generated in the mevalonate (Mev) pathway of mammalian cells. Isopentenyl pyrophosphate (IPP) is the prototypic pAg recognized by Vγ9Vδ2 T cells. The pAgs-reacitivity of Vγ9Vδ2 T cells can be tested *in vivo* and *in vitro* by stimulating monocytes or dendritic cells (DC) with aminobisphosphonates like pamidronate or zoledronate (ZA). Both compounds inhibit farnesylpyrophosphate synthase in the Mev pathway (17, 18) and induce intracellular IPP accumulation and extracellular IPP release that are detected by Vγ9Vδ2 T cells. IPP recognition by Vγ9Vδ2 T cells is mediated by the γδ TCR in association with the isoform A1 of the butyrophilin-3 (BTN3A1) protein family (19, 20).

Vγ9Vδ2 T cells are endowed with peculiar functional properties which make them very good candidates for immunotherapy: they do not require MHC restriction and co-stimulation; they produce pro-inflammatory cytokines (IFN-γ and TNF-α); they recognize antigens shared by a variety of stressed and tumor cells; they behave as professional antigen-presenting cells (21); they can provide help to B cells to produce antibodies (22); and they can induce DC maturation boosting αβ T cell priming and MHC-restricted antigen-specific T-cell responses (23). We believe that this multifaceted array of immune functions gives a unique predisposition to Vγ9Vδ2 T cells to behave as very sentitive biosensors of the immune suppressive TME commitment occurring in the BM of MGUS and MM patients (24).

We have previously shown in a large series of patients (MGUS: $n = 10$; MM at diagnosis: $n = 70$; MM in remission: $n = 52$; MM in relapse: $n = 24$) that BM MM Vγ9Vδ2 T cells are unable to properly react to pAgs stimulation in terms of proliferation, CD107 expression and IFN-γ production. This is an early and long-lasting immune dysfunction, already detectable in MGUS individuals, largely anticipating that of CD8+ T cells and not disappearing even when most of tumor cells have been cleared by ASCT as in MM in remission. The investigation of pAgs reactivity of BM MM Vγ9Vδ2 T cells has been instrumental to show that the frequency of immune suppressor cells in the TME [bone marrow stromal cells (BMSC), regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC)] are similar in the BM of MGUS, MM at diagnosis and MM in remission.

ROLE OF IMMUNE CHECKPOINTS (ICP) AND ICP-LIGANDS (ICP-L) IN THE IMMUNE SUPPRESSIVE TME COMMITMENT OF MGUS AND MM PATIENTS

Immune checkpoints (ICP) are key regulators of immune activation, immune homeostasis, and autoimmunity driven by interactions with the corresponding ligands (ICP-L) expressed by surrounding cells (25). In cancer, the ICP/ICP-L network is often hijacked by tumor cells to suppress anti-tumor immune responses. This has led to the development of anti-ICP/ICP-L monoclonal antibodies (mAbs) to treat a variety of cancers with heterogenous results.

Among the ICP/ICP-L pairs identified so far, the PD-1/PD-L1 axis plays a major role in the generation of the immune suppressive TME in MM. PD-L1 expression in myeloma cells is higher in MM and SMM than in MGUS and predicts an increased risk of disease progression (26, 27). Paiva et al. have shown a significant upregulation of PD-L1 expression in residual myeloma cells of MM patients who are in first complete

remission (27). PD-L1 expression can protect residual myeloma cells from the immune modulation driven by lenalidomide and promote their immune escape and regrowth. Beside myeloma cells, MDSC, and BMSC also express high levels of PD-L1 cells in the BM microenvironment [24 and our unpublished data], underlining a redundancy of immune suppressor cells exploiting the ICP/ICP-L circuitry to hamper anti-myeloma immunity in the TME.

PD-L1 expression is paired by PD-1 overexpression in $CD4^+$ and $CD8^+$ T cells, and NK cells (28–30) isolated from PB and BM of MM patients creating a very effective network to protect myeloma cells from immune recognition and killing. Preliminary data from our laboratory indicate that multiple ICP can be expressed by effector cells, as already reported by Koyama's group in solid tumors (31).

These and other pre-clinical evidences (30, 32, 33) have been the groundwork to introduce anti-PD-1/PD-L1 treatment in MM patients, but clinical results have not met clinical expectations (34–36). These data have confirmed the complexity of the ICP/ICP-L and shown that single PD-1/PD-L1 blockade is insufficient to recover anti-tumor immune responses in MM patients. Investigating the defective pAg reactivity of BM MM Vγ9Vδ2 T cells represent a unique opportunity to identify potential partners and strategies to improve the efficacy of ICP/ICP-L blockade and immune interventions in MGUS and MM.

LESSONS FROM BM MM Vγ9Vδ2 T CELLS

The unsatisfactory results of anti-PD-1/PD-L1 monotherapy have stimulated the hunt for combinatorial treatment including lenalidomide (28, 37), elotuzumab (anti-SLAMF7) (38), histone deacetylase inhibitors, oncolytic reovirus (39), and radiation therapy (40). Lenalidomide and pomalidomide in combination with pembrolizumab (anti-PD-1) and dexamethasone have progressed up to phase III first-line trials, but unexpected toxicity in the pembrolizumab arm has led to the temporary discontinuation of these trials (https://www.onclive.com/web-exclusives/fda-discloses-data-on-halted-pembrolizumab-myeloma-trials). These hitches are paradigmatic examples how difficult is to carry on immunotherapy studies without a full knowledge about the TME landscape and the local conundrum of tumor-host interactions.

We have shown that a significant fraction of Vγ9Vδ2 T cells that are anergic to pAg stimulation in the TME of MGUS individuals and MM patients are PD-1+ (24). The attempts to fully recover anti-myeloma BM Vγ9Vδ2 T-cell activity *in vitro* by single PD-1 blockade has failed (24). Investigating the mechanisms of resistance to PD-1 blockade in $PD\text{-}1^+$ BM MM Vγ9Vδ2 T cells can provide useful hints to improve the potency of ICP blockade in MM and other diseases.

Multiple ICP expression by immune cells, paired by multiple ICP-L expression in tumor cells and surrounding cells in the TME is emerging as a general mechanism of cancer resistance to ICP blockade. Our preliminary results show that BM MM Vγ9Vδ2 T cells express multiple ICP engaged by the corresponding ICP-L expressed by myeloma cells and bystander cells. ICP-L overexpression in MDSC reinforces their intrinsic immune suppressive commitment, but ICP-L overexpression in endothelial cells and BMSC reflects a contranatural protumoral recruitment operated by myeloma cells in the TME. Our data showing that anergic $PD\text{-}1^+$ Vγ9Vδ2 T cells up-regulate PD-1 and express alternative ICP (TIM3, LAG3; that we have defined super-anergic state), if stimulated with pAgs in the presence of single PD-1 blockade, indicates that the TME is reprogrammed to resist any mild and/or insufficient attempt to recover antitumor immune function (**Figure 2**). This is not very different from what we have learned from chemotherapy when polychemotherapy has replaced single-agent chemotherapy (i.e., ABVD for Hodgkin's disease, R-CHOP for diffuse large B-cell lymphoma, ICE for acute myeloid leukemia etc).

Currently, the most common strategies to overcome the onset of alternative ICP are combinations of multiple anti-ICP antibodies. This approach, supported by *in vitro* and *in vivo* data, is impeded by the prohibitive costs and increased side effects and toxicity in the clinical setting. The analysis of the molecular interactions between different ICP (PD-1, TIM-3, LAG-3) in anergic Vγ9Vδ2 T cells could help to identify mechanistic interventions to prevent alternative ICP uregulation and boost the immune potency of ICP inhibitors.

POTENTIAL CONTRIBUTION OF Vγ9Vδ2 T CELLS TO NOVEL IMMUNE TREATMENTS

The spectrum of immune interventions has significantly broadened in MM over the last few years thanks to novel findings and technical advances. Immune responses mediated by non-conventional T cells like Vγ9Vδ2 T cells, NKT cells, and CD1a-restricted T cells have gained significant consideration similar to MHC-restricted immune responses mediated by $CD8^+$ cells. The characterization of suppressor cells like MDSC, Tregs, BMSC, and very importantly, the discovery of the ICP/ICP-L network have been other important steps to promote the renaissance of immunotherapy in MM. The identification of additional targets other than Id has led to an unprecedented surge of mAbs directed against myeloma cells (CD38, CD138, SLAMF7, CD138, BCMA), the TME (ICP/ICP-L), or both (CD38, SLAMF7, anti-PD-L1) (41, 42). Notably, CD38-targeted therapy with daratumumab has emerged as of the most effective passive immunotherapy ever developed in MM (43).

Current adoptive immunotherapy approaches under preclinical or clinical investigation include *ex-vivo* (CAR-T, TCR-engineered T cells) or *in vivo* redirected T cells [bispecifc T-cell engager (BiTEs)] (44, 45). Clinical trials testing BCMA-redirected CAR-T cells are producing impressive results in heavily pretreated relapsed and/or refractory MM patients (44–49).

TCR-engineered T cells are genetically modified in order to express αβ TCR with enhanced affinity for selected TAA. In contrast to CAR, *αβTCR* gene transferred cells retain HLA restriction of Ag recognition and are sensitive to intracellular

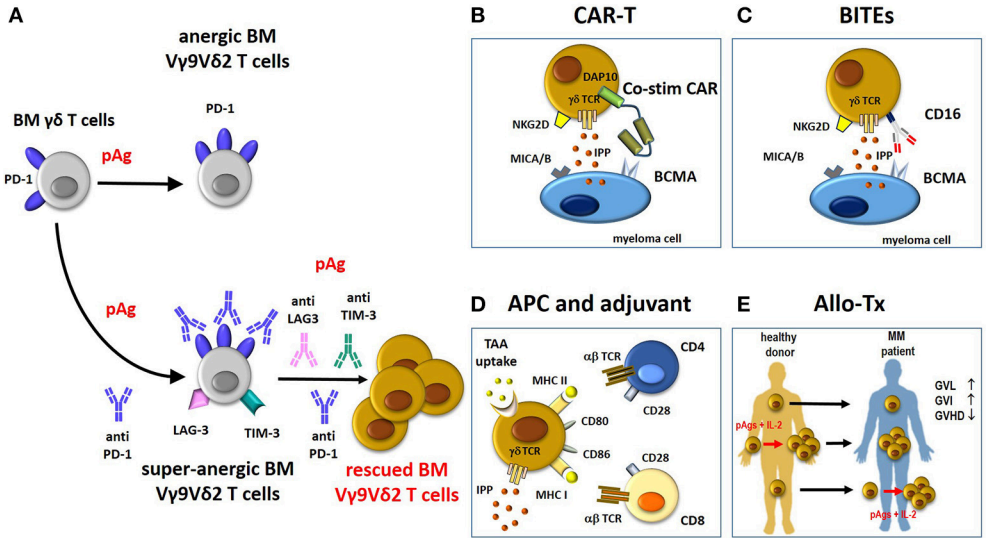

FIGURE 2 | Potential contribution of rescued Vγ9Vδ2 T cells to immune treatments in MM. **(A)** BM Vγ9Vδ2 T cells in the TME are PD-1$^+$ and anergic to pAg-stimulation. This anergy is not overcome by pAg stimulation in the presence of anti-PD-1. Paradoxically, the pAg + anti-PD-1 combination deepens the anergy of BM MM Vγ9Vδ2 T cells (super-anergic state). One possible hallmark of super-anergic immune effector cells is the expression of multiple ICP (i.e., LAG-3. TIM-3) on the same cells. Combinations of multiple anti-ICP antibodies (anti-PD-1/anti-LAG3/anti-TIM-3) is necessary to overcome the super-anergic state. If rescued from the anergic or super-anergic state, Vγ9Vδ2 T cells can become attractive candidates for immune interventions as proposed in panels B-E. Compared to conventional T cells, Vγ9Vδ2 T cells are not MHC-restricted and can be activated by pAgs (IPP), and stress-induced self ligands (i.e., MICA/B) other than CARs, tumor-specific transferred *αβ TCR* genes, and BITEs (see also text). **(B)** The "costimulatory only" CAR Vγ9Vδ2 T cells approach (58). In these cells, the cytotoxic capacity of CD3 (signal 1) is mediated through the native γδ-TCR recognition of IPP, whereas costimulation (signal 2) is provided by a CAR recognizing BCMA with an endodomain consisting of the innate NKG2D signaling molecule, DAP10. The cytotoxic ability of CAR Vγ9Vδ2 T cells is improved by the recognition of other molecules expressed by myeloma cells like MICA/B via NKG2D. **(C)** BITEs can be used to re-direct CD16-expressing Vγ9Vδ2 T cells against MM antigen (i.e., BCMA) and enhance their cytotoxic anti-tumor activity. **(D)** Vγ9Vδ2 T cells can act as APC to present TAA to MHC-restricted CD4+ and CD8$^+$ T cells. APC-Vγ9Vδ2 T cells express many APC-related cell surface receptors like MHC-I and II, and co-stimulatory proteins (CD80, CD86). **(E)** The beneficial activity of Vγ9Vδ2 T cells in the allo-tx settings can be exploited as follows: i) direct infusion of unmanipulated grafts containing small amounts of donor circulating Vγ9Vδ2 T cells; these cells can increase in number after infusion depending on several factors like infections etc; (ii) donor Vγ9Vδ2 T cells from healthy donors are expanded *ex-vivo* before reinfusion in graft recipients using pAg like zoledronic acid and IL-2 (73); (iii) donor Vγ9Vδ2 T cells are expanded *in vivo* in the recipient after allo-tx with pAgs like zoledronic acid and IL-2. BM, bone marrow; pAg, phosphoantigen; CAR-T, chimeric antigen receptor-T; co-stim-CAR, "costimulatory only" CAR; BITEs, bispecifc T-cell engager; APC, antigen-presenting cell; TAA, tumor-associated-antigen; allo-Tx, allogenic-transplantation; GVHD, graft-vs.-host disease; GVL, graft-vs.-leukemia; GVI, graft-vs.-infections; IL-2, interleukin-2.

peptides (44, 45). Cancer testis antigens are under investigation as potential TAA in MM patients (50, 52).

Despite a growing enthusiasm, immunotherapy progresses are still facing many hurdles. The majority of MM treated with anti-CD38 mAbs (daratumumab) eventually progress and the mechanisms involved in resistance to daratumumab are largely unknown. CAR T cells also are not free from handicaps like reduced expression of BCMA on myeloma cells, short persistence or loss *in vivo* of functional CAR T cells (44–49). Bispecific CAR T cells targeting simultaneously two myeloma associated antigens may compensate the decreased BCMA expression, but it may also increase on-target off-tumor toxicity. MHC down-regulation on tumor cells may compromise the therapeutic efficacy of *αβTCR* gene transferred T cells, whereas the eventual recognition of cross reactive epitopes from alternative target antigens may account for considerable on-target off-tumor toxicity. Autoimmune fatal complications have occurred with MAGE-A3 enhanced affinity *αβTCR* gene transferred T cells (51). Another drawback of *αβTCR* gene transfer to conventional CD3$^+$ αβ T cells is the formation of mixed TCR dimers with unknown specificities due to pairing of endogenous and introduced α and β TCR chains (53).

BM Vγ9Vδ2 T cells can be very attractive candidates to deliver antitumor responses in MM, provided that they are rescued from the immune dysfunction they are afflicted. These cells recognize a broader range of targets (including metabolic targets like IPP and self-induced stress ligands) and possess a more favorable safety profile than conventional T cells (16). This unique feature has been exploited to reduce the potential "off target" toxicity of CAR Vγ9Vδ2 T cells (54–57). Fisher et at (58) have designed "costimulatory only" CAR Vγ9Vδ2 T cells in which activation signals 1 and 2 are provided by separate receptors. In these dual-receptor CAR Vγ9Vδ2 T cells, the cytotoxic capacity of CD3 (signal 1) is mediated via the native γδ-TCR recognizing IPP, whereas costimulation (signal 2) is provided by a CAR-mediated recognition of TAA mediated by DAP10, the endodomain consisting of the NKG2D receptor (**Figure 2B**). Normal healthy tissues which do not express IPP do not activate Vγ9Vδ2 TCR and are spared from Vγ9Vδ2 T cell cytotoxicity. Interestingly, these "costimulation only" CAR Vγ9Vδ2 T cells express lower

levels of PD1 and TIM3 than traditional CAR Vγ9Vδ2 T cells after long term culture (58).

Vγ9Vδ2 T cells are excellent candidates for *αβTCR* gene transfer without the risk of expression of undesired mixed TCR dimers (59). Another interesting approach is to engineer αβ T cells to express tumor-specific Vγ9Vδ2 TCRs (TEGs) to redirect αβ T cells against cancer cells (60). Vγ9Vδ2 TCR-redirected αβ T cells very efficiently kill cancer cell lines *in vitro* and primary acute myeloid leukemia blasts in a humanized mouse model. Very recently, TEGs have also been generated in MM patients and shown to be able to recognize and kill myeloma cells in a 3D model (61). Vγ9Vδ2 T cells can also be redirected against myeloma cells with BITEs (**Figure 2C**). The bispecific antibody [(HER2)2xCD16] has been used to re-direct $CD16^+$ Vγ9Vδ2 T cells against $Her2^+$ tumor cells that were killed with very high efficiency (62). HLA-independent recognition of TAA by tumor-redirected CAR Vγ9Vδ2 T cells or BITEs-activated Vγ9Vδ2 T cells may prelude to the development of allogeneic "off the shelf" CAR products.

Another unique feature of Vγ9Vδ2 T cells is their capacity to act as antigen-presenting cells (APC) to boost antigen-specific immune responses mediated by $CD8^+$ cells (21, 63) (**Figure 2D**). Combination therapy of Vγ9Vδ2 T-APC-based vaccines with ICP blockade may have synergistic activity leading to enhanced anti-tumor immune responses and long-lived immuno-surveillance (64, 65). These adjuvant properties are not lost even after chimerization of Vγ9Vδ2 T cells as demonstrated by Capsomidis A. (57)

Lastly, the multifunctional properties of Vγ9Vδ2 T cells may also be beneficial in the allo-tx setting (allo-tx) (**Figure 2E**) (66). Vγ9Vδ2 T cells have been reported to cause less graft-vs.-host disease (GVHD) than αβ T cells while retaining graft-vs.-leukemia activity (GVL) (67, 68). A protective effect of Vγ9Vδ2 T cells against both leukemia cell regrowth and infections has been reported in haploidentical HSCT depleted of TCR-αβ/CD19 lymphocytes (69). Lastly, recent studies suggest an overall favorable effect of high Vγ9Vδ2 T cells immune reconstitution after HSCT; patients with elevated numbers of Vγ9Vδ2 T cells had a significantly higher overall survival rate and a decreased rate of acute GVHD compared to patients with low Vγ9Vδ2 T cell counts (70).

CONCLUSIONS

Investigation of BM MM Vγ9Vδ2 T cells has been useful to gather a faithfully picture of the immune suppressive TME in MGUS and MM. Understanding the mechanisms that are responsible for BM Vγ9Vδ2 T-cell dysfunction, with special regard to resistance to PD-1 blockade, can help to overcome ICP resistance and safely integrate ICP/ICP-L blockade in the immune treatments of MGUS and MM patients. The use of nanotechnologies may improve delivery of antagonistic antibodies to block ICP inhibitory receptors compared to free antibodies and improve T cell activation (71).

Finally, the functional rescue of BM Vγ9Vδ2 T cells is an attractive opportunity to exploit their multifaceted immune functions to carry on *ex-vivo* and *in vivo* adoptive immunotherapy interventions.

AUTHOR CONTRIBUTIONS

All authors have made a substantial contributions to text and figures and have approved the manuscript for submission

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REFERENCES

1. Van Nieuwenhuijzen N, Spaan I, Raymakers R, Peperzak V. From MGUS to multiple myeloma, a paradigm for clonal evolution of premalignant cells. *Cancer Res.* (2018) 78:2449–56. doi: 10.1158/0008-5472.CAN-17-3115
2. Brendan M Weiss. Advances in understanding monoclonal gammopathy of undetermined significance as a precursor of multiple myeloma. *Expert Rev Hematol.* (2010) 3:165–74. doi: 10.1586/ehm.10.13
3. Bianchi G, Munshi NC. Pathogenesis beyond the cancer clone(s) in multiple myeloma. *Blood* (2015) 125:3049–58. doi: 10.1182/blood-2014-11-568881
4. Ghobrial IM, Detappe A, Anderson KC, Steensma DP. The bone-marrow niche in MDS and MGUS: implications for AML and MM. *Nat Rev Clin Oncol.* (2018) 15:219–33. doi: 10.1038/nrclinonc.2017.197
5. Massaia M, Ma DD, Boccadoro M, Golzio F, Gavarotti P, Dianzani U, et al. Decreased ecto-5'nucleotidase activity of peripheral blood lymphocytes in human monoclonal gammopathies: correlation with tumor cell kinetics. *Blood* (1985) 65:530–4.
6. Massaia M, Borrione P, Battaglio S, Mariani S, Beggiato, Napoli P, et al. Idiotype Vaccination in human myeloma: generation of tumor-specific immune responses after high-dose chemotherapy. *Blood* (1999) 94:673–83.
7. Rosenblatt J, Avigan D. Role of immune therapies for myeloma. *JNCCN* (2015) 13:1440–7 doi: 10.6004/jnccn.2015.0168
8. Fostier K, Caers J, Meuleman N, Broos K, Corthals J, Thielemans K, et al. Impact of lenalidomide maintenance on the immune environment of multiple myeloma patients with low tumor burden after autologous stem cell transplantation. *Oncotarget* (2018) 9:20476–89. doi: 10.18632/oncotarget.24944
9. Calcinotto A, Ponzoni M, Ria R, Grioni M, Cattaneo E, Villa I, et al. Modifications of the mouse bone marrow microenvironment favor angiogenesis and correlate with disease progression from asymptomatic to symptomatic multiple myeloma. *Oncoimmunology* (2015) 4:e1008850. doi: 10.1080/2162402X.2015.1008850
10. Das R, Strowig T, Verma R, Koduru S, Hafemann A, Hopf S, et al. Microenvironment-dependent growth of preneoplastic and malignant plasma cells in humanized mice. *Nat Med.* (2016) 22:1351–7. doi: 10.1038/nm.4202
11. Zheng MM, Zhang Z, Bemis K, Belch AR, Pilarski LM, Shively JE, et al. The systemic cytokine environment is permanently altered in multiple myeloma. *PLoS ONE* (2013) 8:e58504. doi: 10.1371/journal.pone.0058504
12. Foglietta M, Castella B, Mariani S, Coscia M, Godio L, Ferracini R, et al. The bone marrow of myeloma patients is steadily inhabited by a normal-sized pool of functional regulatory T cells irrespective of the disease status. *Haematologica* (2014) 99:1605–10. doi: 10.3324/haematol.2014.105866
13. Feng X, Acharya C, An G, Wen, Li Zhang K, Kalbasi A, et al. Targeting CD38 suppresses induction and function of T regulatory cells to

reverse immunosuppression in multiple myeloma. *Blood* (2016) 128:2106. doi: 10.1158/1078-0432.CCR-16-3192
14. Toubai T, Hirate D, Shono Y, Ota S, Ibata M, Mashiko S, et al.. Chimerism and T-cell receptor repertoire analysis after unrelated cord blood transplantation with a reduced-intensity conditioning regimen following autologous stem cell transplantation for multiple myeloma. *Int J Lab Hematol*. (2008) 30:75–81. doi: 10.1111/j.1751-553X.2007.00903
15. Chung D J, Katherine B. Pronschinske, Justin A. Shyer, Sneh Sharma, et al. Young. T cell exhaustion in multiple myeloma relapse after autotransplant: optimal timing of immunotherapy. *Cancer Immunol Res.* (2016) 4:61–71. doi: 10.1158/2326-6066
16. Castella B, Vitale C, Coscia M, Massaia M. Vc9Vd2 T cell-based immunotherapy in hematological malignancies: from bench to bedside. *Cell. Mol. Life Sci*. (2011) 68:2419–2432. doi: 10.1007/s00018-011-0704-8
17. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasmacell activity in multiplemyeloma. *Blood* (2000) 96:384–92.
18. Thompson K, Rogers MJ. Statins prevent bisphosphonate-induced gammadelta-T-cell proliferation and activation in vitro. *J Bone Miner Res*. (2004) 19:278–88. doi: 10.1359/JBMR.0301230
19. Riganti C, Castella B, Massaia M. ABCA1, apoA-I, and BTN3A1: a legitimate ménage à trois in dendritic cells. *Front Immunol.* (2018) 9:1246. doi: 10.3389/fimmu.2018.01246
20. Castella B, Kopecka J, Sciancalepore P, Mandili G, Foglietta M, Mitro N, et al. The ATP-binding cassette transporter A1 regulates phosphoantigen release and Vγ9Vδ2 T cell activation by dendritic cells. *Nat Commun.* (2017) 8:15663. doi: 10.1038/ncomms15663
21. Brandes M, Willimann K, Bioley G, Lévy N, Eberl M, Luo M, et al. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proc Natl Acad Sci USA* (2009) 106:2307–12. doi: 10.1073/pnas.0810059106
22. Born WK, Huang Y, Reinhardt RL, Huang H, Sun D, O'Brien RL. γδ T cells and B cells. *Adv Immunol.* (2017) 134:1–45. doi: 10.1016/bs.ai.2017.01.002
23. Petrasca A, Doherty DG. Human Vδ2(+) γδ T cells differentially induce maturation, cytokine production, and alloreactive T cell stimulation by dendritic cells and B cells. *Front Immunol.* (2014) 5:650. doi: 10.3389/fimmu.2014.00650
24. Castella B, Foglietta M, Sciancalepore P, Rigoni M, Coscia M, Griggio V, et al. Anergic bone marrow V9V2 T cells as early and long-lasting markers of PD-1-targetable microenvironment-induced immune suppression in human myeloma. *Oncoimmunology* (2015) 4:e1047580. doi: 10.1080/2162402X.2015.1047580
25. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
26. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* (2007) 110:296–304. doi: 10.1182/blood-2006-10-051482
27. Paiva B, Azpilikueta A, Puig N, Ocio EM, Sharma R, Oyajobi BO, et al. PD-L1/PD-1 presence in the tumor microenvironment and activity of PD-1 blockade in multiple myeloma. *Leukemia* (2015) 29:2110–3. doi: 10.1038/leu.2015.79
28. Görgün G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res.* (2015) 21:4607–18. doi: 10.1158/1078-0432.CCR-15-0200
29. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother*. (2011) 34:409–18. doi: 10.1097/CJI.0b013e31821ca6ce
30. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
31. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* (2016) 7:10501. doi: 10.1038/ncomms10501
32. Hallett WH, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. *Biol Blood Marrow Transplant*. (2011) 17:1133–45. doi: 10.1016/j.bbmt.2011.03.011
33. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29:1441–4. doi: 10.1038/leu.2015.11
34. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib Study. *J Clin Oncol*. (2016) 34:2698–704. doi: 10.1200/JCO.2015.65.9789
35. Suen H, Brown R, Yang S, Ho PJ, Gibson J, Joshua D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* (2015) 29:1621–2. doi: 10.1038/leu.2015.104
36. Rosenblatt J, Avigan D. Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality? *Blood* (2017) 129:275–79. doi: 10.1182/blood-2016-08-731885
37. Giuliani M, Janji B, Berchem G. Activation of NK cells and disruption of PD-L1/PD-1 axis: two different ways for lenalidomide to block myeloma progression. *Oncotarget* (2017) 8:24031–44. doi: 10.18632/oncotarget.15234
38. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K et al, PD-1 blockade enhances elotuzumab efficacy in mouse tumor models. *Blood Adv.* (2017) 1:753–65. doi: 10.1182/bloodadvances.2017004382
39. Tremblay-LeMay R, Rastgoo N, Chang H. Modulating PD-L1 expression in multiple myeloma: an alternative strategy to target the PD-1/PD-L1 pathway. *J Hematol Oncol.* (2018) 11:46. doi: 10.1186/s13045-018-0589-1
40. Binder DC, Fu YX, Weichselbaum RR. Radiotherapy and immune checkpoint blockade: potential interactions and future directions. *Trends Mol Med*. (2015) 463–5. doi: 10.1016/j.molmed.2015.05.007
41. Kumar S. Emerging options in multiple myeloma: targeted, immune, and epigenetic therapies. *Hematology Am Soc Hematol Educ Program*. (2017) 2017:518–24. doi: 10.1182/asheducation-2017.1.518
42. Köhler M, Greil C, Hudecek M, Lonial S, Raje N, Wäsch R, et al. Current developments in immunotherapy in the treatment of multiple myeloma. *Cancer* (2018) 124:2075–85. doi: 10.1002/cncr.31243
43. Lokhorst HM, Plesner T, Laubach JP, Nahi H, Gimsing P, Hansson M, et al. Targeting CD38 with Daratumumab Monotherapy in Multiple Myeloma. *N Engl J Med.* (2015) 373:1207–19. doi: 10.1056/NEJMoa1506348
44. Vallet S, Pecherstorfer M, Podar K. Adoptive cell therapy in multiple Myeloma. *Expert Opin Biol Ther*. (2017) 17:1511–22. doi: 10.1080/14712598.2017.1375095
45. Danhof S, Hudecek M, Smith EL. CARs and other T cell therapies for MM: The clinical experience. *Best Pract Res Clin Haematol*. (2018) 31:147–57. doi: 10.1016/j.beha.2018.03.002
46. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, et al. T cells expressing an anti-B-cell 460 maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. *Blood* (2016) 128:1688–700. doi: 10.1182/blood-2016-04-711903
47. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT et al. B-Cell Maturation Antigen (BCMA)-Specific Chimeric Antigen Receptor T Cells (CART-BCMA) for Multiple Myeloma (MM): initial safety and efficacy from a phase I study. *Blood* (2016) 128:1147.
48. Fan F (X), Zhao W, Liu J, He A, Chen Y, Cao X,et al. Durable remissions with BCMA-specific chimeric antigen receptor (CAR)-modified T cells in patients with refractory/relapsed multiple myeloma. *J Clin Oncol*. (2017) 35:abstr LBA3001. doi: 10.1200/JCO.2017.35.18_suppl.LBA3001
49. Berdeja JG, Yi Lin, Raje NS, DiCapua Siegel DS, Munshi NC, Liedtke M, et al. First-in-human multicenter study of bb2121 anti-BCMA CAR T-cell therapy for relapsed/refractory multiple myeloma: updated results. *J Clin Oncol*. (2017) 15:3010. doi: 10.1200/JCO.2017.35.15_suppl.3010
50. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1engineered T cells mediate sustained antigen-specific chimeric antigen receptor engineered T cells for the treatment of antitumor effects in myeloma. *Nat Med*. (2015) 21:914–21 doi: 10.1038/nm.3910

51. Amos SM, Duong CP, Westwood JA, Ritchie DS, Junghans RP, Darcy PK, et al. Autoimmunity associated with immunotherapy of cancer. *Blood* (2011) 118:499–509 doi: 10.1182/blood-2011-01-325266
52. Caballero OL, Chen YT. Cancer-testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci.* (2009) 100:2014–21. doi: 10.1111/j.1349-7006.2009.01303.x
53. van der Veken LT, Hagedoorn RS, van Loenen MM, Willemze R, Falkenburg JH, Heemskerk MH. αβ T-cell receptor engineered γδ T cells mediate effective antileukemic reactivity. *Cancer Res.* (2006) 66:3331–7. doi: 10.1158/0008-5472.CAN-05-4190
54. Rischer M, Pscherer S, Duwe S, Vormoor J, Jurgens H, Rossig C. Human gammadelta T cells as mediators of chimaeric-receptor redirected anti-tumour immunity. *Br J Haematol.* (2004) 126:583–92. doi: 10.1111/j.1365-2141.2004.05077.x
55. Deniger DC, Switzer K, Mi T, Maiti S, Hurton L, Singh H, et al. Bispecific T-cells expressing polyclonal repertoire of endogenous γδ T-cell receptors and introduced CD19-specific chimeric antigen receptor. *Mol Ther.* (2013) 21:638–47. doi: 10.1038/mt.2012.267
56. Du SH, Li Z, Chen C, Tan WK, Chi Z, Kwang TW, et al. Co-expansion of cytokine-induced killer cells and Vγ9Vδ2 T cells for CAR T-cell therapy. *PLoS ONE* (2016) 11:e0161820. doi: 10.1371/journal.pone.0161820
57. Capsomidis A, Benthall G, Van Acker HH, Fisher J, Kramer AM, Abeln Z, et al. Chimeric antigen receptor-engineered human gamma delta T cells: enhanced cytotoxicity with retention of cross presentation. *Mol Ther.* (2018) 26:354–65. doi: 10.1016/j.ymthe.2017.12.001
58. Fisher J, Wisidagamage Don ND, Flutter B, Capsomidis A, Cheung GW, Gustafsson K, Anderson J. Avoidance of on-target off-tumor activation using a co-stimulation-only chimeric antigen receptor. *Mol Ther.* (2017) 25:1234–47. doi: 10.1016/j.ymthe.2017.03.002
59. van der Veken LT, Coccoris M, Swart E, Falkenburg JH, Schumacher TN, Heemskerk MH. αβ T cell receptor transfer to γδ T cells generates functional effector cells without mixed TCR dimers *in vivo*. *J Immunol.* (2009) 182:164–70. doi: 10.4049/jimmunol.182.1.164
60. Marcu-Malina V, Heijhuurs S, van Buuren M, Hartkamp L, Strand S, Sebestyen Z, et al. Redirecting αβ T cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor. *Blood* (2011) 118:50–9. doi: 10.1182/blood-2010-12-325993
61. Braham MVJ, Minnema MC, Aarts T, Sebestyen Z, Straetemans T, Vyborova A, et al. Cellular immunotherapy on primary multiple myeloma expanded in a 3D bone marrow niche model. *Oncoimmunology* (2018) 7:e1434465. doi: 10.1080/2162402X.2018.1434465
62. Oberg HH, Kellner C, Gonnermann D, Sebens S, Bauerschlag D , Gramatzki M, et al. Tribody [(her2)2xcD16] is More effective Than Trastuzumab in enhancing γδ T cell and natural Killer cell cytotoxicity against her2-expressing cancer cells. *Front Immunol.* (2018) 9:814. doi: 10.3389/fimmu.2018.00814
63. Meuter S, Eberl M, Moser B. Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells. *Proc Natl Acad Sci USA.* (2010) 107:8730–5. doi: 10.1073/pnas.1002769107
64. Khan MWA, Eberl M, Moser B. Potential use of V9V2 T cells-based vaccines in cancer immunotherapy. *Front Immunol.* (2014) 5 :512. doi: 10.3389/fimmu.2014.00512
65. van Beek JJ, Wimmers F, Hato SV, de Vries IJ, Sköld AE. Dendritic cell cross talk with innate and innate-like effector cells in antitumor immunity: implications for DC vaccination. *Crit Rev Immunol.* (2014) 34:517–36.
66. de Witte MA, Sarhan D, Davis Z, Felices M, Vallera DA, Hinderlie P, et al. Early Reconstitution of NK and γδ T Cells and Its Implication for the Design of Post-Transplant Immunotherapy. *Biol Blood Marrow Transplant.* (2018) 24:1152–62. doi: 10.1016/j.bbmt.2018.02.023
67. Minculescu L, Sengeløv H. The role of gamma delta T cells in haematopoietic stem cell transplantation. *Scand J Immunol.* (2015) 81:459–68. doi: 10.1111/sji.12289
68. Scheper W1, Gründer C, Straetemans T, Sebestyen Z1, Kuball J1. Hunting for clinical translation with innate-like immune cells and their receptors. *Leukemia* (2014) 28:1181–90. doi: 10.1038/leu.2013.37
69. Irma Airoldi, Alice Bertaina, Ignazia Prigione, Alessia Zorzoli, Daria Pagliara, Claudia Cocco, et al. gd T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-ab1/CD191 lymphocytes. *Blood* (2015) 125:2349–58 doi: 10.1182/blood-2014-09-599423
70. Lamb LS, Pillai S, Langford S, Bowersock J, Stasi AD, Saad A. Clinical-scale manufacturing of γδ T cells for protection against infection and disease recurrence following haploidentical peripheral blood stem cell transplantation and cyclophosphamide gvhd prophylaxis. *Bone Marrow Transplant.* (2018) 53:766–9. doi: 10.1038/s41409-018-0130-8
71. Hagan CT, Medik YB, Wang AZ. Nanotechnology approaches to improving cancer immunotherapy. *Adv Cancer Res.* (2018) 139:35–56. doi: 10.1016/bs.acr.2018.05.003
72. Chabannon C, Kuball J, Bondanza A, Dazzi F, Pedrazzoli P, Toubert A, et al. Hematopoietic stem cell transplantation in its 60s: a platform for cellular therapies. *Sci Transl Med.* (2018) 10:eaap9630. doi: 10.1126/scitranslmed.aap9630
73. M Smetak, B Kimmel, J Birkmann, K Schaefer-Eckart, H Einsele, M Wilhelm, Kunzmann V. Clinical-scale single-step CD4+ and CD8+ cell depletion for donor innate lymphocyte infusion (DILI). *Bone Marrow Transplant.* (2008) 41:643–50. doi: 10.1038/sj.bmt.1705942

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REVIEW
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Blood Transfusion Management for Patients Treated With Anti-CD38 Monoclonal Antibodies

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Daratumumab has proven to be highly efficacious for relapsed and refractory multiple myeloma (MM) and has recently been approved in the frontline setting for MM patients ineligible for transplantation. In the future, expanded indications are possible for daratumumab and other anti-CD38 monoclonal antibodies in development. For several years, it has been recognized that these therapies interfere with blood bank testing by binding to CD38 on red blood cells and causing panagglutination on the Indirect Antiglobulin Test. This can lead to redundant testing and significant delays in patient care. Given the anticipated increase in utilization of anti-CD38 monoclonal antibodies, as well as the transfusion needs of MM patients, it is critical to understand the nature of this interference with blood bank testing and to optimize clinical and laboratory procedures. In this review, we summarize the pathophysiology of this phenomenon, examine the clinical data reported to date, describe currently available methods to resolve this issue, and lastly provide a guide to clinical management of blood transfusions for patients receiving anti-CD38 monoclonal antibodies.

Keywords: CD38, monoclonal antibody, daratumumab, isatuximab, transfusion

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INTRODUCTION

In 2015, daratumumab became the first anti-CD38 monoclonal antibody to be approved by the United States Food and Drug Administration. Its high efficacy and favorable safety profile in recent trials led to expanding indications for relapsed or refractory multiple myeloma (MM) (1–3), and it has recently become the first monoclonal antibody approved in the front-line setting for MM, in transplant-ineligible patients along with bortezomib, melphalan, and prednisone (4).

In addition, there may be further approvals of anti-CD38 monoclonal antibodies in the future. Daratumumab is being tested in various stages of development across a wide variety of cancers as well as in a subcutaneous formulation. Other anti-CD38 monoclonal antibodies are also under investigation including isatuximab, with several ongoing phase 3 trials in MM, as well as MOR202 and TAK079 in early clinical trials for MM (5). A bispecific monoclonal antibody targeting CD38 and CD3, GBR-1342, has also begun a phase 1 trial for MM.

From early on, it was recognized that daratumumab interfered with blood compatibility testing by causing panagglutination in the Indirect Antiglobulin Test (IAT) (6). This was also been shown to be true of isatuximab and MOR-202 surrogates, and indeed is likely to be a class effect rather than specific to any one antibody (7). With increasing numbers of patients receiving anti-CD38 therapy, it is important to recognize this issue in order to prevent delays in obtaining red blood cells and to reduce laboratory costs.

Given the frequency of anemia in patients with myeloma due to marrow replacement by plasma cells, comorbidities (e.g., infections, myelodysplastic syndrome), and various myelosuppressive treatments, blood transfusions are an important part of the supportive care of patients with MM. In this review, we will summarize the pathophysiology of anti-CD38 interference with blood bank testing, review published clinical data, examine various solutions to this problem, and lastly propose a clinical decision algorithm to optimize transfusion management.

PATHOPHYSIOLOGY

CD38 is a transmembrane glycoprotein with various receptor and enzymatic functions (8). It is found in low levels on many cells of both hematopoietic and non-hematopoietic lineages, but has high expression on normal plasma cells. Furthermore, CD38 is highly expressed in nearly all myeloma cells, making it an attractive target for therapy (9). Anti-CD38 antibodies work through a variety of mechanisms, including complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, induction of apoptosis, and incompletely understood immunomodulatory functions affecting regulatory cells and cytotoxic T cells (8, 10, 11).

The indirect antiglobulin test (IAT) utilizes a secondary antibody, antihuman globulin (AHG), directed against the Fc portion of the immunoglobulin molecule to detect antibodies bound to the red blood cell (RBC) membrane. This test is used as part of the RBC antibody screen, RBC antibody identification testing, phenotyping of RBCs for RBC antigens, and in the full crossmatch. CD38 is expressed at low levels on RBCs (12–14), therefore, leading to positive results (agglutination) when plasma from patients on anti-CD38 monoclonal antibodies is used in the IAT. When antibody screening and antibody identification panels are performed as part of pretransfusion testing, anti-CD38 antibody in the patient's serum binds to CD38 on reagent RBCs to cause a weak, usually 1+ panagglutination, in all testing using AHG (gel, tube, solid phase) (see **Figure 1**) (6). Additional testing is required to identify if RBC alloantibodies are present, leading to delays in the provision of RBCs to the patient for routine transfusions. This cannot be assumed to be a false-positive as many patients have received multiple transfusions in the context of relapsed and refractory MM and may in fact have RBC alloantibodies, necessitating the identification of antigen-negative RBCs for transfusion. Importantly, anti-CD38 antibodies do not affect ABO and Rh(D) typing.

This phenomenon does not routinely occur with a patient's own RBCs, and Direct Antiglobulin Testing (DAT) and autocontrol (AC) on the RBC antibody identification panel are often both negative. In the DAT, the patient's RBCs are reacted with AHG to identify the presence of *in vivo* bound antibodies or complement, while in the AC the patient's own plasma is reacted against their own RBCs to detect both antibodies bound to the red cell membrane and self-reactive antibodies. The lack of detection of antibodies in either test has been shown to be due, at least in part, to downregulation of CD38 on RBCs after exposure to daratumumab (12). Even in the period immediately following infusion of daratumumab, there has been no evidence of any clinically significant hemolysis (15), potentially due to the low expression of CD38 on RBCs.

CLINICAL DATA ON IMPACT OF ANTI-CD38 ANTIBODIES ON BLOOD TYPING

To our knowledge, 10 published studies have reported on the results of blood bank testing of patient samples after initiation of anti-CD38 therapy (6, 7, 12, 15–21). In aggregate, these studies provide results for 91 patients (88 treated with daratumumab and 3 treated with isatuximab). All 91 (100%) demonstrated a positive IAT after receiving therapy. In patients who were tested prior to initiating therapy, 5/65 (7.7%) had an RBC alloantibody; the specific antibodies are detailed in **Table 1**. Six of 43 (14.0%) patients had a positive autocontrol IAT, and 13/67 (19.4%) had positive DAT. One study performed long-term follow-up DAT on three patients who previously had a positive result, and all became negative (17). Three studies reported on time to resolution of positive IAT after cessation of therapy; the durations were 2–6 months (range), median 5 months (range 1–9 months), and median 3.4 months (range 2.1–6.3) (7, 15, 17). Data from these individual studies are presented in **Table 1**.

SOLUTIONS TO ANTI-CD 38 ANTIBODY INTERFERENCE WITH IAT

Overcoming this interference is possible through a variety of methods, each with its own benefits and downsides. There is no universal solution that can be practically applied in all scenarios, and therefore it is necessary to understand the available options. In this section, we will discuss the methodology, applicability, optimal use, and relative cost of each, as well as the supporting clinical data. A summary is provided in **Table 2**.

Dithiothreitol and Proteolytic Enzymes

The most common method of interrupting the binding of anti-CD38 antibody with CD38 receptor is to treat reagent RBCs with dithiothreitol (DTT). The precise laboratory technique has been described in detail elsewhere (22). DTT is a reducing agent that cleaves disulfide bonds present on CD38 receptors. As a result, it can denature CD38 antigen and prevent the antibody from binding. However, this technique also denatures other clinically significant RBC antigens, most notably Kell, but also less immunogenic antigens including those in the Lutheran, Yt, JMH, LW, Cromer, Indian, Dombrock, and Knops systems (23). Therefore, to reduce the risk of a possible hemolytic transfusion reaction due to an unidentified anti-Kell antibody, all patients need to be transfused with Kell-negative blood, unless they are known to be Kell-positive. Clinically significant antibodies in patients with antibodies against the DTT-sensitive antigens such at the Cartwright blood group system (Yt) would be missed

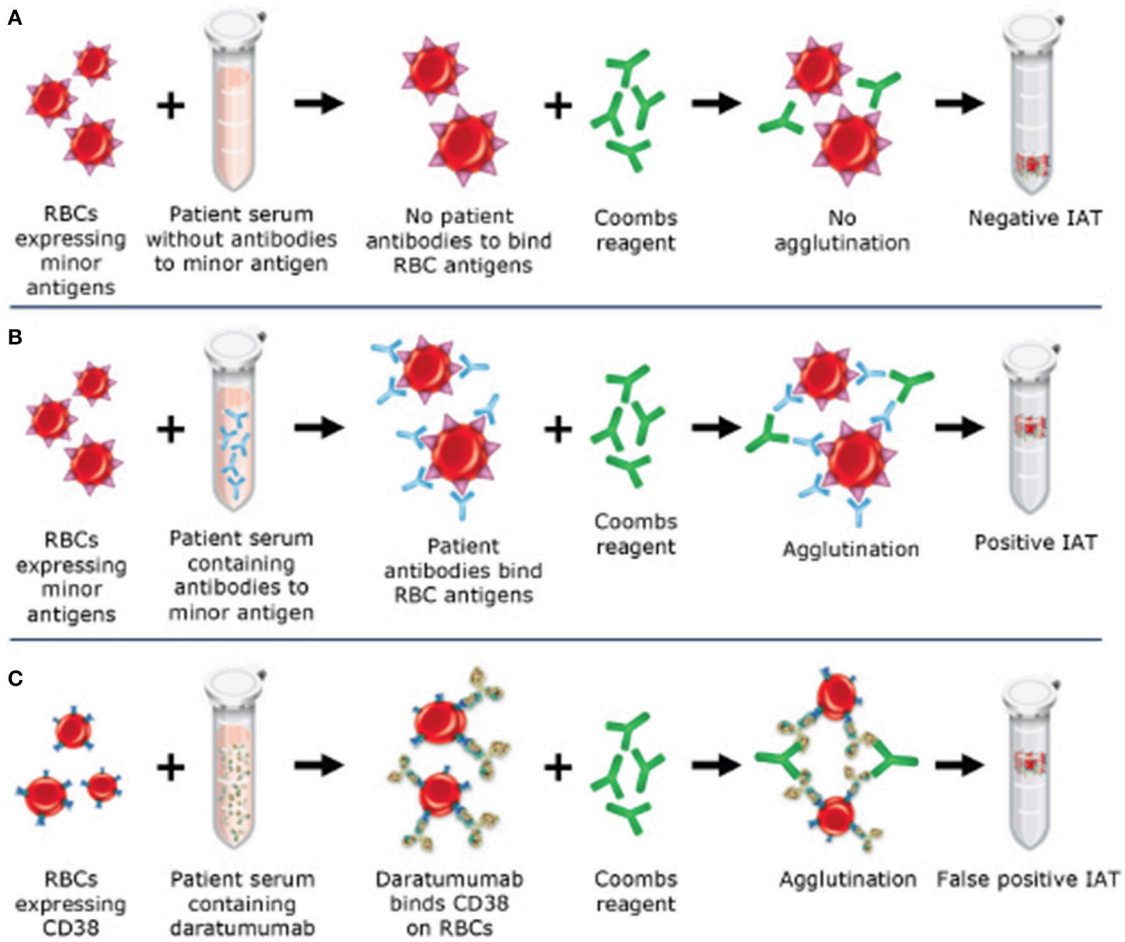

FIGURE 1 | Mechanism of daratumumab interference with the indirect antiglobulin test (IAT). **(A)** Negative IAT in a sample without RBC alloantibodies or daratumumab. **(B)** Positive IAT due to RBC alloantibodies in the sample. **(C)** Positive IAT due to interference from daratumumab, which binds to CD38 on RBCs and causes agglutination with the addition of Coombs reagent.

using this test method and put the patient at risk of a hemolytic transfusion reaction.

Although DTT treatment is a technically straightforward method to perform in the laboratory, it can require more than 2–4 h to perform manually, requires properly trained medical technologists using standardized procedures, and DTT is noxious and should be used under a hood. The cost of the reagent is minimal, but implementation is usually encumbered by the absence of resources that are not routinely available in many hospital blood banks.

Treating RBCs with DTT is very reliable and has been validated in an international multi-center study (22). In this study, 25 centers each received two blood samples, one spiked with daratumumab and another spiked with daratumumab plus a clinically significant RBC alloantibody (either anti-s, anti-D, or anti-Fy^a). 24/25 (96%) of centers observed daratumumab interference in the first sample with their routine tests, and all of them could resolve this using DTT-treated reagent RBCs. In the second part of the study, 100% of the study sites could identify the RBC antibody after removal of daratumumab interference with DTT. Most of the centers surveyed at the conclusion of the study found the method to be simple and reported they would use it in the future as their standard of care. However, it is important to note that these were all academic medical centers or blood center reference laboratories, and feasibility for smaller community laboratories may differ.

Treatment of RBCs with the proteolytic enzymes trypsin or papain has not been studied to the same extent as DTT and these methods are unlikely to replace DTT at this time. Chapuy et al. demonstrated that 2% trypsin reduced daratumumab binding to CD38-transduced HL-60 cells by 40%, compared to 92% for 10 mmol/L DTT (6). Trypsin does not degrade Kell antigens, but does destroy a number of other clinically significant antigens including M, N, En^{a}TS, and the less immunogenic Ge2, Ge3, Ge4, Ch/Rg, and Lutheran antigens (24). Additional studies are needed to determine the advantages in clinical utility and laboratory operations using trypsin compared to DTT.

TABLE 1 | Clinical data on anti-CD38 monoclonal antibody interference with blood bank testing.

Study	No. of patients	Anti-CD38 MoAb	Pre-existing alloantibodies	Positive IAT	Positive auto-control IAT	Positive DAT	Duration IAT positivity
Bub et al. (16)	5	Dara	n/a	5/5	2/5	2/5	n/a
Carreño-Tarragona et al. (17)	33	30 Dara 3 ISA	anti-D and anti-C ($n = 2$), anti-E and anti-C ($n = 1$)	33/33	n/a	5/21 for Dara 1/2 for ISA	Median 5 months (range 1–9 months)
Chapuy et al. (6)	5	Dara	n/a	5/5	3/5	3/5	n/a
Chari et al. (15)	7	Dara	anti-D and anti-E ($n = 1$), anti-E, K, Jkb, Fya, Fyb S, Knops ($n = 1$)	7/7	1/7	1/7	Median 3.4 months (range 2.1–6.3)
Deneys et al. (18)	14	Dara	None	14/14	n/a	n/a	n/a
Oostendorp et al. (7)	11	Dara	None	11/11	0/11	0/11	Range 2–6 months
Sullivan et al. (12)	13	Dara	n/a	13/13	0/13	0/13	n/a
Subramaniyan et al.(19)	1	Dara	n/a	1/1	0/1	0/1	n/a
Lin et al. (20)	1	Dara	n/a	1/1	0/1	0/1	n/a
Setia et al. (21)	1	Dara	n/a	1/1	n/a	1/1	n/a

MoAb, monoclonal antibody; IAT, indirect antiglobulin test; DAT, direct antiglobulin test; Dara, Daratumumab; ISA, isatuximab; n/a, not available.

Papain was successfully used to eliminate IAT interference from daratumumab and isatuximab in all 33 patients in one study (16). The authors were able to identify Rh group antibodies in all three patients with pre-existing alloantibodies. Hypothetically, papain could be used for quick identification of Kell antibodies as it does not denature Kell antigen, but this was not tested in the study. Papain does degrade antigens from the Duffy and MNS blood group systems, as well as several minor antigens including Ch/Rg, Ge2, and Ge4 (24), so its use would mainly be complementary to other approaches. In the study, papain was used in conjunction with phenotypically matched RBCs for safe provision of transfusions. If used in parallel with DTT, it could overcome the limitation of having to provide Kell-negative blood when using DTT alone. This would, however, increase the complexity of laboratory procedures and may not be practical in a routine setting.

Typing of RBCs

Extended phenotyping and genotyping of patient RBCs are effective, albeit expensive, methods for safely providing compatible blood. Phenotyping must be done prior to initiation of anti-CD38 antibody therapy and in the absence of both RBC transfusion in the prior 3 months and positive DAT. Extended phenotyping assesses, at a minimum, the most common immunogenic antigens, namely those in the Rh, Duffy, Kidd, Kell, and MNS blood group systems (18). Genotyping for RBC antigens can be performed at any time during therapy and can provide more comprehensive detail than phenotyping, particularly regarding minor antigens (25). Genotyping is expensive and requires at least 1 week of turnaround time in most cases.

Information collected from RBC typing is stored and then used to provide phenotypically matched blood for future transfusions. Providing antigen-matched RBCs can be a challenge for blood banks with smaller inventories and may unnecessarily use scarce resources that are better utilized for patients more likely to have broad alloimmunization (e.g., sickle cell anemia). Use of extensively matched RBCs may not be necessary if DTT treatment is used; a negative antibody screen after DTT treatment would only require RhD and Kell compatible blood.

In practice, transfusion with phenotypically matched RBCs has been very safe. Chari et al. (15) reported on transfusion outcomes in SIRIUS, a phase 2 trial of single agent daratumumab for treatment-refractory MM (26). In this study, 47 patients received a total of 147 units of packed RBCs without any transfusion reactions or evidence of hemolysis (15). In-depth analysis of two clinical sites showed that exclusively using phenotypically-matched RBCs resulted in no adverse transfusion reactions, hemolysis, or development of new alloantibodies.

Deneys et al. reported on 11 patients at their institution who had been enrolled in daratumumab clinical trials and required transfusion (18). These patients were assigned phenotypically-matched RBCs that were cross-match compatible when DTT treated donor RBCs were tested against the patients' serum. Patients received between 2 and 44 units of RBCs and there were only 2 mild transfusion reactions (1 fever, 1 erythema). Another short letter reported five patients in a daratumumab clinical trial who received between 1 and 20 units of RBCs on the basis of extended phenotyping and/or genotyping; again, no adverse events were reported (16).

It should be noted that these data, while encouraging, were obtained from clinical trial contexts in large academic centers. The feasibility of RBC-typing and provision of antigen-matched RBCs in the community setting should be assessed on a case-by-case basis.

Anti-Idiotype Antibody and Soluble CD38 Receptor

The most direct approach to prevent IAT panreactivity is to neutralize the anti-CD38 antibody in the patient's serum prior to conducting the IAT. This can be done with a reagent antibody

TABLE 2 | Approaches for overcoming anti-CD38 monoclonal antibody interference with IAT.

Method	Mechanism	Advantages	Disadvantages
DTT	Denatures CD38 antigen on reagent RBCs	Cheap Easy to apply Well-validated and reliable	Denatures Kell antigen; must give K-negative RBCs (unless Kell status known) Destroys other clinically significant minor antigens (Lutheran, YT, JMH, LW, Cromer, Indian, Dombrock, and Knops systems)
Trypsin	Cleaves CD38 antigen on reagent RBCs	Cheap Easy to apply	Denatures several significant antigens (M, N, En^aTS, Ge2, Ge3, Ge4, Ch/Rg, and Lutheran) Not validated Less reliable than DTT at removing CD38 from reagent RBCs
Papain	Cleaves CD38 antigen on reagent RBCs	Cheap Easy to apply Reliable	Destroys many significant antigens, including MNS and Duffy systems as well as Ch/Rg, Ge2, and Ge4 Due to above, can only be used as a complementary method
RBC phenotype	Antigen profiling of patient RBCs	Only needs to be performed once Provides reliable information for future use Does not require future IAT testing if matched units available	Cannot be done if already started anti-CD38 therapy, or blood transfusion within 3 months Requires extended match to ensure no antibodies or future alloantibody formation Extended-match units may be scarce and better utilized for patients with known alloantibodies
RBC genotype	Antigen profiling of patient RBCs	Only needs to be performed once Provides reliable information for future use Does not require future IAT testing if matched units available Can be performed at any time	Expensive Requires extended match to ensure no antibodies or future alloantibody formation Extended-match units may be scarce and better utilized for patients with known alloantibodies
Anti-idiotype antibody	Neutralizes anti-CD38 antibody prior to IAT	Simple and would allow for normal blood bank testing once anti-CD38 antibody removed Commercially available (for daratumumab)	Expensive Not typically available in blood bank inventory Would require different reagent for each anti-CD38 monoclonal antibody
Soluble CD38 antigen	Neutralizes anti-CD38 antibody prior to IAT	Simple and would allow for normal blood bank testing once anti-CD38 antibody removed Applicable to any anti-CD38 monoclonal antibody Commercially available	Expensive Not typically available in blood bank inventory May be less efficacious than anti-idiotype antibody Would require large amount of soluble CD38 to neutralize therapeutic monoclonal antibodies
$F(ab')_2$ fragments	Fragments preferentially bind CD38 and do not cause IAT positivity	Simple and would allow for routine blood bank testing after application	Not validated Not commercially available
Cord blood/In (Lu) RBCs	Reagent cells lack CD38 antigen	Easy to perform; no additional steps required	In (Lu) RBCs are rare Cord blood cell antigen expression differs from reagent RBCs; therefore, would need to be typed prior to use

directed at the specific anti-CD38 monoclonal antibody or a decoy soluble CD38 receptor. The efficacy of these techniques was demonstrated in two studies in which blood samples were spiked with daratumumab and then treated with anti-daratumumab antibody or soluble CD38 receptor (6, 7). Both were able to eliminate the interference with the IAT, although in one study the soluble CD38 receptor was perhaps slightly less effective (6). Importantly, these did not interfere with known alloantibodies present in the spiked serum.

Recently an anti-daratumumab antibody was approved to similarly mitigate the interference of daratumumab on serum immunofixation tests (27). However, these reagents are expensive and a specific anti-idiotype antibody would have to be designed for each new anti-CD38 monoclonal antibody. For soluble CD38 receptor, large quantities would need to be used to reliably overcome the anti-CD38 antibody concentration in the patient's serum. A recent correspondence reported the use of $F(ab')_2$ fragments, produced by digestion of daratumumab with pepsin, to preferentially bind CD38 on reagent RBCs and prevent daratumumab interference (28). The authors were able to detect known RBC alloantibodies in several samples using this technique. This method may prove useful but requires further validation and widespread commercial availability. For the foreseeable future, these are likely to be utilized only in a research setting.

CD38-Negative RBCs

RBCs lacking CD38 antigens do not bind anti-CD38 monoclonal antibodies and therefore do not demonstrate panagglutination in the IAT. It has been shown that cord blood may lack CD38 antigen, which was exploited in pre-transfusion testing to successfully screen for antibodies at one clinical site; 17 units of RBCs were transfused without adverse events (29). However, cord blood may have different antigen expression than RBCs routinely used in blood bank testing (e.g., P1, Lewis), and would need to be typed prior to use (30). Similarly, the rare In(Lu) RBCs which are Lu(a– b–) do not react with daratumumab (31). However, these are not readily available and therefore not a practical solution.

CLINICAL DECISION-MAKING

As of yet, there is no universal solution to the problem of anti-CD38 antibody interference in pre-transfusion and RBC compatibility testing. As a result, clinicians must understand the various techniques described and factor in local practices, cost, and availability when approaching this issue. This section will serve as a general guide to approaching blood transfusions in patients receiving anti-CD38 monoclonal antibodies. An algorithm for clinical management is presented in **Figure 2**.

Prior to Initiation of Therapy

Prior to the first dose of an anti-CD38 monoclonal antibody, all patients should get a type and screen to identify any alloantibodies present at baseline. They should also get either an extended phenotype or genotype, depending on the resources available. Incorporating a baseline type and screen and phenotype/genotype into the electronic or written chemotherapy order set can ensure that a type and screen sample is collected and sent to the blood bank along with notification of impending anti-CD38 therapy. Importantly, phenotyping may be inaccurate if a patient has received a blood transfusion in the prior 3 months or if they have a positive DAT; genotyping is unaffected.

This baseline testing ensures that the patient can receive appropriately matched blood even if their local blood bank does not perform DTT or trypsin testing. This is especially valuable in patients who are anticipated to require many transfusions in the

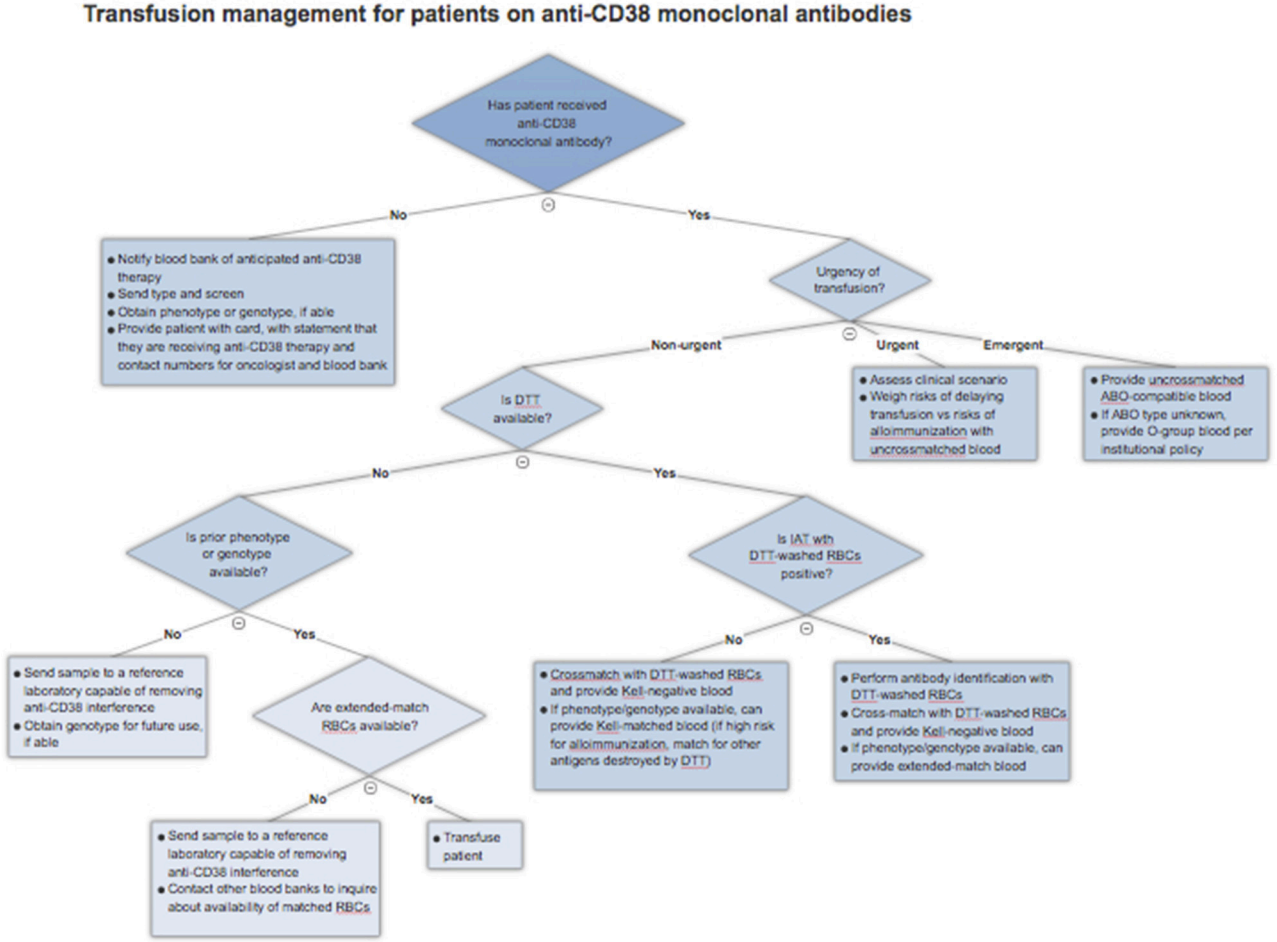

FIGURE 2 | Blood transfusion management algorithm for patients treated with anti-CD38 monoclonal antibodies.

future. Ideally, the blood bank would provide the patients with a card detailing their blood type and RBC antigens. However, at a minimum, patients should always carry a card indicating they are receiving anti-CD38 therapy that could interfere with blood typing along with contact information for the treating oncologist and blood bank. This allows other professionals to readily obtain this information in the event of travel or an emergency requiring care away from a cancer center.

Patients on Therapy Who Require a Blood Transfusion

If a patient has already received therapy and requires a blood transfusion, the blood bank should be explicitly notified that the patient is on a CD38 monoclonal antibody and requires special testing. Given near universal IAT positivity in this scenario, the blood bank can proceed with DTT treatment of RBCs, if available, as this is currently the most validated method. If there is a negative RBC antibody screen after application of DTT, the patient can be assumed to have no alloantibodies, bearing in mind those antigens, particularly Kell, denatured by the reagent. The blood bank can then crossmatch DTT-treated Kell-negative RBCs with the patient's serum and safely issue compatible blood. There remains a very slim possibility of a transfusion reaction to one of the minor antigens destroyed by DTT treatment, so clinicians should remain alert of this possibility. If the IAT is positive even after application of DTT, this suggests a true alloantibody, and antibody identification should be performed using DTT-treated RBCs.

The availability of an extended RBC phenotype or genotype can simplify this process. Matched RBCs can be issued by the blood bank depending on the extent of matching required and the availability of those units. Not all blood banks will have sufficient inventory to provide extended matches and may wish to preserve these scarce resources for patients known to have multiple alloantibodies. In this case, the blood bank can proceed with DTT-treated RBC antibody screen, with the advantage of knowing ahead of time the patient's Kell status. The blood bank can then provide crossmatched and Kell-compatible blood without the need for extensive antigen matching.

In the case that the blood bank is unable to perform testing with DTT and there is no phenotype or genotype available, a sample should be sent to a reference laboratory that has methods for removing anti-CD38 interference. This will increase the time required to procure appropriate blood, but it is a necessary step to ensure patient safety, provided that there is no urgency for transfusion.

Emergent and Urgent Transfusions

Patients who require blood transfusion in a life-threatening situation should receive uncrossmatched ABO and Rh-compatible blood. If the patient's blood type is unknown, they should be given group O red cells, as with other patients. There should not be any delay due to IAT positivity.

In the case of urgent, but not life-threatening, need for transfusions, the clinician must weigh the risks and benefits of providing uncrossmatched blood. In general, the acute risk to the patient is quite low as the likelihood of prior alloimmunization is low. In a large study of emergency-release transfusions involving 1,407 patients and 4,144 units of RBCs, 3% of patients developed alloantibodies after the acute event, while only 0.3% had received incompatible blood and 0.02% had a delayed hemolytic transfusion reaction, with no acute hemolytic reactions reported (32). These rates are likely to be higher in MM patients who have received transfusions previously. The turnaround time required to provide phenotypically-matched RBCs (if patient has a known phenotype or genotype) or performing DTT testing must be considered in the context of the urgency of the clinical situation and the risks of alloimmunization.

CONCLUSION

Anti-CD38 monoclonal antibodies are highly efficacious therapies for MM, and their use is likely to increase as they continue to gain new indications. Daratumumab has now been approved in the frontline setting for transplant-ineligible MM and is in late-stage testing for many other malignancies, while isatuximab is in phase three trials for MM. As use of these antibodies becomes routine and expands into the community setting, the challenges and costs associated with blood compatibility testing will continue to grow. It will become imperative for laboratories and medical centers to streamline processes and maintain open communication. To date, there have been no safety issues for these patients receiving transfusions, and it must remain a priority to ensure that patient safety is preserved. This will require the awareness and education of patients, clinicians, and blood bank personnel.

AUTHOR CONTRIBUTIONS

GL designed and drafted the manuscript; SA, JJ, HC, SJ, DM, SP, and JR critically revised the manuscript; AC conceived, designed, and revised the manuscript. All authors agreed on the final version to be submitted.

REFERENCES

1. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:1319–31. doi: 10.1056/NEJMoa1607751
2. Palumbo A, Chanan-Khan A, Weisel K, Nooka AK, Masszi T, Beksac M, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:754–66. doi: 10.1056/NEJMoa1606038
3. Chari A, Suvannasankha A, Fay JW, Arnulf B, Kaufman JL, Ifthikharuddin JJ, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. *Blood* (2017) 130:974–81. doi: 10.1182/blood-2017-05-785246
4. Mateos MV, Dimopoulos MA, Cavo M, Suzuki K, Jakubowiak A, Knop S, et al. Daratumumab plus bortezomib, melphalan, and prednisone for untreated myeloma. *N Engl J Med.* (2018) 378:518–28. doi: 10.1056/NEJMoa1714678

5. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood* (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
6. Chapuy CI, Nicholson RT, Aguad MD, Chapuy B, Laubach JP, Richardson PG, et al. Resolving the daratumumab interference with blood compatibility testing. *Transfusion* (2015) 55(6 Pt 2):1545–54. doi: 10.1111/trf.13069
7. Oostendorp M, Lammerts van Bueren JJ, Doshi P, Khan I, Ahmadi T, Parren PW, et al. When blood transfusion medicine becomes complicated due to interference by monoclonal antibody therapy. *Transfusion* (2015) 55(6 Pt 2):1555–62. doi: 10.1111/trf.13150
8. van de Donk NW, Janmaat ML, Mutis T, Lammerts van Bueren JJ, Ahmadi T, Sasser AK, et al. Monoclonal antibodies targeting CD38 in hematological malignancies and beyond. *Immunol Rev.* (2016) 270:95–112. doi: 10.1111/imr.12389
9. Stevenson GT. CD38 as a therapeutic target. *Mol Med.* (2006) 12:345–6. doi: 10.2119/2006-00082.Stevenson
10. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol.* (2011) 186:1840–8. doi: 10.4049/jimmunol.1003032
11. Overdijk MB, Verploegen S, Bogels M, van Egmond M, Lammerts van Bueren JJ, Mutis T, et al. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs* (2015) 7:311–21. doi: 10.1080/19420862.2015.1007813
12. Sullivan HC, Gerner-Smidt C, Nooka AK, Arthur CM, Thompson L, Mener A, et al. Daratumumab (anti-CD38) induces loss of CD38 on red blood cells. *Blood* (2017) 129:3033–7. doi: 10.1182/blood-2016-11-749432
13. Zocchi E, Franco L, Guida L, Benatti U, Bargellesi A, Malavasi F, et al. A single protein immunologically identified as CD38 displays NAD+ glycohydrolase, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities at the outer surface of human erythrocytes. *Biochem Biophys Res Commun.* (1993) 196:1459–65. doi: 10.1006/bbrc.1993.2416
14. Mehta K, Shahid U, Malavasi F. Human CD38, a cell-surface protein with multiple functions. *FASEB J.* (1996) 10:1408–17. doi: 10.1096/fasebj.10.12.8903511
15. Chari A, Arinsburg S, Jagannath S, Satta T, Treadwell I, Catamero D, et al. Blood transfusion management and transfusion-related outcomes in daratumumab-treated patients with relapsed or refractory multiple myeloma. *Clin Lymphoma Myeloma Leuk.* (2018) 18:44–51. doi: 10.1016/j.clml.2017.09.002
16. Bub CB, Reis IND, Aravechia MG, Santos LD, Bastos EP, Kutner JM, et al. Transfusion management for patients taking an anti-CD38 monoclonal antibody. *Rev Bras Hematol Hemoter.* (2018) 40:25–9. doi: 10.1016/j.bjhh.2017.09.003
17. Carreno-Tarragona G, Cedena T, Montejano L, Alonso R, Miras F, Valeri A, et al. Papain-treated panels are a simple method for the identification of alloantibodies in multiple myeloma patients treated with anti-CD38-based therapies. *Transfus Med.* (2018). doi: 10.1111/tme.1250. [Epub ahead of print].
18. Deneys V, Thiry C, Frelik A, Debry C, Martin B, Doyen C. Daratumumab: Therapeutic asset, biological trap! *Transfus Clin Biol.* (2018) 25:2–7. doi: 10.1016/j.tracli.2017.12.001
19. Subramaniyan R, Satheshkumar R, Pereira KR. Role of daratumumab in transfusion medicine: a must know entity. *Rev Bras Hematol Hemoter.* (2017) 39:375–8. doi: 10.1016/j.bjhh.2017.07.002
20. Lin MH, Liu FY, Wang HM, Cho HC, Lo SC. Interference of daratumumab with pretransfusion testing, mimicking a high-titer, low avidity like antibody. *Asian J Transfus Sci.* (2017) 11:209–11. doi: 10.4103/0973-6247.214358
21. Setia R, Dogra M, Sachdeva P, Handoo A, Choudhary D, A A. Daratumumab (Anti-CD38) interference with serological testing: an emerging challenge for blood banks in developing countries. *Global J Transfus Med.* (2017) 2:163–5. doi: 10.4103/GJTM.GJTM_42_17
22. Chapuy CI, Aguad MD, Nicholson RT, AuBuchon JP, Cohn CS, Delaney M, et al. International validation of a dithiothreitol (DTT)-based method to resolve the daratumumab interference with blood compatibility testing. *Transfusion* (2016) 56:2964–72. doi: 10.1111/trf.13789
23. Bub CB. Dithiothreitol treatment of red blood cells. *Immunohematology* (2017) 33:170–2.
24. Reid ME, Lomas-Francis C, Olsson ML. *The Blood Group Antigen Factsbook.* 3rd ed. Amsterdam: Elsevier/AP (2012).
25. Anani WQ, Marchan MG, Bensing KM, Schanen M, Piefer C, Gottschall JL, et al. Practical approaches and costs for provisioning safe transfusions during anti-CD38 therapy. *Transfusion* (2017) 57:1470–9. doi: 10.1111/trf.14021
26. Lonial S, Weiss BM, Usmani SZ, Singhal S, Chari A, Bahlis NJ, et al. Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. *Lancet* (2016) 387:1551–60. doi: 10.1016/S0140-6736(15)01120-4
27. McCudden C, Axel AE, Slaets D, Dejoie T, Clemens PL, Frans S, et al. Monitoring multiple myeloma patients treated with daratumumab: teasing out monoclonal antibody interference. *Clin Chem Lab Med* (2016) 54:1095–104. doi: 10.1515/cclm-2015-1031
28. Selleng K, Gebicka PD, Thiele T. F(ab')2 fragments to overcome daratumumab interference in transfusion tests. *N Engl J Med.* (2018) 379:90–1. doi: 10.1056/NEJMc1804751
29. Schmidt AE, Kirkley S, Patel N, Masel D, Bowen R, Blumberg N, et al. An alternative method to dithiothreitol treatment for antibody screening in patients receiving daratumumab. *Transfusion* (2015) 55:2292–3. doi: 10.1111/trf.13174
30. Anani WQ, Duffer K, Kaufman RM, Denomme GA. How do I work up pretransfusion samples containing anti-CD38? *Transfusion* (2017) 57:1337–42. doi: 10.1111/trf.14144
31. Velliquette R, Shakarian G, Jhang J, Rodriguez M, Morrison M, Adamy J, et al. Daratumumab-derived anti-CD38 can be easily mistaken for clinically significant antibodies to Lutheran antigens or to Knops antigens [abstract]. *Transfusion* (2015) 55:26A.
32. Mulay SB, Jaben EA, Johnson P, Badjie K, Stubbs JR. Risks and adverse outcomes associated with emergency-release red blood cell transfusion. *Transfusion* (2013) 53:1416–20. doi: 10.1111/j.1537-2995.2012.03922.x

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REVIEW
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Update on PD-1/PD-L1 Inhibitors in Multiple Myeloma

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The treatment of cancer, especially of various types of solid tumors, has been revolutionized by the blockade of the PD-1/PD-L1 pathway by immune checkpoint inhibitors. Their success amongst hematologic malignancies, however, has been limited so far to the treatment of classic Hodgkin's lymphoma, which portrays a typical overexpression of PD-1 ligands (PD-L1, PD-L2) as a consequence of changes in chromosome 9p24.1. Their current application in multiple myeloma (MM) is rather uncertain, as discordant results have been reported by distinct research groups concerning especially the expression of PD-1/PD-L1 molecules on malignant plasma cells or on the responsible immune effector cell populations, respectively. In MM it seems that an approach based on combination treatment might be appropriate as unsatisfactory results have been yielded by monotherapy with PD-1/PD-L1 inhibitors. Immunomodulatory drugs, which are the current cornerstone of MM treatment, are the most logical partners as they possess many possibly synergistic effects. Nevertheless, the initially optimistic results have become disappointing due to the excessive and unpredictable toxicity of the combination of pembrolizumab with lenalidomide or pomalidomide. The FDA has suspended or put on hold several phase 3 trials in relapsed as well as in newly diagnosed myeloma patients. There are also other potentially synergistic and promising combinations, such as the anti-CD38 monoclonal antibody daratumumab, irradiation, etc. Not only the effective partner but also the correct timing of the initiation of the PD-1/PD-L1 inhibitors treatment seems to be of utmost importance. These strategies are currently being examined in various stages of myeloma such as during consolidation post autologous stem cell transplantation, targeting minimal residual disease or even in high risk smoldering myeloma.

Keywords: multiple myeloma, PD-1, PD-L1, pembrolizumab, nivolumab, durvalumab, safety, toxicity

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INTRODUCTION

Multiple myeloma (MM) is a genetically heterogeneous clonal plasma cell disorder which is virtually always preceded by monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic premalignant stage (1, 2). An increased threat of progression to symptomatic disease is represented by smoldering multiple myeloma (SMM), the transitional clinical stage between MGUS and MM (3). MM represents ∼1% of all cancers with the estimated incidence of 6 cases per 100,000 persons per year (1, 4, 5) and is the second most common hematologic malignancy. Though still believed to be incurable by many authors, recent progress in its treatment

has indicated that the so-called operational cure can be achieved by at least a small proportion of these patients (6). Proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) have become the standard of care and newer generations of these agents (pomalidomide, carfilzomib, ixazomib) have been recently approved (7–9). Moreover, there has been recent implementation in routine clinical practice of molecules that possess distinct mechanisms of action like monoclonal antibodies (daratumumab [anti-CD38], elotuzumab [anti-CS1]) and histone deacetylase inhibitors [panobinostat] (10, 11). Daratumumab and isatuximab (both anti-CD38 mAbs) have especially demonstrated exceptional results in relapsed as well as in newly diagnosed myeloma (12–17).

Immunotherapy has proven to be very encouraging in the therapy of many cancers. Its objective is the identification of malignant cells and their annihilation by the process of stimulating and provoking the body's own immune system (18). In MM, immunotherapy efficacy depends on the observation that while allogeneic stem cell transplantation is limited by its toxicity, it is curative for a subset of patients with MM due to the graft-versus-myeloma effect (19). In order to develop new tools to elicit the myeloma-specific immune response, the mAbs targeting surface antigens on malignant plasma cells such as the above-mentioned daratumumab, elotuzumab, isatuximab, and others have been introduced. Cellular therapy, including dendritic cell vaccines, bi-specific mAbs (especially BCMA—T cell bi-specific antibody), chimeric antigen receptor T cells—CAR T cells [the most promising being CAR-T cells targeting BCMA (B cell maturation antigen)] is another form of immunotherapy (20–22). This review aims to describe a group of mAbs targeting immune checkpoints that represent a novel group of immunotherapeutic agents.

TARGETING IMMUNE CHECKPOINTS

Immune checkpoints, a plethora of inhibitory or stimulatory pathways, are encoded in the immune system and are essential for self-tolerance and also for the modulation of physiological immune responses. The processes of activation, maturation and expansion of T lymphocytes, and inhibition of their apoptosis are supported by stimulatory checkpoints and their ligands (e.g., CD137/CD137L, CD28/CD80, and CD86, CD27/CD70, CD40/CD40L, OX40/OXO4OL, GITR/GITRL, ICOS/ICOSL), while an opposite effect is elicited by inhibitory checkpoints with their ligands (PD-1/PD-L1 and PD-L2, CTLA-4/CD80 and CD86, A2AR/adenosine, KIR/MHC class I, LAG3/MHC class II) (18). They are crucial against the activation of autoimmunity and for the protection against damage of tissue when the immune system is activated against an infection under normal circumstances (23). Nevertheless, there is a possibility that tumor cells may become invisible to the host's immune system when they start to express ligands of checkpoint receptors on their surface and thus abuse and hijack these native pathways (24). Inhibitory immune checkpoint blockade with blocking mAbs (immune checkpoint inhibitors) has consequently emerged as a novel option for cancer treatment. Indeed, checkpoint inhibitors are now a conventional part of the treatment of numerous types of solid tumors (melanoma, non-small cell lung cancer, renal cell carcinoma, head and neck carcinoma) and Hodgkin's lymphoma (25–28).

PD-1/PD-L1 PATHWAY

There are two chief, well-described inhibitory pathways: (i) cytotoxic T-lymphocyte associated protein 4 (CTLA-4, CD152) as a checkpoint receptor and its cognate ligands B7–1 (CD80) and B7–2 (CD86), and (ii) programmed-death 1 (PD-1, CD279) receptor with its two ligands PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC). PD-1, a 288 amino acid type I transmembrane protein, is a part of the CD28 receptor family and is expressed on antigen-activated and exhausted T and B cells (29). Two ligands, PD-L1 and PD-L2, are expressed on antigen binding cells (macrophages and dendritic cells) as well as on a subset of activated B lymphocytes and microvascular endothelial cells. Furthermore, there has been a detection of a constitutive level of PD-L1 expression on the cells of various tissues (heart, lung, liver, pancreatic islet cells, astrocytes, etc.) (30, 31). As described earlier, engagement of the PD-1 receptor with its ligands PD-L1 or PD-L2 prompts the temporary down-regulation of T cell function (24, 30, 32). It was recently discovered that not only T cells, but tumor-associated macrophages and NK cells too are involved in the PD-1/PD-L1 pathway (33, 34). Tumor cell visibility for the host immune system is regained by targeting either PD-1 or its receptors (PD-L1/PD-L2) by blocking mAbs, thus leading to the annihilation of the cancer cells.

Many of these checkpoint inhibitors are examined also in MM. This review provides a comprehensive update mainly focused on the clinical efficacy and toxicity of these drugs and their combinations.

PRECLINICAL DATA AND RATIONALE FOR COMBINATIONS

Expression of PD-L1 on Myeloma Plasma Cells

There are discrepancies between many research groups concerning PD-L1 expression on plasma cells (PCs). It has been demonstrated by several studies that PD-L1 expression is limited to PCs of MM patients, and is absent on those of healthy donors (HD) (35–38). Likewise, PD-L1 expression was reported to be higher on PCs in MM and SMM than in MGUS (35, 39). On the other hand, no differences have been found in the expression of PD-L1 in MM, MGUS and HD by Paiva et al. and Kelly et al. studies (40, 41). The Dhodapkar et al. study has also shown that PD-L1 expression on malignant PCs was associated with an increased risk of progression from SMM to MM (39). Interestingly, the Paiva et al. study has revealed statistically higher PD-L1 expression on clonal PCs from MRD positive MM patients compared to PCs from HD (40).

Expression of PD-L1 and PD-1 on Immune Cell Subsets

A crucial role in regulating the response of T cells against tumors is played by dendritic cells (DCs). The BM of MM patients was found to have increased levels of plasmocytoid DCs (pDCs). Their diminished ability to trigger T cell response contributed to immune dysfunction (42). The over-expression of PD-L1 on pDCs in MM patients has been demonstrated by several authors (37, 43).

Numerous studies have demonstrated that there is an overexpression of PD-1 on CD4+ and CD8+ T cells from MM patients compared to HD (38, 44). Paiva et al. have shown a significant surge in PD-1 expression on CD4+ and CD8+ T cells only in relapsed or relapsed/refractory MM (RRMM) and MRD positive MM patients (40). PD-1 absence on normal CD56+CD3- NK cells from HD has been confirmed by all published studies. A markedly increased expression of PD-1 on NK cells from MM patients compared to HD was reported by the Benson et al. and Görgün et al. studies; whereas Paiva et al. found no difference between them (34, 38, 40).

Rationale for the Combination of PD-1/PD-L1 Inhibitors and IMiDs

Immunomodulatory drugs are back-bone agents in the treatment of newly diagnosed MM (NDMM) as well as in RRMM patients (45). IMiDs, possessing many potentially synergistic properties, could enhance the efficacy of PD-1/PD-L1 blockade. Lenalidomide has been shown to: (i) directly down-regulate PD-L1 expression on MM PCs, (ii) decrease the levels of regulatory T cells (Tregs), (iii) co-stimulate T and NK cells and (iv) down-regulate PD-1 expression on T cells (34, 38, 46–48). This combination, however, has the potential to unleash the immune response, leading to severe toxicity.

The Boston group pioneered preclinical work testing the combination of PD-1/PD-L1 blockade with lenalidomide. FACS sorted T cells and NK cells were separately co-cultured with CD138+ MM cells from RRMM patients in addition to anti-PD-1, anti-PD-L1, alone or in combination, and with lenalidomide. They have demonstrated that effector cell-mediated anti-myeloma cytotoxicity is induced by the blockade of PD-1 and PD-L1 alone, and more significantly, in combination with each other. They found that checkpoint blockade-mediated cytotoxicity is further enhanced by lenalidomide (38).

Rationale for the Combination of PD-1/PD-L1 Inhibitors and Elotuzumab

There has been recent demonstration that elotuzumab and anti-PD-1 mAb in combination lead to antitumor efficacy enhancement in the myeloma mouse model in the study published by Bezman et al. In these mouse models, combination treatment with elotuzumab and anti–PD-1 promoted tumor-infiltrating NK and $CD8^+$ T-cell activation, as well as increased intra-tumoral cytokine and chemokine release. The rationale for the clinical investigation of elotuzumab/anti–PD-1 combination therapy in patients with MM has been supported by these observations (49).

Rationale for the Combination of PD-1/PD-L1 Inhibitors and Anti-CD38 mAbs

Anti-CD38 mAbs such as daratumumab or isatuximab are highly effective breakthrough agents for the treatment of MM, with daratumumab already being approved in several indications. As was demonstrated by Chen et al. in the lung cancer mouse model, CD38 could act as a mechanism of resistance in the context of anti-PD-1/PD-L1 therapy. The combination therapy of anti-PD-L1 and anti-CD38 demonstrated a dramatic therapeutic benefit on primary lung cancer tumor growth and metastasis (50). This finding was confirmed also in the MM mouse model J558 by Bezman et al. who demonstrated that combined treatment of anti-CD38 and anti-PD-1 mAb was more effective than each of them alone (51). Thus, based on these preclinical data this combination stands to yield promising results.

Rationale for the Combination of PD-1/PD-L1 Inhibitors and Irradiation

Radiation therapy (RT), a procedure already renowned for its synergism with checkpoint inhibitors, is a promising candidate for combination treatment due to its capacity for the induction of cancer cell death as well as the mobilization of immune responses for tumor control (52, 53). Radiation appears to intensify cancer cell annihilation and the release of DNA, with a resulting augmentation of T cell priming mediated by dendritic cells. Immune-mediated tumor regression in specific locations out of the irradiated field can be a result of localized RT through the so-called "abscopal effect" (54–56). Preclinical evidence of the efficacy of PD-1/PD-L1 inhibitors plus irradiation is based on the study in the myeloma mouse model. The administration of PD-L1 blockade post lymphodepleting irradiation led to the survival of approximately 66% of mice, compared to 0% in the control group (irradiation without anti-PD-L1 mAb). Interestingly, there was evidence of complete abrogation of the therapeutic efficacy of irradiation plus anti–PD-L1 due to the depletion of either CD4+ or CD8+ T cells. Depletion of NK cells, on the other hand, did not cause any marked effect on therapeutic efficacy (57). This hypothesis has been further confirmed also in the clinical trial phase 1, when only one patient with nivolumab monotherapy reached CR which was only after therapeutic RT on the rib plasmocytoma (58).

Rationale for the Use of PD-1/PD-L1 Inhibitors After ASCT

Administration of checkpoint inhibitors after ASCT as consolidation therapy has an immunologic merit. On the basis of the study by Chung et al., it is believed that during this period the Tregs numbers drop, tumor burden reaches nadir, and CD8+ cytototoxic lymphocytes increase in number and express checkpoint inhibitory molecules such as PD-1 and others (59). There is also preclinical evidence of this approach based on the myeloma mouse model when anti-PD-L1 mAbs were administered with cell vaccination. PD-L1 blockade was used after ASCT and administration of whole cell vaccination. This exhibited an improvement in survival from 0 to 40% of myeloma bearing mice in comparison to ASCT and whole cell

vaccination alone (60). Indeed, initiation of immunotherapy at this point may be clinically relevant and several studies are already ongoing.

CLINICAL DATA

Monoclonal antibodies targeting the PD-1/PD-L1 axis can be essentially separated into two groups: (i) those against the PD-1 receptor and (ii) those against the ligands (PD-L1/PD-L2). Nivolumab (OPDIVO, MDX1106, BMS-936558, Bristol-Myers Squibb)—a fully human IgG4 mAb; pembrolizumab (KEYTRUDA, MK-3475, Merck)—a highly selective humanized IgG4 mAb, pidilizumab (MDV9300, CT-011, Medivation/Pfizer)—an IgG1 mAb, cemiplimab (REGN-2810, Sanofi), PDR001 (Novartis), and JNJ-63723283 (Janssen) are the main anti-PD-1 mAbs in use. The most promising anti-PD-L1 mAbs are durvalumab (Imfinzi, AstraZeneca), atezolizumab (Tecentriq, Roche), and BMS-936559 (Bristol-Myers Squibb). All available clinical results are summarized in **Table 1**.

Pembrolizumab

Pembrolizumab in Relapsed or Relapsed/Refractory Multiple Myeloma

Monotherapy with pembrolizumab was examined in RRMM patients in the KEYNOTE-013 phase 1b clinical trial. Pembrolizumab at the rate of 10 mg/kg every 2 weeks or at a set dose of 200 mg every 3 weeks in total was administered in 30 patients with a median of 4 previous lines of therapy. None of the subjects experienced response and 57% (17/30) had stable disease (SD). Only one grade 3 adverse event (AE) related to treatment occurred—myalgia (61). At the 2017 EHA (European Hematology Association) meeting, updated preliminary results of the phase 1 study (KEYNOTE-023) of pembrolizumab plus lenalidomide and dexamethasone were presented. In total, 51 RRMM patients with a median of 4 previous lines of therapy received 200 mg of pembrolizumab every 2 weeks, 25 mg of lenalidomide orally on days 1–21, and 40 mg of weekly oral dexamethasone in each 28-day cycle. Responses occurred in 50% (20/40) (1 sCR, 5 VGPR, 14 PR) of patients and there was evidence of progressive disease (PD) in 1 patient. The disease control rate (sCR + CR + VGPR + PR + SD) was 39/40 (98%) in the efficacy population and 28/29 (97%) in the lenalidomide-refractory population with ORR being 38% (11/29) in this subgroup of patients. The most frequent grade $\geq$3 treatment-related AEs were neutropenia (33%), thrombocytopenia (18%), and anemia (12%); AE related death occurred in 2 patients (4%) (ischemic stroke, hepatic failure). Five (10%) patients suffered from immune-related AEs (irAEs). No incidence of pneumonitis was reported (62). Another combination of pembrolizumab plus pomalidomide and dexamethasone was examined in a single-center phase 2 study. Twenty-eight-day cycles of pembrolizumab 200 mg every 2 weeks, pomalidomide 4 mg daily for 21 days, and dexamethasone 40 mg weekly were administered to 48 RRMM patients with a median of 3 previous lines of therapy. ORR was 60% (29/48), including sCR/CR (8%), VGPR (19%) and PR (33%). Progression-free survival (PFS) was 17.4 months at the median follow-up of 15.6 months, and overall survival (OS) had not yet been reached. Grade 3/4 adverse events occurred in 40% (19/48) of patients. These included pneumonia (15%), hyperglycemia (25%) and hematologic toxicities (40%). Immune-related AEs included pneumonitis (13%) and hypothyroidism (10%), mostly $\leq$ grade 2 (63). In June 2017, FDA suspended a randomized phase 3 trial (KEYNOTE-183) of pomalidomide and low-dose dexamethasone with or without pembrolizumab in patients with RRMM who had received at least two prior lines of therapy. Two hundred and forty-nine randomized patients were included in a complete evaluation of safety and efficacy of the trial. The ORR in the investigation arm was 34 vs. 40% in the control arm. The median time to progression (TTP) for the pembrolizumab arm was 8.1 vs. 8.7 months in the control arm (HR: 1.14). Median PFS for the pembrolizumab arm was 5.6 vs. 8.4 months in the control arm; HR, 1.53 (95% CI, 1.05–2.22); $P = 0.98$. Median OS was not reached vs. 15.2 months; HR, 1.61 (95% CI, 0.91–2.85); $P = 0.95$ (71). The toxicity issue has particularly been addressed in a specific paragraph of this manuscript[1]

Pembrolizumab as Consolidation After Autologous Stem Cell Transplantation

Pembrolizumab has also been tested as a part of consolidation strategy during the lymphodepleted state post autologous stem cell transplantation (ASCT) for the eradication of the residual clone of plasma cells. First, pembrolizumab monotherapy [200 mg every 3 weeks starting day +14 after ASCT (upon engraftment) for 9 doses (day+180)] was examined in a two-site, single arm phase 2 study. Twenty-nine patients who had not reached CR after induction treatment were enrolled in the study, and the CR rate in the evaluable patients was 31% (7/23). Among 29 patients, there were two grade 3 toxicities (colitis, infusion reaction), and one grade 2 toxicity (radiculopathy) which led to treatment discontinuation. Overall, another 12 irAEs not leading to discontinuation included grade 1–2 events including 4 cases of infusion reactions, 2 cases of hypothyroidism, skin rashes and colitis each and grade 3 events of acute kidney injury and hepatitis. The authors concluded that the administration of pembrolizumab after ASCT was safe. Nevertheless, the results must be interpreted carefully, as it is not possible to distinguish between the effect of HD melphalan and pembrolizumab (65). Another phase 2, single center study was performed in high risk MM patients during the 3–6 months after ASCT. Patients were administered pembrolizumab 200 mg on day 1; lenalidomide 25 mg daily on days 1–14; and dexamethasone 40 mg daily on days 1, 8 and, 15 of a 21-day cycle for a total of 2 cycles and then an additional 2 cycles of pembrolizumab + lenalidomide without corticosteroids at the same dose and frequency. ORR to upfront therapy was 100% with 1 (8.3%) achieving CR, 5 (41.6%) achieving VGPR and 6 (50%) achieving PR to induction. Four patients (33%) achieved stringent CR after the study treatment. 2 patients suffered from non-hematologic grade 3 AEs including hypoxia and maculopapular rash. This study was suspended as of 7th May 2017 after the FDA placed Merck studies using the combination of pembrolizumab and IMiDs on hold (64).

[1]https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm

TABLE 1 | Available results of clinical trials with PD-1/PD-L1 inhibitors in multiple myeloma.

Title (author)	*N*	Con.	T (*n*)	Experimental arm	ORR *n* (%)	CR *n* (%)	SD *n* (%)	Identifier Phase
PEMBROLIZUMAB								
A Trial of Pembrolizumab (MK-3475) in Participants With Blood Cancers (MK-3475-013/KEYNOTE-013) (61)	100	RRMM	4	Pembrolizumab	0/30 (0)	0/30 (0)	17/30 (57)	NCT01953692 1
A Study of Pembrolizumab (MK-3475) in Combination With Standard of Care Treatments in Participants With Multiple Myeloma (MK-3475-023/KEYNOTE-023) (62)	115	RRMM	4	Pembrolizumab/ Lenalidomide/ Dexamethasone	20/40 (50)	1/40 (3)	19/40 (48)	NCT02036502 1
1454GCC: Anti-PD-1 (MK-3475) and IMiD (Pomalidomide) Combination Immunotherapy in Relapsed/ Refractory Multiple Myeloma (63)	48	RRMM	3	Pembrolizumab/ Pomalidomide/ Dexamethasone	29/48 (60)	4/48 (8)	14/48 (30)	NCT02289222 1, 2
Pembrolizumab + Lenalidomide Post Autologous Stem Cell Transplant (ASCT) in High-risk Multiple Myeloma (MM) (64)	43	NDMM RRMM	NA	Pembrolizumab/ Lenalidomide/ Dexamethasone	12/12 (100)	1/12 (8)	NA	NCT02906332 2 suspended
Phase 2 Multi-center Study of Anti-PD-1 During Lymphopenic State After HDT/ASCT for Multiple Myeloma (65)	50	NDMM	0	Pembrolizumab/ Lenalidomide	29/29 (100)	7/23(31)	NA	NCT02331368 2
Pembrolizumab (MK-3475) in MM Patients With Residual Disease (66)	20	NDMM RRMM	0 1	Pembrolizumab	3/14 (21)	2/14 (14) sCR 1/14 (7) iCR	5/11 (42)	NCT02636010 2
Study of Lenalidomide and Dexamethasone With or Without Pembrolizumab (MK-3475) in Participants With Newly Diagnosed Treatment Naive Multiple Myeloma (MK-3475-185/KEYNOTE-185) #	640	NDMM	0	a) Lenalidomide/ Dexamethasone b) Pembrolizumab/ Lenalidomide/ Dexamethasone	93/150 (62) 97/151 (64)	NA NA	NA NA	NCT02579863 3 suspended
Study of Pomalidomide and Low Dose Dexamethasone With or Without Pembrolizumab (MK-3475) in Refractory or Relapsed and Refractory Multiple Myeloma (rrMM) (MK-3475-183/KEYNOTE-183) #	300	RRMM	NA	a) Pembrolizumab/ Pomalidomide/ Dexamethasone b) Pomalidomide/ Dexamethasone	50/124 (40) 43/125 (34)	NA NA	NA NA	NCT02576977 3 suspended
NIVOLUMAB								
An Investigational Immuno-Therapy Study to Determine the Safety and Effectiveness of Nivolumab and Daratumumab, With or Without Pomalidomide and Dexamethasone, in Patients With Multiple Myeloma (58, 67)	375	RRMM	3 5	Nivolumab Nivolumab/ Ipilimumab	0/27 (0) 0/7 (0)	0/27 (0) 0/7 (0)	17/27 (63) 1/7 (14)	NCT01592370 1 put on hold (enrolment resumed)
Check Point Inhibition After Autologous Stem Cell Transplantation in Patients at High Risk of Post Transplant Recurrence (CPIT001) (68)	42	NDMM RRMM	NA	Nivolumab/ Ipilimumab	NA 4/4 (100)	NA 4/4 (100)	NA 0/4 (0)	NCT02681302 1, 2
PIDILIZUMAB								
Lenalidomide and Pidilizumab in Treating Patients With Relapsed/Refractory Multiple Myeloma (69)	53	RRMM	2	Pidilizumab/ Lenalidomide	4/12 (33)	NA	4/12 (33)	NCT02077959 1/2
Blockade of PD-1 in Conjunction With the Dendritic Cell/Myeloma Vaccines Following Stem Cell Transplantation (70)	35	NDMM	0	Pidilizumab Pidilizumab/ Dendritic Cell Fusion Vaccine	NA 12/44 (54)	NA 6/22 (27)	NA NA	NCT01067287 2

Pembrolizumab - mAb anti-PD-1; Nivolumab - mAb anti-PD-1; Pidilizumab - mAb anti-PD-1; mAb - monoclonal antibody; N -estimated enrolment; Con. - condition; RRMM - relapsed or refractory multiple myeloma; NDMM - newly diagnosed multiple myeloma; T - prervious therapies; m - median number of previous therapies; NA, not available; ORR - overall response rate; CR - complete response; sCR - stringent CR; iCR - immunophenotypic CR; SD - stable disease, n - number of assessed patients. # data were presented on https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm

GEM-Pembresid is a Spanish phase 2 clinical trial evaluating pembrolizumab monotherapy as consolidation after ASCT in those patients that achieved at least VGPR but with persistent residual disease. A dose of 200 mg of pembrolizumab was given every 3 weeks for 12 months. Amongst the 14 patients that were evaluable, 3 (21%) upgraded their response, 2 patients in VGPR converted into sCR, and 1 CR patient achieved MRD negativity. There was ongoing reduction of the FLC and MRD

levels, respectively, in two additional patients. Treatment with pembrolizumab showed good tolerance with no related AEs. Another objective of the study was to identify biological markers of response or resistance to pembrolizumab. Flow cytometric studies revealed that early progressions were related to lower basal NK numbers and a lower PD1 expression in effector memory CD8+ T cells (66).

Pembrolizumab in Newly Diagnosed Multiple Myeloma

KEYNOTE-185 was a phase 3, randomized, clinical trial of lenalidomide and low-dose dexamethasone with or without pembrolizumab in patients with NDMM who were ineligible for ASCT. The safety and efficacy analysis included 301 randomized patients. Based on this evaluation, FDA suspended this trial in June 2017 due to excessive toxicity that is further discussed in detail in a specific paragraph of this manuscript. In the investigation arm, the ORR was 64% compared to 62% in the control arm; median TTP was not reached in both arms[2] Median PFS was not reached in either arm; HR, 1.22 (95% CI, 0.67–2.22); $P = 0.75$ as well as median OS; HR, 2.06 (95% CI, 0.93–4.55); $P = 0.97$ (72). The toxicity issue has particularly been addressed in a specific paragraph of this manuscript.

Pembrolizumab in Smoldering Multiple Myeloma

At the ASH (American Society of Hematology) 2017 annual meeting, the preliminary results of a pilot study of pembrolizumab for immunoprevention in smoldering MM were presented. The study included patients with intermediate—high risk smoldering multiple myeloma (I-HR-SMM) according to the PETHEMA, Mayo, or SWOG criteria. Pembrolizumab doses were given at 200 mg every 21 days for up to 8 cycles. Those patients that achieved ≥ minor response after 8 cycles were eligible to continue treatment for up to 24 cycles. The target ORR was 25%. Twelve patients with I-HR-SMM were enrolled. Stringent CR was achieved by one patient (8%), 10 patients had SD (83%), and one patient had PD (8%). Therapy had to be discontinued in five patients as a result of related AEs due to elevated liver function tests ($n = 2$), acute kidney injury ($n = 2$), and myalgia ($n = 1$) (73).

Ongoing clinical trials with pembrolizumab are summarized in **Table 3**.

Nivolumab

Nivolumab in Relapsed or Relapsed/Refractory Multiple Myeloma

Leshokin et al. have published the results of a phase 1 clinical trial assessing nivolumab as a single-agent in patients with relapsed or refractory T- or B-cell lymphoma or MM. Of the 27 RRMM patients evaluated (median of 3 previous lines of therapy), 63% (17/27) had reached SD as a best response with the exception of one patient who reached CR but only after irradiation of a focal plasmacytoma. Nivolumab's safety profile was similar to that observed in solid tumors. Thirty-four percentage of patients suffered from irAEs, with pneumonitis being the most frequent (11%) (58). The phase 1 study's preliminary results of nivolumab plus ipilimumab were presented at ASH 2016. There was no response in any of the 7 enrolled RRMM patients (with a median of 5 previous therapies), and 14% (1/7) had SD (67).

Nivolumab as Consolidation After Autologous Stem Cell Transplantation

At the 2017 ASH meeting, the initial safety and efficacy data for the combination of nivolumab and ipilimumab as consolidation following ASCT for high-risk hematologic malignancies were presented. 25 patients with different diagnoses including diffuse large B cell lymphoma and peripheral T cell lymphoma were enrolled in the study. 11 MM patients (7 newly diagnosed, 4 relapsed after the first ASCT within 3 years) were also enrolled in the study in total. All 4 relapsed patients (100%) were in sCR after consolidation (before ASCT only 2 had been in sCR). There have been a significant number of irAEs (80%). The nivolumab plus ipilimumab combination was discontinued after six patients (24% total: colitis 12%, pneumonitis 4%, adrenal crisis 4%, and hepatotoxicity 4%) presented with AEs of any grade related to treatment. One case of death that could be attributed to experimental treatment occurred (due to recurrent pneumonitis complicated by parainfluenza) (68).

Table 2 summarizes ongoing clinical trials with nivolumab and other anti-PD-1 mAbs.

Pidilizumab

Pidilizumab in Relapsed or Relapsed/Refractory Multiple Myeloma

The initial results of the phase 1/2 study of pidilizumab with lenalidomide in RRMM patients were presented at ASH 2015. Of the 12 patients that were evaluable (median of 2 prior lines of therapy), 33% (4/12) responded/had responses and another 33% of patients reached SD (69).

Pidilizumab as Consolidation After Autologous Stem Cell Transplantation

The combination of pidilizumab with a dendritic cell/myeloma fusion cell vaccination was administered post ASCT. Of the 22 RRMM patients that were enrolled, VGPR was reached by 27% (6/22) and CR was reached by another 27% (6/22). We must interpret these results carefully, however, as the type of treatment that led to these outcomes is not clear (70).

IMMUNE-RELATED TOXICITY

The therapeutic usage of mAbs such as PD-1 or CTLA-4 that block inhibitory checkpoint molecules may serve to enhance the specific (dominantly T cell) immune response which activates the immune system against the tumor (74). Functional disruption of immune checkpoint molecules, however, can lead to immunologic tolerance imbalances and thus an uncontrolled immune response, which may present clinically with autoimmune-like/inflammatory side-effects, leading to collateral damage of normal tissues and organ systems. Such

[2]https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm

TABLE 2 | Ongoing clinical trials with Nivolumab and other anti-PD-1 monoclonal antibodies (PDR001, JNJ-63723283, Cemiplimab) in multiple myeloma.

Title	*N*	Con.	Experimental arm	Identifier Phase
Nivolumab Role in the Treatment of Patients With Refractory or Relapse Multiple Myeloma	40	RRMM	a) Nivolumab/Pomalidomide/ Dexamethasone b) Nivolumab/Elotuzumab/ Pomalidomide/Dexamethasone	NCT03023527 1 put on hold (enrolment resumed)
An Investigational Immuno-Therapy Study to Determine the Safety and Effectiveness of Nivolumab and Daratumumab, With or Without Pomalidomide and Dexamethasone, in Patients With Multiple Myeloma	375	RRMM	a) Nivolumab b) Nivolumab/Ipilimumab[§] c) Nivolumab/Daratumumab d) Nivolumab/Daratumumab/ Pomalidomide/Dexamethasone	NCT01592370 1 put on hold (enrolment resumed)
ASCT With Nivolumab in Patients With Multiple Myeloma	30	NDMM*	Nivolumab	NCT03292263 1/2
An Exploratory Study to Evaluate the Combination of Elotuzumab and Nivolumab With and Without Pomalidomide in Relapsed Refractory Multiple Myeloma	70	RRMM	a) Nivolumab/Elotuzumab b) Nivoluma/Elotuzumab/ Pomalidomide/Dexamethasone	NCT03227432 2
A Study of Elotuzumab in Combination With Pomalidomide and Low Dose Dexamethasone and Elotuzumab in Combination With Nivolumabin Patients With Multiple Myeloma Relapsed or Refractory to Prior Treatment With Lenalidomide	95	RRMM	a) Elotuzumab/Pomalidamide/Dexamethasone b) Nivolumab/Elotuzumab	NCT02612779 2 put on hold (enrolment resumed)
Nivolumab Combined With Daratumumab With or Without Lenalidomide	60	RRMM	a) Nivolumab/Daratumumab b) Nivolumab/Daratumumab/ Lenalidomide/Dexamethasone	NCT03184194 2
An Investigational Immuno-therapy Study of Nivolumab, Elotuzumab, Pomalidomide and Dexamethasone Combinations in Patients With Multiple Myeloma (CheckMate 602)	406	RRMM	a) Nivolumab/ Pomalidomide/Dexamethasone b) Pomalidomide/ Dexamethasone c) Nivolumab/Elotuzumab/ Pomalidomide/Dexamethasone	NCT02726581 3 put on hold
Study of Single Agent CJM112, and PDR001 in Combination With LCL161 or CJM112 in Patients With Multiple Myeloma	70	RRMM	a) CJM112 b) PDR001/ CJM112 c) PDR001/LCL161	NCT03111992 1
A Study of JNJ-63723283, an Anti-programmed Death-1 Monoclonal Antibody, Administered in Combination With Daratumumab, Compared With Daratumumab Alone in Participants With Relapsed or Refractory Multiple Myeloma	386	RRMM	a) Daratumumab b) JNJ-63723283/Daratumumab	NCT03357952 1
Isatuximab in Combination With Cemiplimab in Relapsed/Refractory Multiple Myeloma (RRMM) Patients	105	RRMM	Cemiplimab/Isatuximab	NCT03194867 1/2

*N - estimated enrolment; Con. - condition; RRMM - relapsed or refractory multiple myeloma; NDMM - newly diagnosed multiple myeloma; mAb - monoclonal antibody; PDR001; mAb anti-PD1 - JNJ-63723283 - mAb anti-PD1; Cemiplimab - mAb anti-PD1; Ipilimumab - mAb anti-CTLA-4; Lirilumab - mAb anti-KIR; Elotuzumab - mAb anti-SLAMF7; CJM112 - mAb anti-IL-17A; LCL161 - mitochondrial-derived activator of caspases mimetic and inhibitor of apoptosis antagonists *NDMM who achieved partial remission, stable disease or progression disease after autologous stem cell transplantation, [§]Ipilimumab or Lirilumabb.*

adverse events are termed 'immune-related adverse events' (irAEs) and are thought to be principally T-cell mediated (75, 76). The safety data comes dominantly from the studies performed in solid oncology. IrAEs generally occur quite early. They mostly present within weeks to 3 months after the initiation of treatment with immune checkpoint blockers. The most commonly reported AE with anti-PD-1/PD-L1 is fatigue. Across monotherapy studies the incidence of fatigue is 16–37% for anti-PD-1 and 12–24% for anti-PD-L1 (76). The most frequent and typical organ specific irAEs are: (i) dermatologic toxicities, (ii) diarrhea/colitis, (iii) endocrine toxicities, (iv) hepatic toxicities, (v) pneumonitis, and (vi) rare toxicities such as neurologic syndromes, renal toxicity, myocarditis, and others. Standard treatment algorithms for irAEs utilizing immune-modulating medications that include high-dose corticosteroids, antihistamines, anti-tumor necrosis factor α (TNFα) mAbs, and calcineurin inhibitors have been developed (76–78).

Pembrolizumab and nivolumab monotherapy in RRMM patients exhibited safety profiles which were consistent with those observed in other cancers (58, 61). However, concerns have recently been raised regarding the excessive toxicity of the combination of pembrolizumab with IMiDs used specifically in myeloma trials. A move based on safety concerns identified in KEYNOTE-183 and KEYNOTE-185 was made by the FDA. Pembrolizumab in combination with dexamethasone and pomalidomide or lenalidomide for the treatment of RRMM or NDMM patients, respectively, was evaluated by two phase three clinical trials. The discontinuation of both trials was directed by the agency on July 3rd 2017, as according to interim results, an added risk of death was linked to pembrolizumab. In KEYNOTE-183 ($N = 249$, pembrolizumab, pomalidomide, dexamethasone) there were 29 deaths in the pembrolizumab arm vs. 21 deaths in the control arm at the median follow-up of 8.1 months. In the pembrolizumab group the hazard ratio (HR) for overall survival (OS) compared with the control

TABLE 3 | Ongoing clinical trials with Pembrolizumab in multiple myeloma including smoldering multiple myeloma.

Title	*N*	Con.	Experimental arm	Identifier Phase
Pembrolizumab for Smoldering Multiple Myeloma (SMM)	16	SMM	Pembrolizumab	NCT02603887 1
NY-ESO-1^{c259}T Alone and in Combination With Pembrolizumab for Multiple Myeloma	20	RRMM	a) NY-ESO-1^{c259}T cells/Pembrolizumab b) NY-ESO-1^{c259}T cells	NCT03168438 1
A Study of Pembrolizumab (MK-3475) in Combination With Standard of Care Treatments in Participants With Multiple Myeloma (MK-3475-023/KEYNOTE-023)	84	RRMM	a) Pembrolizumab/Lenalidomide/Dexamethasone b) Pembrolizumab/Carfilzomib/Dexamethasone	NCT02036502 1
Pembrolizumab and Radiation Therapy in Patients With Relapsed or Refractory Multiple Myeloma	24	RRMM	Pembrolizumab/RT	NCT03267888 1
ACP-196 (Acalabrutinib) in Combination With Pembrolizumab, for Treatment of Hematologic Malignancies (KEYNOTE145)	159	RRMM	Pembrolizumab/Acalabrutinib	NCT02362035 1, 2
Efficacy and Safety Study of Pembrolizumab (MK-3475) in Combination With Daratumumab in Participants With Relapsed Refractory Multiple Myeloma (MK-3475-668/KEYNOTE-668)	57	RRMM	Pembrolizumab/Daratumumab	NCT03221634 2
Pembrolizumab, Ixazomib Citrate, and Dexamethasone in Treating Participants With Relapsed Multiple Myeloma	42	RRMM	Pembrolizumab/Ixazomib/Dexamethasone	NCT03506360 2

N - estimated enrolment; Con. - condition; RRMM - relapsed or refractory multiple myeloma; SMM - smoldering myeloma; Pembrolizumab - mAb anti-PD-1; Acalabrutinib - Bruton's tyrosine kinase inhibitor; NY-ESO-1^{c259}T cells - autologous genetically modified T Cells.

arm was 1.61 (95% CI, 0.91–2.85), meaning an increase of >50% in the relative risk of death. Severe grade 3–5 toxicity was increased by 18% (83 vs. 65%, investigational vs. control arm). The incidence of serious AEs was 63% compared to 46% in the control arm. In the pembrolizumab arm the following non-disease progression causes of death were identifiable: myocarditis, Stevens-Johnson syndrome, myocardial infarction, pericardial hemorrhage, cardiac failure, respiratory tract infection, neutropenic sepsis, sepsis, multiple organ dysfunction, respiratory failure, and unknown (71, 79).

The KEYNOTE-185 (pembrolizumab, lenalidomide, dexamethasone) safety and efficacy analysis included 301 patients. Nineteen deaths were reported at the median follow-up of 6.6 months (HR for OS, 2.06; 95% CI, 0.93–4.55) in the pembrolizumab group compared with 9 in the control arm. The relative risk of death in the pembrolizumab arm was more than double the risk in the control group, the safety analysis saw a 22% increase of severe, grade 3–5 toxicity (72 vs. 50%, investigational vs. control arm) and there was an incidence of 54% of serious AEs compared to 39% in the control arm. The following causes of death, not related to disease progression, were identifiable in the pembrolizumab arm: intestinal ischemia, cardio-respiratory arrest, suicide, pulmonary embolism, cardiac arrest, pneumonia, sudden death, myocarditis, large intestine perforation, and cardiac failure (72, 79)[3] The discrepancy between positive phase two trials with no safety signals and suspended phase 3 trials is provoking. It may be partially explained by the imbalance between the investigational and control arm at least in the Keynote-185 study. The investigators probed baseline features of patients who had died: age over 80 (42 vs. 33% in the control arm), ISS III disease (31.6 vs. 22.2%), renal impairment (10.5 vs. 0%), hypercalcemia at presentation (21 vs. 11%), and high-risk cytogenetics (26.3 vs. 0%) were more prevalent in the pembrolizumab arm as was stated by Dr. Usmani[4].

In September 2017 the FDA placed on partial holds three clinical trials that assessed nivolumab-based combinations (the phase 3 CheckMate-602, phase 1 CheckMate-039, and phase 2 CA204142 trials) in patients with RRMM. At the same time, the agency also put a full hold on MEDI4736-MM-002, a phase 1b study which had the aim of establishing an appropriate dose and regimen for the durvalumab and lenalidomide combination with and without low-dose dexamethasone in NDMM patients, as well as on MM-005, a phase 2 study evaluating the combination of durvalumab and daratumumab in RRMM patients.[5] Analogically two phase 1/2 trials with atezolizumab and lenalidomide or pomalidomide in RRMM patients were put on partial clinical hold at this time-point. Nevertheless, several studies, mainly with nivolumab and atezolizumab, were resumed in December 2017 after a successful safety review observed no increased toxicity. All trials that were suspended or put on hold by FDA are summarized in **Table 5**.

Recently, an alarming case report describing lethal fulminant myocarditis after a single pembrolizumab dose in a newly diagnosed myeloma patient enrolled in the Keynote-185 trial has been published. The authors also discuss the role of pre-existing occult autoimmunity that may have played a part in such a severe and rapid course of myocarditis leading to death within a few days (80).

The combination of pembrolizumab with IMiDs seems to be toxic indeed and immune-related AEs are severe and unpredictable.

[3]https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm

[4]https://am.asco.org/analyses-data-halted-keynote-trials-presented-poster-sessions

[5]https://www.onclive.com/web-exclusives/fda-places-holds-on-several-durvalumab-combination-trials

TABLE 4 | Ongoing clinical trials with PD-L1 inhibitors in multiple myeloma (Atezolizumab, Durvalumab, BMS-936559).

Title	*N*	Con.	Experimental arm	Identifier Phase
ATEZOLIZUMAB				
Study of Atezolizumab (Anti-Programmed Death-Ligand 1 [PD-L1] Antibody) Alone or in Combination With an Immunomodulatory Drug and/or Daratumumab in Participants With Multiple Myeloma (MM)	288	RRMM	a) Atezolizumab b) c*) Atezolizumab/Lenalidomide d) Atezolizumab/Daratumumab e) Atezolizumab/Daratumumab/Lenalidomide f) Atezolizumab/Daratumumab/Pomalidomide	NCT02431208 1 put on hold (enrolment resumed)
Pilot Study Of Anti-Programmed Death Ligand-1 (Anti-PD-L1, Atezolizumab) In Asymptomatic Myeloma	20	SMM	Atezolizumab	NCT02784483 1 suspended
A Study of Cobimetinib Administered as Single Agent and in Combination With Venetoclax, With or Without Atezolizumab, in Participants With Relapsed and Refractory Multiple Myeloma	72	RRMM	a) Atezolizumab/Cobimetinib b) Cobimetinib/Venetoclax c) Atezolizumab/Cobimetinib/Venetoclax	NCT03312530 1, 2
DURVALUMAB				
A Study to Determine Dose and Regimen of Durvalumab as Monotherapy or in Combination With Pomalidomide With or Without Dexamethasone in Subjects With Relapsed and Refractory Multiple Myeloma	138	RRMM	a) Durvalumab b) Durvalumab/Pomalidomide c) Durvalumab/Pomalidomide/Dexamethasone	NCT02616640 1 put on hold
A Study of Durvalumab in Combination With Lenalidomide With and Without Dexamethasone in Subjects With Newly Diagnosed Multiple Myeloma	138	NDMM	a) Durvalumab/Lenalidomide b) Durvalumab/Lenalidomide/ Dexamethasone	NCT02685826 1 suspended
A Study of PVX-410, a Cancer Vaccine, and Durvalumab +/- Lenalidomide for Smoldering MM	26	SMM	a) Durvalumab b) Durvalumab/PVX-410 c) Durvalumab/PVX-410/Lenalidomide	NCT02886065 1
Phase 1 Study to Assess Safety & Tolerability of Tremelimumab & Durvalumab, Administered With High Dose Chemotherapy and Autologous Stem Cell Transplant	24	RRMM§	Durvalumab/Tremelimumab	NCT02716805 1suspended
A Study to Determine the Safety and Efficacy for the Combination of Durvalumab and Daratumumab in Relapsed and Refractory Multiple Myeloma (FUSIONMM-003)	144	RRMM	a) Durvalumab/Daratumumab b) Durvalumab/Daratumumab/ Pomalidomid/Dexamethasone	NCT02807454 2 put on hold
A Study to Determine the Efficacy of the Combination of Daratumumab (DARA) Plus Durvalumab (DURVA) (D2) in Subjects With Relapsed and Refractory Multiple Myeloma (RRMM) (FUSION-MM-005)	180	RRMM	Durvalumab/Daratumumab	NCT03000452 2 suspended
BMS-936559				
Safety Study of Anti-Programmed Death-Ligand 1 in Hematologic Malignancy	110	RRMM	BMS-936559	NCT01452334 1 withdrawn

*RRMM - relapsed or refractory multiple myeloma; NDMM - newly diagnosed multiple myeloma; SMM - smoldering multiple myeloma; N - estimated enrolment; Con.- condition; mAb - monoclonal antibody; autoHSCT - autologous stem cell transplantation; Atezolizumab - mAb anti-PD-L1; Durvalumab - mAb anti-PD-L1; BMS-936559 - mAb anti-PD-L1; Tremelimumab - mAb anti-CTLA-4; PVX-410, tetra-peptide vaccine against XBP1, CD138, and CS1, *Atezolizumab/Lenalidomide is administrated to patients who have measurable disease after autoHSCT, §Tremelimumab or Tremelimumab/Durvalumab is administrated prior to and for 2 cycles post autoHSCT followed by up to 6 additional monthly cycles of durvalumab alone.*

CONCLUSION AND FUTURE PERSPECTIVES

The PD-1/PD-L1 axis blockade in MM represents a "hot topic," as there are plenty of ongoing clinical trials summarized in **Tables 1**–**4**. Single agent PD-1 blockade is not effective in MM and does not induce any responses in contrast to many solid tumors and Hodgkin's lymphoma (58, 61). It may be partially explained by senescent rather than exhausted phenotype of T cells in MM, thus the PD-1 blockade is not able to re-invigorate their function (81). A combination-based approach is needed and IMiDs as backbone agents in MM possess many potentially synergistic properties (52). Promising results of the pembrolizumab plus either lenalidomide or pomalidomide and dexamethasone combination in heavily pretreated RRMM patients have been recently reported, reaching ORR in about 50–60% (62, 63). However, safety concerns have been raised regarding this combination and FDA suspended two phase 3 trials with pembrolizumab (Keynote-183, Keynote-185) in June 2017. Based on this analysis many other trials including any PD-1/PD-L1 inhibitors in combination with IMiDs have been put on hold, but several of them, especially with nivolumab and atezolizumab, have been restarted after the safety review. Immune-related toxicity is severe and unpredictable. Indeed, from the clinical point of view, this unfavorable toxic profile makes the position of PD-1/PD-L1 inhibitors in frontline treatment or even in smoldering myeloma questionable. Another interesting strategy is to administer anti-PD-1/PD-L1 mAbs after ASCT as a part of consolidation as it also has an immunological merit. Further investigation and randomized

TABLE 5 | Suspended and put on hold clinical trials with PD-1/PD-L1 inhibitors in multiple myeloma.

Title	*N*	Con.	Experimental arm	Identifier Phase
Pembrolizumab + Lenalidomide Post Autologous Stem Cell Transplant (ASCT) in High-risk Multiple Myeloma (MM)	43	NDMM RRMM	Pembrolizumab/Lenalidomide/Dexamethasone	NCT02906332 2 suspended
Study of Lenalidomide and Dexamethasone With or Without Pembrolizumab (MK-3475) in Participants With Newly Diagnosed Treatment Naive Multiple Myeloma (MK-3475-185/KEYNOTE-185)	640	NDMM	a) Lenalidomide/Dexamethasone b) Pembrolizumab/Lenalidomide/Dexamethasone	NCT02579863 3 suspended
Study of Pomalidomide and Low Dose Dexamethasone With or Without Pembrolizumab (MK-3475) in Refractory or Relapsed and Refractory Multiple Myeloma (rrMM) (MK-3475-183/KEYNOTE-183)	300	RRMM	a) Pembrolizumab/Pomalidomide/Dexamethasone b) Pomalidomide/Dexamethasone	NCT02576977 3 suspended
An Investigational Immuno-Therapy Study to Determine the Safety and Effectiveness of Nivolumab and Daratumumab, With or Without Pomalidomide and Dexamethasone, in Patients With Multiple Myeloma	375	RRMM	a) Nivolumab b) Nivolumab/Ipilimumab[§] c) Nivolumab/Daratumumab d) Nivolumab/Daratumumab/ Pomalidomide/Dexamethasone	NCT01592370 1 put on hold (enrolment resumed)
A Study of Elotuzumab in Combination With Pomalidomide and Low Dose Dexamethasone and Elotuzumab in Combination With Nivolumabin Patients With Multiple Myeloma Relapsed or Refractory to Prior Treatment With Lenalidomide	95	RRMM	a) Elotuzumab/Pomalidamide/Dexamethasone b) Nivolumab/Elotuzumab	NCT02612779 2 put on hold (enrolment resumed)
An Investigational Immuno-therapy Study of Nivolumab, Elotuzumab, Pomalidomide and Dexamethasone Combinations in Patients With Multiple Myeloma (CheckMate 602)	406	RRMM	a) Nivolumab/Pomalidomide/Dexamethasone b) Pomalidomide/Dexamethasone c) Nivolumab/Elotuzumab/ Pomalidomide/Dexamethasone	NCT02726581 3 put on hold
Study of Atezolizumab (Anti-Programmed Death-Ligand 1 [PD-L1] Antibody) Alone or in Combination With an Immunomodulatory Drug and/or Daratumumab in Participants With Multiple Myeloma (MM)	288	RRMM	a) Atezolizumab b) c*) Atezolizumab/Lenalidomide d) Atezolizumab/Daratumumab e) Atezolizumab/Daratumumab/Lenalidomide f) Atezolizumab/Daratumumab/Pomalidomide	NCT02431208 1 put on hold (enrolment resumed)
Pilot Study Of Anti-Programmed Death Ligand-1 (Anti-PD-L1, Atezolizumab) In Asymptomatic Myeloma	20	SMM	Atezolizumab	NCT02784483 1 suspended
A Study to Determine Dose and Regimen of Durvalumab as Monotherapy or in Combination With Pomalidomide With or Without Dexamethasone in Subjects With Relapsed and Refractory Multiple Myeloma	138	RRMM	a) Durvalumab b) Durvalumab/Pomalidomide c) Durvalumab/Pomalidomide/Dexamethasone	NCT02616640 1 put on hold
A Study of Durvalumab in Combination With Lenalidomide With and Without Dexamethasone in Subjects With Newly Diagnosed Multiple Myeloma	138	NDMM	a) Durvalumab/Lenalidomide b) Durvalumab/Lenalidomide/Dexamethasone	NCT02685826 1 suspended
A Study to Determine the Safety and Efficacy for the Combination of Durvalumab and Daratumumab in Relapsed and Refractory Multiple Myeloma (FUSIONMM-003)	144	RRMM	a) Durvalumab/Daratumumab b) Durvalumab/Daratumumab/ Pomalidomid/Dexamethasone	NCT02807454 2 put on hold
A Study to Determine the Efficacy of the Combination of Daratumumab (DARA) Plus Durvalumab (DURVA) (D2) in Subjects With Relapsed and Refractory Multiple Myeloma (RRMM) (FUSION-MM-005)	180	RRMM	Durvalumab/Daratumumab	NCT03000452 2 suspended

N - estimated enrolment; Con. - condition; RRMM - relapsed or refractory multiple myeloma; NDMM - newly diagnosed multiple myeloma; SMM - smoldering myeloma; mAb - monoclonal antibody; Pembrolizumab - mAb anti-PD-1; Pidilizumab - mAb anti-PD-1; Nivolumab - mAb anti-PD-1; Atezolizumab - mAb anti-PD-L1; Durvalumab - mAb anti-PD-L1.

trials are needed to prove the effectiveness of this approach. There are many efforts to combine checkpoint inhibitors with other agents or procedures. The most promising seem to be: (i) mAbs targeting surface antigens such as daratumumab or elotuzumab (49), (ii) irradiation because of its abscopal effect and many others that are still under investigation (56, 82). Not only the right partner for the combination but also the right timing of the initiation of treatment seems to be of utmost importance. Finally, the checkpoint inhibitors possess very distinct toxicity profiles from the routinely used agents in MM and thus physicians should be aware of these immune-related adverse events and of the management of these sometimes very complicated situations as well. Either way, blockade of PD-1/PD-L1 pathway may still be a hope for a specific subset of myeloma patients because of its capacity to induce durable responses where other treatment strategies have failed.

AUTHOR CONTRIBUTIONS

TJ wrote the manuscript, concept, work coordination. BP and RH overall proofread.

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REFERENCES

1. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
2. Rajkumar SV, Dimopoulos MA, Palumbo A, Blade J, Merlini G, Mateos M-V, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* (2014) 15:e538–48. doi: 10.1016/S1470-2045(14)70442-5
3. Kyle RA, Remstein ED, Therneau TM, Dispenzieri A, Kurtin PJ, Hodnefield JM, et al. Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. *N Engl J Med.* (2007) 356:2582–90. doi: 10.1056/NEJMoa070389
4. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin.* (2014) 64:9–29. doi: 10.3322/caac.21208
5. Maluskova D, Svobodová I, Kucerova M, Brozova L, Muzik J, Jarkovský J, et al. Epidemiology of multiple myeloma in the Czech Republic. *Klin Onkol Cas Ceske Slov Onkol Spolecnosti* (2017) 30:35–42. doi: 10.14735/amko20172S35
6. Lahuerta J-J, Paiva B, Vidriales M-B, Cordón L, Cedena M-T, Puig N, et al. Depth of response in multiple myeloma: a pooled analysis of three PETHEMA/GEM clinical trials. *J Clin Oncol.* (2017) 35:2900–10. doi: 10.1200/JCO.2016.69.2517
7. Stewart AK, Rajkumar SV, Dimopoulos MA, Masszi T, Špička I, Oriol A, et al. Carfilzomib, lenalidomide, and dexamethasone for relapsed multiple myeloma. *N Engl J Med.* (2015) 372:142–52. doi: 10.1056/NEJMoa1411321
8. Moreau P, Masszi T, Grzasko N, Bahlis NJ, Hansson M, Pour L, et al. Oral ixazomib, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 374:1621–34. doi: 10.1056/NEJMoa1516282
9. San Miguel J, Weisel K, Moreau P, Lacy M, Song K, Delforge M, et al. Pomalidomide plus low-dose dexamethasone versus high-dose dexamethasone alone for patients with relapsed and refractory multiple myeloma (MM-003): a randomised, open-label, phase 3 trial. *Lancet Oncol.* (2013) 14:1055–66. doi: 10.1016/S1470-2045(13)70380-2
10. Lonial S, Dimopoulos M, Palumbo A, White D, Grosicki S, Spicka I, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Med.* (2015) 373:621–31. doi: 10.1056/NEJMoa1505654
11. San-Miguel JF, Hungria VTM, Yoon S-S, Beksac M, Dimopoulos MA, Elghandour A, et al. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol.* (2014) 15:1195–206. doi: 10.1016/S1470-2045(14)70440-1
12. Lokhorst HM, Plesner T, Laubach JP, Nahi H, Gimsing P, Hansson M, et al. Targeting CD38 with daratumumab monotherapy in multiple myeloma. *N Engl J Med.* (2015) 373:1207–19. doi: 10.1056/NEJMoa1506348
13. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:1319–31. doi: 10.1056/NEJMoa1607751
14. Mateos M-V, Dimopoulos MA, Cavo M, Suzuki K, Jakubowiak A, Knop S, et al. Daratumumab plus bortezomib, melphalan, and prednisone for untreated myeloma. *N Engl J Med.* (2017) 378:518–28. doi: 10.1056/NEJMoa1714678
15. Jelinek T, Hajek R. Monoclonal antibodies - A new era in the treatment of multiple myeloma. *Blood Rev.* (2016) 30:101–10. doi: 10.1016/j.blre.2015.08.004
16. Jelínek T, Korístka M, Cermáková Z, Hájek R. Daratumumab-hope for myeloma patients, a challenge for clinical laboratories. *Klin Onkol Cas Ceske Slov Onkol Spolecnosti* (2017) 30:13–9. doi: 10.14735/amko201713
17. Martin T, Baz R, Benson DM, Lendvai N, Wolf J, Munster P, et al. A phase 1b study of isatuximab plus lenalidomide and dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 129:3294–303. doi: 10.1182/blood-2016-09-740787
18. Jelinek T, Mihalyova J, Kascak M, Duras J, Hajek R. PD-1/PD-L1 inhibitors in haematological malignancies: update 2017. *Immunology* (2017) 152:357–71. doi: 10.1111/imm.12788
19. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. *Blood* (1996) 87:1196–8.
20. Cohen AD, Garfall AL, Stadtmauer EA, Lacey SF, Lancaster E, Vogl DT, et al. Safety and efficacy of B-Cell Maturation Antigen (BCMA)-Specific Chimeric Antigen Receptor T Cells (CART-BCMA) with cyclophosphamide conditioning for refractory Multiple Myeloma (MM). *Blood* (2017) 130:505. Available online at: www.bloodjournal.org/content/130/Suppl_1/505
21. Berdeja JG, Lin Y, Raje N, Munshi N, Siegel D, Liedtke M, et al. Durable clinical responses in heavily pretreated patients with relapsed/refractory multiple myeloma: updated results from a multicenter study of bb2121 Anti-Bcma CAR T cell therapy. *Blood* (2017) 130:740. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/740
22. Seckinger A, Delgado JA, Moser S, Moreno L, Neuber B, Grab A, et al. Target expression, generation, preclinical activity, and pharmacokinetics of the BCMA-T cell bispecific antibody EM801 for multiple myeloma treatment. *Cancer Cell* (2017) 31:396–410. doi: 10.1016/j.ccell.2017.02.002
23. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
24. Armand P. Immune checkpoint blockade in hematologic malignancies. *Blood* (2015) 125:3393–400. doi: 10.1182/blood-2015-02-567453
25. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WEE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med.* (2015) 373:123–35. doi: 10.1056/NEJMoa1504627
26. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med.* (2015) 373:23–34. doi: 10.1056/NEJMoa1504030
27. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med.* (2015) 373:1803–13. doi: 10.1056/NEJMoa1510665
28. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med.* (2015) 372:311–9. doi: 10.1056/NEJMoa1411087
29. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* (1992) 11:3887–95.
30. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
31. Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, et al. Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. *Eur J Immunol.* (2003) 33:3117–26. doi: 10.1002/eji.200324270
32. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol.* (2005) 25:9543–53. doi: 10.1128/MCB.25.21.9543-9553.2005

33. Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature* (2017) 545:495–9. doi: 10.1038/nature22396
34. Benson DM, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
35. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* (2007) 110:296–304. doi: 10.1182/blood-2006-10-051482
36. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. *Leukemia* (2013) 27:464–72. doi: 10.1038/leu.2012.213
37. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29:1441–4. doi: 10.1038/leu.2015.11
38. Görgün G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide Enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res.* (2015) 21:4607–18. doi: 10.1158/1078-0432.CCR-15-0200
39. Dhodapkar MV, Sexton R, Das R, Dhodapkar KM, Zhang L, Sundaram R, et al. Prospective analysis of antigen-specific immunity, stem-cell antigens, and immune checkpoints in monoclonal gammopathy. *Blood* (2015) 126:2475–8. doi: 10.1182/blood-2015-03-632919
40. Paiva B, Azpilikueta A, Puig N, Ocio EM, Sharma R, Oyajobi BO, et al. PD-L1/PD-1 presence in the tumor microenvironment and activity of PD-1 blockade in multiple myeloma. *Leukemia* (2015) 29:2110–3. doi: 10.1038/leu.2015.79
41. Kelly KR, Espitia CM, Zhao W, Wu K, Visconte V, Anwer F, et al. Oncolytic reovirus sensitizes multiple myeloma cells to anti-PD-L1 therapy. *Leukemia* (2018) 32:230–3. doi: 10.1038/leu.2017.272
42. Chauhan D, Singh AV, Brahmandam M, Carrasco R, Bandi M, Hideshima T, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell* (2009) 16:309–23. doi: 10.1016/j.ccr.2009.08.019
43. Sponaas A-M, Moharrami NN, Feyzi E, Standal T, Holth Rustad E, Waage A, et al. PDL1 Expression on plasma and dendritic cells in myeloma bone marrow suggests benefit of targeted anti PD1-PDL1 therapy. *PLoS ONE* (2015) 10:e0139867. doi: 10.1371/journal.pone.0139867
44. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances *ex vivo* T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother Hagerstown Md 1997* (2011) 34:409–18. doi: 10.1097/CJI.0b013e31821ca6ce
45. Moreau P, San Miguel J, Sonneveld P, Mateos MV, Zamagni E, Avet-Loiseau H, et al. Multiple myeloma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* (2017) 28:iv52–61. doi: 10.1093/annonc/mdx096
46. Jelinek T, Kufova Z, Hajek R. Immunomodulatory drugs in AL amyloidosis. *Crit Rev Oncol Hematol.* (2016) 99:249–60. doi: 10.1016/j.critrevonc.2016.01.004
47. Sedlarikova L, Kubiczkova L, Sevcikova S, Hajek R. Mechanism of immunomodulatory drugs in multiple myeloma. *Leuk Res.* (2012) 36:1218–24. doi: 10.1016/j.leukres.2012.05.010
48. Giuliani M, Janji B, Berchem G. Activation of NK cells and disruption of PD-L1/PD-1 axis: two different ways for lenalidomide to block myeloma progression. *Oncotarget* (2017) 8:24031–44. doi: 10.18632/oncotarget.15234
49. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K, et al. PD-1 blockade enhances elotuzumab efficacy in mouse tumor models. *Blood Adv.* (2017) 1:753–65. doi: 10.1182/bloodadvances.2017004382
50. Chen L, Byers LA, Ullrich S, Wistuba II, Qin X-F, Gibbons DL. *CD38 as a Novel Immune Checkpoint and a Mechanism of Resistance to the Blockade of the PD-1/PD-L1 Axis.* (Orlando, FL), Abstract 79, Oral abstract. Available online at: https://meetinglibrary.asco.org/record/144495/abstract (Accessed August 22, 2018).
51. Bezman NA, Kinder M, Jhatakia AD, Mattson BK, Pizutti D, Thompson EW, et al. Abstract 1727: antitumor activity associated with dual targeting of CD38 and programmed death-1 (PD-1) pathways in preclinical models. *Cancer Res.* (2018) 78:1727. doi: 10.1158/1538-7445.AM2018-1727
52. Jelinek T, Hajek R. PD-1/PD-L1 inhibitors in multiple myeloma: the present and the future. *OncoImmunology* (2016) 5:e1254856. doi: 10.1080/2162402X.2016.1254856
53. Sharabi AB, Lim M, DeWeese TL, Drake CG. Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy. *Lancet Oncol.* (2015) 16:e498–509. doi: 10.1016/S1470-2045(15)00007-8
54. Ngiow SF, McArthur GA, Smyth MJ. Radiotherapy complements immune checkpoint blockade. *Cancer Cell* (2015) 27:437–8. doi: 10.1016/j.ccell.2015.03.015
55. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature* (2015) 520:373–7. doi: 10.1038/nature14292
56. Binder DC, Fu Y-X, Weichselbaum RR. Radiotherapy and immune checkpoint blockade: potential interactions and future directions. *Trends Mol Med.* (2015) 21:463–5. doi: 10.1016/j.molmed.2015.05.007
57. Kearl TJ, Jing W, Gershan JA, Johnson BD. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. *J Immunol Baltim Md 1950* (2013) 190:5620–8. doi: 10.4049/jimmunol.1202005
58. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib study. *J Clin Oncol.* (2016) 34:2698–704. doi: 10.1200/JCO.2015.65.9789
59. Chung DJ, Pronschinske KB, Shyer JA, Sharma S, Leung S, Curran SA, et al. T-cell exhaustion in multiple myeloma relapse after autotransplant: optimal timing of immunotherapy. *Cancer Immunol Res.* (2016) 4:61–71. doi: 10.1158/2326-6066.CIR-15-0055
60. Hallett WHD, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. *Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant* (2011) 17:1133–45. doi: 10.1016/j.bbmt.2011.03.011
61. Ribrag V, Avigan DE, Martinelli G, Green DJ, Trisha W-D, Posada JG, et al. PEMBROLIZUMAB MONOTHERAPY FOR RELAPSED/REFRACTORY MULTIPLE MYELOMA... In Siegel D, Editor. *Poster Presentation, Abstract: P344.* Madrid. (2018). Available online at: https://learningcenter.ehaweb.org/eha/2017/22nd/181631/vincent.ribrag.pembrolizumab.monotherapy.for.relapsed.refractory.multiple.htmlf=m3e1181l15532 (Accessed May 2, 2018).
62. Rodriguez-Otero P, Mateos M-V, Orlowski R, Siegel D, Reece D, Moreau P, Miguel JS. PEMBROLIZUMAB PLUS LENALIDOMIDE AND LOW-DOSE DEXAMETHASONE FOR... In: Rodriguez-Otero P, Editors. *Oral Presentation, Abstract: S783.* Madrid. (2018). Available online at: https://learningcenter.ehaweb.org/eha/2017/22nd/182070/paula.rodriguez-otero.pembrolizumab.plus.lenalidomide.and.low-dose.htmlf=media=3 (Accessed May 2, 2018).
63. Badros A, Hyjek E, Ma N, Lesokhin A, Dogan A, Rapoport AP, et al. Pembrolizumab, pomalidomide, and low-dose dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 130:1189–97. doi: 10.1182/blood-2017-03-775122
64. Biran N, Andrews T, Feinman R, Vesole DH, Richter JR, Zenreich J, Anand P, McBride L, Ivanovski K, Korngold R, et al. A phase II trial of the anti - PD-1 monoclonal antibody pembrolizumab (MK-3475) + lenalidomide + dexamethasone as post autologous stem cell transplant consolidation in patients with high-risk multiple myeloma. *Blood* (2017) 130:1831. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/1831
65. Pawarode A, D'Souza A, Pasquini MC, Johnson B, Braun T, Dhakal B, et al. Phase 2 Study of pembrolizumab during lymphodepleted state after autologous hematopoietic cell transplantation in multiple myeloma patients. *Blood* (2017) 130:339. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/339
66. Ocio EM, Puig N, Corchete L, Pérez JJ, Dávila J, Paíno T, et al. Immune predictors of response to pembrolizumab monotherapy as consolidation in multiple myeloma patients: results of the GEM-Pembresid clinical trial. *Blood* (2017) 130:1874. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/1874

67. Ansell S, Gutierrez ME, Shipp MA, Gladstone D, Moskowitz A, Borello I, Popa-Mckiver M, Farsaci B, Zhu L, Lesokhin AM, et al. A phase 1 study of nivolumab in combination with ipilimumab for relapsed or refractory hematologic malignancies (CheckMate 039). *Blood* (2016) 128:183. Available online at: http://www.bloodjournal.org/content/128/22/183
68. Skarbnik AP, Donato ML, Rowley SD, Goy A, Vesole DH, Munshi PN, et al. Preliminary safety and efficacy data for combined checkpoint inhibition with Ipilimumab (I) and Nivolumab (N) As Consolidation Following Autologous Stem Cell Transplantation (ASCT) for High-Risk Hematologic Malignancies - Cpit-001 trial. *Blood* (2017) 130:340. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/340
69. Efebera YA, Rosko A, Hofmeister CC, Benner J, Bakan C, Benson DM. *Paper: First Interim Results of a Phase I/II Study of Lenalidomide in Combination with Anti-PD-1 Monoclonal Antibody MDV9300 (CT-011) in Patients with Relapsed/Refractory Multiple Myeloma*. Orlando, IL. Available online at: https://ash.confex.com/ash/2015/webprogram/Paper81417.html (Accessed July 25, 2016).
70. Rosenblatt J, Avivi I, Binyamini N, Uhl L, Somayia P, Stroopinsky D, et al. *Paper: Blockade of PD-1 in Combination with Dendritic Cell/Myeloma Fusion Cell Vaccination Following Autologous Stem Cell Transplantation Is Well Tolerated, Induces Anti-Tumor Immunity and May Lead to Eradication of Measureable Disease*. Orlando, IL. Available online at: https://ash.confex.com/ash/2015/webprogram/Paper80695.html (Accessed July 25, 2016).
71. Mateos M-V, Blacklock H, Schjesvold F, Rocafiguera AO, Simpson D, Lonial S. A phase 3 randomized study of pembrolizumab (Pembro) plus pomalidomide (Pom) and dexamethasone (Dex) for relapsed/refractory multiple myeloma (RRMM): KEYNOTE-183. In: *Poster session, Abstract 8021*. Chicago, IL. (2018). Available online at: https://meetinglibrary.asco.org/record/161606/abstract (Accessed August 22, 2018).
72. Usmani SZ, Schjesvold F, Rocafiguera AO, Karlin L, Rifkin RM, San-Miguel J. A phase 3 randomized study of pembrolizumab (pembro) plus lenalidomide (len) and low-dose dexamethasone (Rd) versus Rd for newly diagnosed and treatment-naive multiple myeloma (MM): KEYNOTE-185. In: *Poster Discussion Session, Abstract 8010*. Chicago, IL. (2018). Available online at: https://meetinglibrary.asco.org/record/160702/abstract (Accessed August 22, 2018).
73. Manasanch EE, Mathur R, Lee HC, Weber DM, Patel KK, Thomas SK, et al. Pilot study of pembrolizumab for immunoprevention in smoldering multiple myeloma. *Blood* (2017) 130:3089. Available online at: http://www.bloodjournal.org/content/130/Suppl_1/3089
74. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* (2002) 3:991–8. doi: 10.1038/ni1102-991
75. Weber JS, Yang JC, Atkins MB, Disis ML. Toxicities of immunotherapy for the practitioner. *J Clin Oncol.* (2015) 33:2092–9. doi: 10.1200/JCO.2014.60.0379
76. Naidoo J, Page DB, Li BT, Connell LC, Schindler K, Lacouture ME, et al. Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. *Ann Oncol.* (2015) 26:2375–91. doi: 10.1093/annonc/mdv383
77. Haanen JBAG, Carbonnel F, Robert C, Kerr KM, Peters S, Larkin J, et al. Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* (2017) 28:iv119–42. doi: 10.1093/annonc/mdx225
78. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med.* (2018) 378:158–68. doi: 10.1056/NEJMra1703481
79. Krauss AC, Mulkey F, Shen Y-L, Rosenberg A, Miller B, Spring S. *FDA Analysis of Pembrolizumab Trials in Multiple Myeloma: Immune Related Adverse Events (irAEs) and Response*. Chicago, IL. Available online at: https://meetinglibrary.asco.org/record/160685/abstract (Accessed August 22, 2018).
80. Martinez-Calle N, Rodriguez-Otero P, Villar S, Mejías L, Melero I, Prosper F, et al. Anti-PD1 associated fulminant myocarditis after a single pembrolizumab dose: the role of occult pre-existing autoimmunity. *Haematologica* (2018) 103:e318–21. doi: 10.3324/haematol.2017.185777
81. Suen H, Brown R, Yang S, Weatherburn C, Ho PJ, Woodland N, et al. Multiple myeloma causes clonal T-cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* (2016) 30:1716–24. doi: 10.1038/leu.2016.84
82. Tremblay-LeMay R, Rastgoo N, Chang H. Modulating PD-L1 expression in multiple myeloma: an alternative strategy to target the PD-1/PD-L1 pathway. *J Hematol OncolJ Hematol Oncol.* (2018) 11:46. doi: 10.1186/s13045-018-0589-1

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CD38-Specific Biparatopic Heavy Chain Antibodies Display Potent Complement-Dependent Cytotoxicity Against Multiple Myeloma Cells

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CD38 is overexpressed by multiple myeloma cells and has emerged as a target for therapeutic antibodies. Nanobodies are soluble single domain antibody fragments derived from the VHH variable domain of heavy chain antibodies naturally occurring in camelids. We previously identified distinct llama nanobodies that recognize three non-overlapping epitopes of the extracellular domain of CD38. Here, we fused these VHH domains to the hinge, CH2, and CH3 domains of human IgG1, yielding highly soluble chimeric llama/human heavy chain antibodies (hcAbs). We analyzed the capacity of these hcAbs to mediate complement-dependent cytotoxicity (CDC) to CD38-expressing human multiple myeloma and Burkitt lymphoma cell lines. Combinations of two hcAbs that recognize distinct, non-overlapping epitopes of CD38 mediated potent CDC, in contrast to the hcAb monotherapy with only weak CDC capacity. Similarly, combining daratumumab with a hcAb that recognizes a non-overlapping epitope resulted in dramatically enhanced CDC. Further, introducing the E345R HexaBody mutation into the CH3 domain strongly enhanced the CDC potency of hcAbs to CD38-expressing cells. Exploiting their high solubility, we genetically fused two distinct nanobodies into heteromeric dimers via a flexible peptide linker and then fused these nanobody dimers to the hinge, CH2 and CH3 domains of human IgG1, yielding highly soluble, biparatopic hcAbs. These biparatopic hcAbs elicited CDC toward CD38-expressing myeloma cells more effectively than daratumumab. Our results underscore the advantage of nanobodies vs. pairs of VH and VL domains for constructing bispecific antibodies. Moreover, the CD38-specific biparatopic heavy chain antibodies described here represent potential new powerful therapeutics for treatment of multiple myeloma.

Keywords: complement-dependent cytotoxicity, CD38, multiple myeloma, nanobody, heavy chain antibody, antibody engineering, biparatopic antibodies

Abbreviations: Ab, antibody; bsAb, bispecific Ab; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; Fc, crystallizing fragment; hcAb, heavy chain antibody, Ig immunoglobulin; kDa, kilodalton; NAD^+, nicotinamide adenine dinucleotide; moAb, monoclonal antibody; Nb, nanobody; VH, variable domain of a conventional heavy chain; VHH, variable domain of a camelid heavy chain antibody; scFv, single chain variable fragment.

INTRODUCTION

CD38 is overexpressed by multiple myeloma and other hematological tumors and has attracted interest as a target for therapeutic antibodies (1–4). CD38 is a cell surface ecto-enzyme that metabolizes NAD^+ released from damaged cells in inflammation (5). In concert with CD203 and CD73, CD38 contributes to the conversion of NAD^+ to immunosuppressive adenosine in the tumor microenvironment (6, 7). By suppressing effector T cell responses, CD38 may thereby promote tumor growth (5, 8). The conventional CD38-specific monoclonal antibody daratumumab was generated from CD38-immunized transgenic mice that carry genomic loci encoding human IgH and IgL (9). Daratumumab has proven high therapeutic efficacy in multiple myeloma (3, 10).

Complement-dependent cytotoxicity (CDC) is an important mechanism for the killing of tumor cells (11–13). CDC is initiated when complement factor 1 (C1q) binds to antibodies on the cell surface. It has been recognized that monospecific IgG antibodies are generally ineffective at inducing CDC, while IgM and combinations of non-crossreactive IgG molecules induce potent CDC (14–17). Modeling and mutagenesis studies suggest that IgG hexamer formation facilitates efficient binding and activation of C1q (18). Amino acid substitutions in the CH3 domain of daratumumab that enhanced the formation of IgG hexamers were found to enhance the binding of C1q to CD38 on the cell surface. These so called HexaBody mutations also enhanced CDC by daratumumab (18, 19).

The variable domain of heavy chain antibodies that naturally occur in camelids is called VHH or nanobody (Nanobody® is a trademark of Ablynx). Nanobodies exhibit several advantages over conventional antibodies (20–24). The single domain format of nanobodies greatly facilitates the construction of bispecific and biparatopic dimers by genetically linking two nanobodies with a flexible peptide linker (25–30). Genetic fusion of a nanobody to the hinge, CH2 and CH3 domains of human IgG1 yields highly soluble llama/human chimeric heavy chain antibodies (hcAbs) (31–33). At half the size of a conventional antibody (75 vs. 150 kDa), hcAbs may penetrate tissues better than conventional antibodies (32). To date, more than 2,000 patients and healthy subjects have received nanobodies in clinical studies without any adverse side effects (34–36). The European Commission recently granted marketing authorization for the first nanobody-based drug, Caplacizumab (Cablivi™), a nanobody-dimer directed against von Willebrand factor, for the treatment of acquired thrombotic thrombocytopenic purpura (aTTP), a rare blood clotting disorder (37).

The goal of this study was to assess the capacity of CD38-specific hcAbs to induce CDC to CD38-expressing multiple myeloma cells. Our results show that the combination of two CD38 hcAbs elicits potent CDC, provided the two hcAbs recognize distinct epitopes. We sought to exploit the high solubility of nanobodies to construct highly soluble biparatopic nanobody-based hcAbs that contain a tandem pair of CD38-specific nanobodies recognizing non-overlapping epitopes. Remarkably, these biparatopic hcAbs show higher CDC potency than daratumumab and therefore hold promise as novel therapeutics for the treatment of multiple myeloma.

RESULTS

Individual CD38-Specific hcAbs Induce Little if Any CDC

In order to generate CD38-specific heavy chain antibodies, we genetically fused the nanobody coding sequence to the coding sequence for the hinge, CH2, and CH3 domains of human IgG1

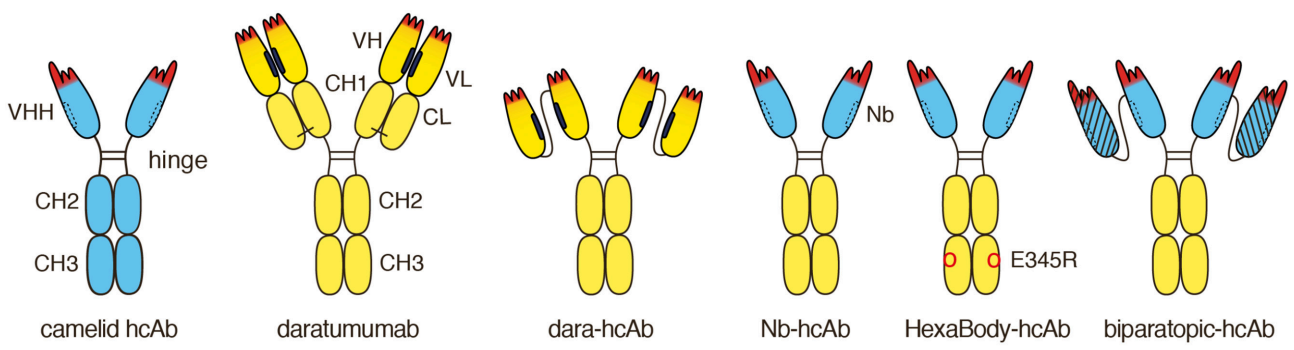

FIGURE 1 | Schematic diagram of heavy chain antibodies (hcAbs) used in this study. Naturally occurring camelid hcAbs lack the CH1 domain and light chains. The antigen binding module of these hcAbs is composed of a single highly soluble variable domain (VHH) that is linked directly to the hinge. Like other conventional antibodies, daratumumab is composed of two IgG1 heavy chains and two kappa light chains. The antigen binding module of daratumumab is composed of two non-covalently associated variable domains, VH, and VL. The proper orientation of these domains is mediated by a hydrophobic interface (indicated in black) and is further stabilized by the disulfide linked CL and CH1 domains. We genetically fused the VH and VL domains of daratumumab via a flexible peptide linker and further fused this single chain variable fragment (scFv) to the hinge, CH2, and CH3 domains of IgG1, generating dara-hcAb, corresponding to the format of camelid hcAbs. The proper orientation of the antigen recognition module in this construct is mediated solely by the hydrophobic interface between the two V domains. We fused distinct CD38-specific VHH domains to the hinge, CH2 and CH3 domains of human IgG1, generating Nb-hcAbs, i.e., chimeric llama/human IgG1 hcAbs. A recombinant VHH domain or nanobody (Nb) is highly soluble and does not show any tendency to associate with light chains or any other hydrophobic proteins (the hydrophilic face corresponding to the hydrophobic VL-interface of conventional antibodies is indicated by a dashed line). We introduced the E345R HexaBody mutation into some hcAbs, generating HexaBody-hcAbs. We further exploited the inherent solubility of VHHs to generate biparatopic hcAbs by fusing two distinct CD38-specific VHHs via a flexible G4Sn linker and further fusing such dimers to the hinge, CH2 and CH3 domains of human IgG1.

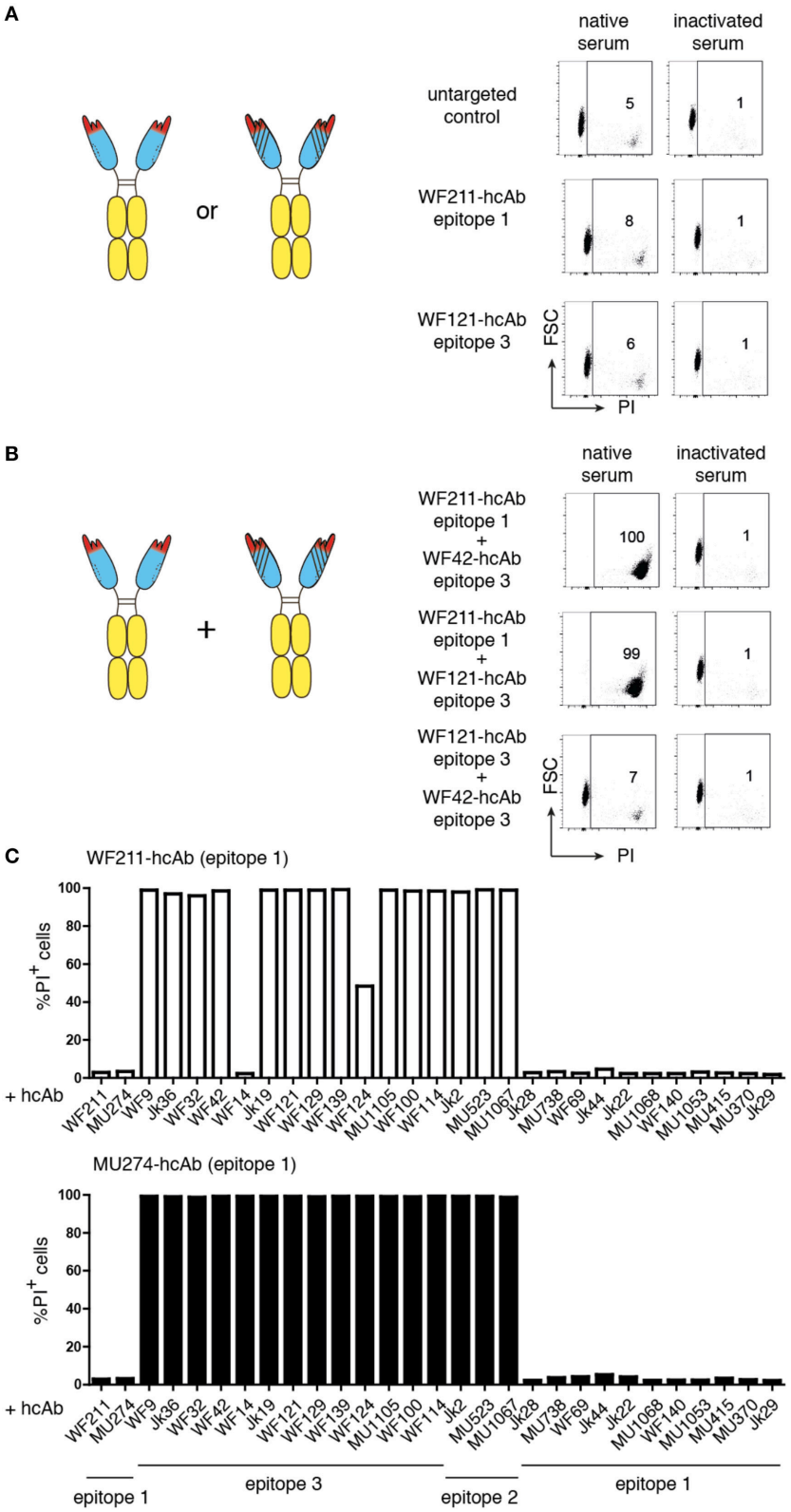

FIGURE 2 | Combinations of two hcAbs recognizing non-overlapping epitopes of CD38 are potent inducers of CDC. CA-46 cells were incubated for 60 min at 37°C in the presence of of saturating amounts (10–30 nM) of individual hcAbs **(A)** or with combinations of two hcAbs **(B)** and native serum or inactivated serum (preincubated for

(Continued)

FIGURE 2 | 30 min at 56°C to inactivate complement components). Cells were washed and resuspended in PBS containing BSA and propidium iodide (PI) before analysis by flow cytometry. **(A,B)** The schematics illustrate the CD38-specific hcAbs used in this experiment. Representative FACS plots illustrate the gating strategy used to determine the percentages of dead cells (PI +, FSC = forward scatter low). **(C)** Bar diagrams showing % of PI-positive cells of samples treated with a combination of either WF211-hcAb or MU274-hcAb and the hcAbs indicated below. Results are representative of three similar experiments.

TABLE 1 | Combinations of two CD38-specific hcAbs recognizing distinct epitopes induce potent CDC.

Epitope	–	JK2-hcAb	MU1067-hcAb	WF211-hcAb	MU274-hcAb	JK36-hcAb	WF100-hcAb
2	JK2-hcAb	5	4	99	100	100	96
2	MU1067-hcAb	4	5	100	99	100	96
2	MU523-hcAb	4	5	100	100	100	96
1	MU738-hcAb	100	100	2	3	100	97
1	JK44-hcAb	100	100	3	4	100	98
1	JK29-hcAb	99	100	1	2	100	97
1	MU1068-hcAb	100	100	1	1	100	96
1	MU415-hcAb	100	100	2	3	100	96
1	JK22-hcAb	99	100	2	4	100	90
1	WF211-hcAb	100	100	3	3	100	37
1	MU1053-hcAb	100	100	2	1	100	54
1	MU370-hcAb	100	100	1	2	100	97
1	MU274-hcAb	100	100	2	2	100	95
1	JK28-hcAb	100	100	2	1	100	53
3	WF124-hcAb	96	100	50	100	4	2
3	WF121-hcAb	99	99	99	100	4	2
3	WF42-hcAb	95	98	99	100	8	2
3	JK19-hcAb	100	100	100	100	5	2
3	JK36-hcAb	100	100	98	100	4	2
3	WF100-hcAb	100	100	99	100	5	2
3	WF9-hcAb	100	100	100	100	4	2
3	WF14-hcAb	81	97	2	100	4	2

Numbers indicate the percentage of PI positive cells, 60 min after incubation of CA-46 cells in the presence of saturating amounts (10–30 nM) of the indicated combinations of hcAbs and human serum. Numbering of binding epitopes is according to Fumey et al. (38).

(**Figure 1**). We tested the capacity of individual CD38-specific hcAbs to induce CDC to CD38-expressing LP-1 or CA-46 tumor cells in the presence of human serum as a source of complement (**Figure 2**). After 1 h incubation at 37°C cells were analyzed by flow cytometry for uptake of the DNA-staining dye propidium iodide as a marker for cell death. The results reveal that individual hcAbs show little if any capacity to induce CDC (**Figure 2A**).

Combinations of Two hcAbs Recognizing Non-Overlapping Epitopes of CD38 Are Potent Inducers of CDC

It has been shown that combinations of non-crossreactive IgG antibodies can induce potent CDC (15–17). We therefore tested whether combinations of two distinct CD38-specific hcAbs could induce CDC. Indeed, certain combinations of hcAbs induced potent CDC, whereas other hcAb combinations were as ineffective as individual hcAbs (**Figures 2B,C**). Pre-incubating the serum for 30 min at 56°C abrogated cytotoxicity, indicating that killing was dependent on active complement components.

We had previously assigned the 22 nanobodies to one of three distinct epitopes of CD38 on the basis of cross-blockade and sequential binning analyses (38). Taking these epitope assignments into consideration, a clear pattern emerges: Any combination of two hcAbs that recognize non-overlapping epitopes elicits very potent CDC whereas any combination of two hcAbs that recognize overlapping epitopes elicits little if any CDC (**Table 1**).

Combination of Daratumumab With a CD38-Specific hcAb That Binds a Distinct Epitope of CD38 Enhances It's CDC Potency

We next tested whether any of our CD38-specific hcAbs could elicit potent CDC also in combination with the benchmark therapeutic antibody daratumumab (**Figure 3**). The results show that only certain hcAbs complement daratumumab to induce potent CDC. Considering our previous assignment of nanobody epitopes relative to that of daratumumab (38), a similar clear pattern again emerges: CD38-specific hcAbs carrying a nanobody that binds independently of daratumumab elicit potent CDC when combined with daratumumab. In contrast, CD38-specific hcAbs carrying a nanobody that binds an epitope overlapping with daratumumab elicit little if any CDC when combined with daratumumab (**Table 2**).

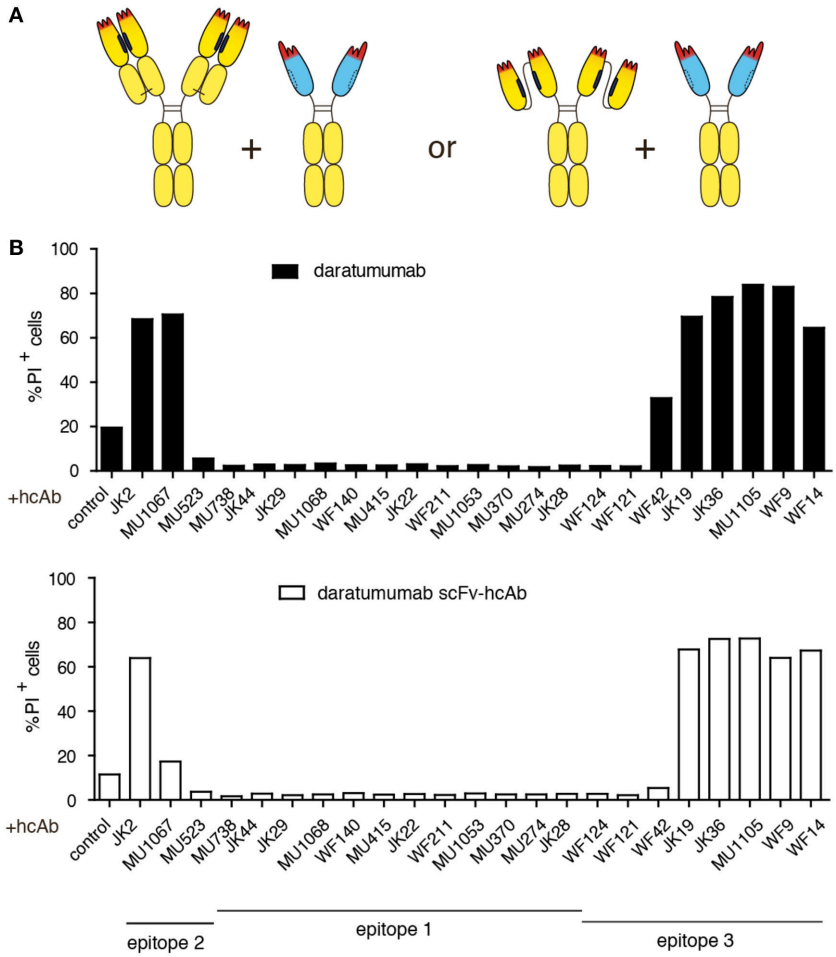

FIGURE 3 | Combination of daratumumab with an anti-CD38 hcAb that binds a distinct epitope on CD38 enhances its CDC potency. CA-46 cells were incubated for 60 min at 37°C in the presence of Ab combinations (50 nM each) containing either daratumumab or a scFv-hcAb form of daratumumab and a CD38-specific hcAb and 15% (v/v) of native human serum. Cells were washed and resuspended in PBS containing BSA and propidium iodide before analysis by flow cytometry. **(A)** Schematic illustrating the constructs used in this experiment. **(B)** Bar diagrams showing % of PI-positive cells ± standard deviation of three samples treated in parallel with the indicated hcAbs. Results are representative of four similar experiments.

In analogy to the nanobody-based heavy chain antibodies, we also constructed a hcAb version of daratumumab by fusing the VH and VL domains of daratumumab via a flexible Gly-Ser linker and further fusing this scFv to the hinge, CH2 and CH3 domains of human IgG1. In CDC assays, the same hcAbs that enhanced the CDC potency of daratumumab also enhanced the CDC potency of this dara-hcAb (**Figure 3B**).

Introduction of the E345R HexaBody Mutation Enhances the CDC Potency of CD38-Specific hcAbs

It has been shown that certain amino acid substitutions in the C1q binding face of daratumumab enhance the tendency of daratumumab to spontaneously form hexamers (18). These so called HexaBody mutations enhanced the CDC potency of daratumumab (19). We aimed to determine whether a HexaBody mutation would similarly enhance the CDC potency of CD38-specific hcAbs. We therefore introduced the E345R mutation into the CH3 domain of our hcAbs and analyzed the capacity of these HexaBody hcAbs to induce CDC. The results, indeed, reveal an enhanced CDC potency of the HexaBody hcAbs over their parental counterparts (**Figure 4**). LP-1 cells in which the CD38 gene had been inactivated by CRISPR/Cas9 technology were resistant to CDC by HexaBody hcAbs, indicating that binding to CD38 is essential for induction of CDC.

Combining Nanobodies Directed to Two Distinct Epitopes on CD38 in a Biparatopic hcAb Induces Potent CDC

The soluble nature of nanobodies allows easy reformatting of nanobodies into homo- and heteromeric dimers by linking the C-terminus of one nanobody to the N-terminus of another nanobody by a flexible peptide linker [e.g., (G4S)n]. Moreover,

TABLE 2 | Combinations of daratumumab with a hcAb recognizing a distinct epitope induce potent CDC.

Epitope	hcAb	Daratumumab
2	JK2-hcAb	86
2	MU1067-hcAb	68
2	MU523-hcAb	60
1	MU738-hcAb	9
1	JK44-hcAb	17
1	JK29-hcAb	11
1	MU1068-hcAb	6
1	MU415-hcAb	8
1	JK22-hcAb	5
1	WF211-hcAb	4
1	MU1053-hcAb	5
1	MU370-hcAb	7
1	MU274-hcAb	6
1	JK28-hcAb	6
3	WF124-hcAb	29
3	WF121-hcAb	30
3	WF42-hcAb	80
3	JK19-hcAb	95
3	JK36-hcAb	65
3	WF100-hcAb	93
3	WF9-hcAb	94
3	WF14-hcAb	65

Numbers indicate the percentage of PI positive cells, 90 min after incubation of LP-1 cells in the presence of the indicated combinations of daratumumab with a CD38-specific hcAb and human serum. Numbering of binding epitopes is according to Fumey et al. (38).

such nanobody dimers can be fused to the hinge, CH2, and CH3 domains of human IgG1 to generate tetravalent bispecific or biparatopic hcAbs (32). In order to determine whether the potent CDC induction capacity of certain hcAb combinations could be combined into a single molecule, we constructed biparatopic hcAbs containing two nanobodies that recognize distinct epitopes of CD38. These biparatopic hcAbs were produced at high yield as soluble proteins in transiently transfected HEK-6E cells. The results of CDC assays reveal that biparatopic hcAbs indeed induce potent CDC as single reagents (**Figure 5**).

Biparatopic CD38-Specific hcAbs Have Higher CDC Potency Than Daratumumab

In order to further compare the CDC potencies of daratumumab and our biparatopic hcAbs, we performed CDC-assays with titrated amounts of antibodies (**Figure 6**). The results show that the biparatopic hcAbs are much more potent than daratumumab at inducing CDC.

DISCUSSION

Our results confirm the finding that combinations of two distinct, non-crossreactive IgG antibodies induce CDC more potently than monospecific IgG (15–17) and provide further insight into the molecular mechanism of this phenomenon. The schematic

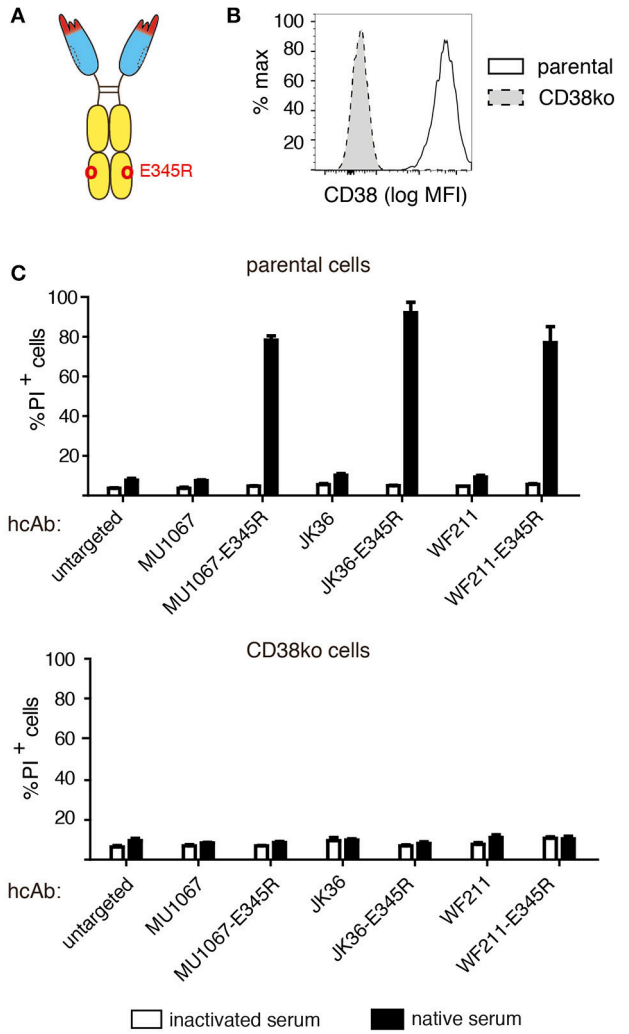

FIGURE 4 | Introduction of the E345R HexaBody mutation enhances the CDC potency of CD38-specific hcAbs. **(A)** Schematic illustration of the hcAb E345R mutant that facilitates Fc-hexamerization used in this experiment. **(B)** The CD38 gene was stably inactivated in LP-1 cells using CRISPR/Cas9 technology. Cells were stained with AF647-conjugated Nb JK36 and analyzed by flow cytometry. **(C)** Parental and CD38ko LP-1 cells were incubated for 60 min at 37°C with the indicated CD38-specific hcAbs (100 nM) and 15% v/v native human serum. Cells were stained with propidium iodide and analyzed by flow cytometry. Bar diagrams showing % of PI-positive cells ± standard deviation of three samples treated in parallel with the indicated hcAbs. Results are representative of four similar experiments.

diagrams shown in **Figure 7** present hypothetical models that need to be tested in more detail: Binding of a monospecific hcAb, moAb, or a combination of Abs that recognize an overlapping epitope of CD38 can maximally crosslink two CD38 molecules on the cell surface (**Figure 7A**). Addition of a second hcAb that binds to an epitope distinct from that of the first hcAb can crosslink two or more CD38 dimers connected by the first hcAb, thereby facilitating the formation of C1q-activating oligomers (**Figure 7B**). The E345R HexaBody mutation (18) enhances the CDC potency of hcAbs by facilitating formation of hexamers on the cell surface (**Figure 7C**). It is not known

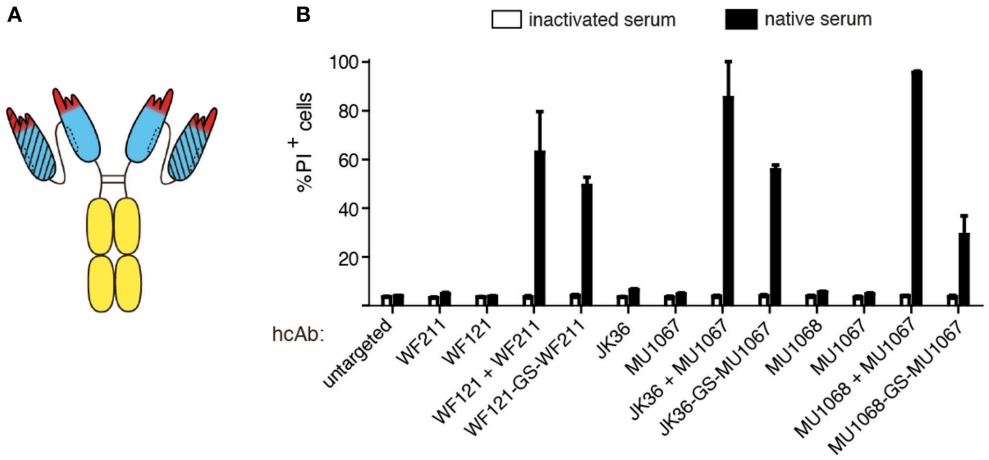

FIGURE 5 | Combining two nanobodies directed to distinct epitopes of CD38 in a biparatopic hcAb induces potent CDC. LP-1 cells were incubated for 60 min at 37°C with an untargeted control hcAb or with the indicated monospecific or biparatopic hcAbs (100 nM) and either native human serum or inactivated serum. Cells were washed, stained with propidium iodide and analyzed by flow cytometry. **(A)** Schematic illustrating the biparatopic hcAbs used in this experiment. **(B)** Induction of CDC by biparatopic CD38-specific hcAbs combining two nanobodies with non-overlapping epitopes ("-GS-") in comparison to the combinations of the respective hcAbs ("+"). Bar diagrams showing % of PI-positive cells ±standard deviation of three samples treated in parallel with the indicated hcAbs. Results are representative of four similar experiments.

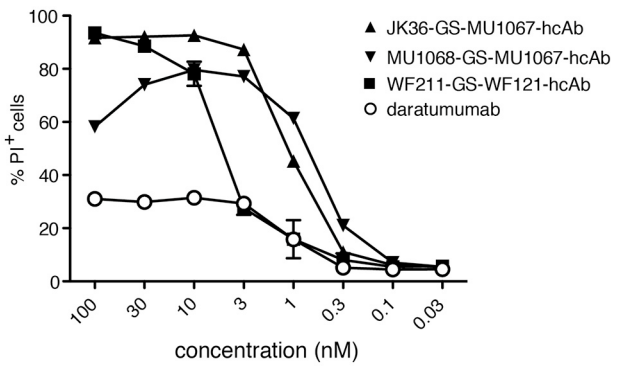

FIGURE 6 | Biparatopic CD38-specific hcAbs combining nanobodies directed to two distinct epitopes on CD38 have higher CDC potency than daratumumab. CA-46 cells were incubated for 60 min at 37°C with titrated amounts of the indicated hcAbs or daratumumab and native human serum. Cells were washed, stained with propidium iodide and analyzed by flow cytometry. Results are representative of three similar experiments. Data are averages ±SD ($n = 3$).

whether HexaBody hcAbs are also effective when binding monovalently as has been demonstrated for daratumumab (18). Remarkably, fusing two CD38-specific nanobodies that recognize distinct epitopes of CD38 into a biparatopic hcAb also results in potent CDC, likely reflecting the capacity of such biparatopic hcAbs to efficiently induce the formation of clusters (**Figure 7D**).

Biparatopic hcAbs have several inherent advantages over conventional bispecific antibodies (bsAbs) (**Figure 8**). Evolution has shaped a remarkably high stability and solubility of camelid VHH domains in the absence of a paired light chain (22, 24, 39). Our study shows that this unique biochemical property can be exploited to construct highly soluble, stable CD38-specific biparatopic hcAbs that induce potent CDC. Owing to their excellent solubility, nanobody-based biparatopic hcAbs are easier to construct, produce and purify at high yield than corresponding constructs based on conventional H + L chain antibodies. Biparatopic hcAbs are composed of two copies of a single polypeptide chain (**Figure 8A**). In contrast, conventional bsAbs are typically composed of two or more distinct polypeptide chains (**Figures 8B,C**) (40). The latter requires careful titration of two or more expression vectors and/or the use of dual cassette vectors in order to ensure expression in the appropriate molar ratios. In contrast, production of a biparatopic hcAb requires transfection of cells with only a single vector encoding a single heavy chain composed entirely of naturally highly soluble protein domains. A key structural advantage of a biparatopic heavy chain antibody over symmetric bsAbs (**Figure 8B**) lies in the high solubility of each VHH vs. the inherent instability of VH-VL pairing. For the proper assembly of bsAbs in the regular IgG format (**Figure 8C**), it is necessary to introduce mutations into the CH3 domains to promote pairing of two distinct H chains, resulting in asymmetric antibodies. Similarly, mutations need to be introduced into the CH1 and CL domains to promote the proper paring of H and L chains (41–43).

A potential advantage of biparatopic hcAbs and symmetric bsAbs over asymmetric bsAbs is their higher valency. Biparatopic hcAbs are tetravalent, i.e., they carry four antigen binding modules, each composed of a single highly soluble Ig-domain (**Figure 8A**). In contrast, bsAbs in the regular IgG format are bivalent, i.e., they carry only two antigen binding modules, each composed of two or more Ig-domains (**Figure 8C**). It is likely that oligomers are induced more effectively by tetravalent than by bivalent Abs.

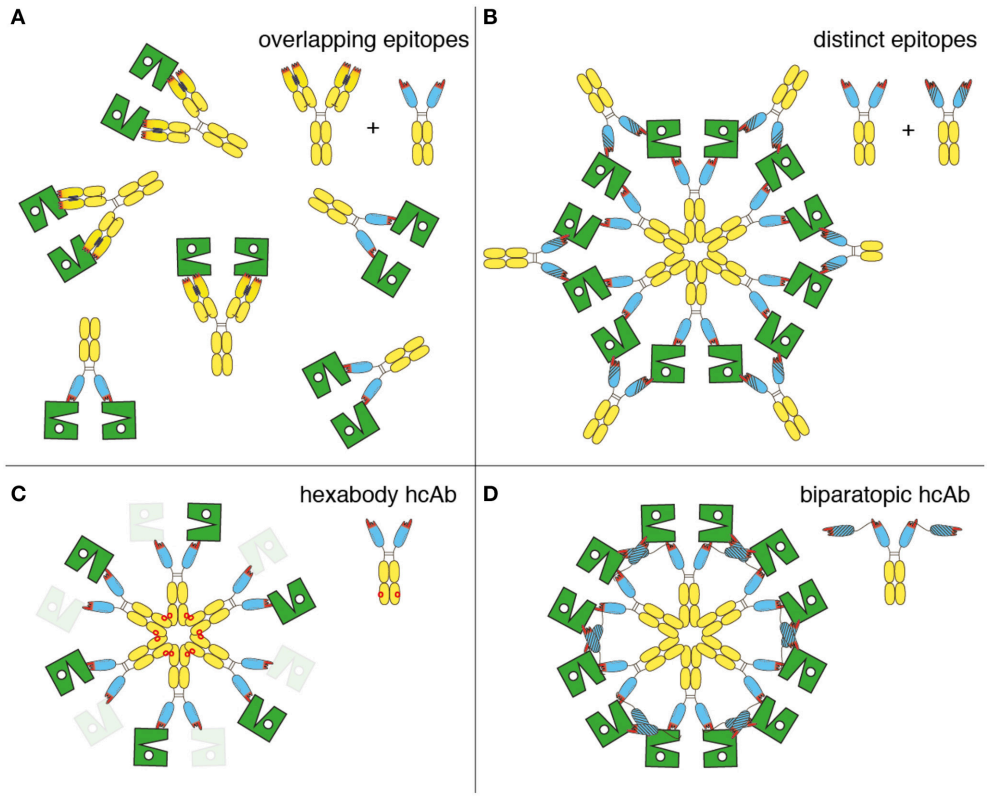

FIGURE 7 | Schematic diagram illustrating the proposed molecular mechanism for the enhanced CDC by antibody combinations, HexaBody hcAbs, and biparatopic hcAbs. The extracellular enzymatic domain of CD38 is illustrated schematically in green, daratumumab and nanobody-based hcAbs are represented by the symbols used in other figures. **(A)** Single antibodies or combinations of antibodies (conventional or heavy chain) that recognize an overlapping epitope of CD38 can maximally crosslink two CD38 molecules. These CD38 dimers are not assembled into higher order oligomers and therefore show little if any capacity to bind C1q and to induce CDC. **(B)** Combinations of antibodies recognizing distinct epitopes of CD38 (conventional or heavy chain) facilitate formation of oligomers. **(C)** Introduction of the E345R hexabody mutation facilitates formation of hexameric antibody clusters on the cell surface. It remains to be determined whether HexaBody hcAbs are effective when binding monovalently (indicated by masked CD38), as demonstrated for daratumumab (18). **(D)** Biparatopic heavy chain antibodies containing nanobodies that recognize distinct epitopes of CD38 facilitate the formation of oligomers, since the two genetically fused VHH domains can bind to two different molecules of CD38.

Biparatopic hcAbs also have inherent advantages over HexaBody mutants. A mutated Fc domain carries a higher risk of inducing an antibody response than the parental WT IgG. Moreover, some HexaBody mutants show a tendency to spontaneously assemble into hexamers (19). Such spontaneous aggregation could result in enhanced uptake of these complexes by the reticuloendothelial system, thereby reducing their *in vivo* half-life. Therefore, HexaBody mutants E430G and E345K that do not induce any hexamerization in solution and whose hexamerization is fully dependent on target binding were selected for clinical use (19).

Our study has potential clinical relevance for multiple myeloma patients: The observation that circulating myeloma cells in patients that develop resistance to daratumumab express increased levels of complement inactivating cell surface proteins (CD55, CD59), suggests that CDC is an important tumor cytotoxic mechanism *in vivo* (44). Indeed, the finding that daratumumab displays higher CDC-inducing potency than other CD38-specific moAbs accelerated its path to clinical use (9, 18). Here, we demonstrate that the CDC-potency of daratumumab can be enhanced by complementation with a CD38-specific hcAb, provided that the latter recognizes a distinct, non-overlapping epitope of CD38. Future studies are needed to assess whether this enhancing effect by a CD38-specific hcAb also renders myeloma cells of patients that have become refractory to daratumumab susceptible to CDC. Moreover, we demonstrate that CD38-specific biparatopic hcAbs recognizing two distinct epitopes of CD38 display more potent CDC than daratumumab.

A potential limitation for biparatopic hcAbs with increased complement activation potential is the risk for killing CD38-expressing normal cells and for generating off-target cytotoxicity. CD38 is highly expressed by multiple myeloma plasma cells and a small subpopulation of regulatory T cells (Tregs) (45). CD38 is also found on natural killer (NK) cells, monocytes, B cells, and T cells of healthy donors (45). Treatment with daratumumab results in a preferential depletion of CD38+ immunosuppressive cells, with a concomitant increase in functional T-helper and cytotoxic T cells. It will be important to determine whether biparatopic hcAbs can mediate similar beneficial effects by preferentially killing CD38+ immunosuppressive cells.

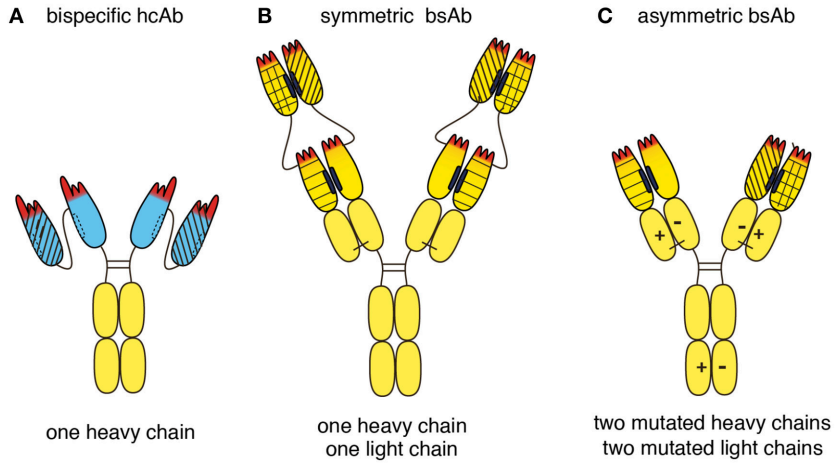

FIGURE 8 | Schematic diagram illustrating the structural advantages of a biparatopic hcAb over a bispecific conventional moAb. **(A)** Llama VHH domains are depicted in blue, human Ig domains are depicted in yellow. Biparatopic nanobody-based hcAbs are composed of two identical heavy chains, each carrying two soluble VHH domains connected by a peptide linker. Biparatopic hcAbs therefore do not have any chain-pairing problem. **(B)** Symmetric bsAbs such as dual-variable-domain bsAbs are composed of two identical heavy chains and two identical light chains. Each of these chains is N-terminally extended by an additional V domain. The structural advantage of a biparatopic heavy chain antibody over such a dual-variable-domain bsAb lies in the inherent high stability and solubility of each VHH vs. the greater instability of each VH-VL pair. **(C)** Asymmetric bsAbs with the regular IgG architecture typically are composed of two distinct heavy chains and two distinct light chains. Mutations need to be introduced into both heavy chains to avoid unwanted homomeric pairing of heavy chains. Such mutations facilitate heteromeric pairing of heavy chains, e.g., by electrostatic pairing as indicated here ("+" and "–"), knob in hole, or CH3-repulsion. Similarly, unwanted pairing of light chains to the 'wrong' heavy chain can be minimized by introducing mutations into both light chains, e.g., electrostatic pairing as indicated here. Other strategies to minimize mispairing of light and heavy chains include swapping of CH1 and CL domains in one of the antibodies, using a fixed light chain, or by separate expression of the two antibodies (each containing a different mutant H chain), followed by mixing of the purified antibodies under mild reducing conditions that preferentially reduce the disulfide bridges in the hinge region rather than the disulfide bond linking the CL and CH1 domains. Under carefully controlled conditions, properly assembled bsAbs can be produced at high yield, yet additional purification steps are usually needed to remove contaminating mispaired variants. Biparatopic hcAbs carry four antigen binding modules and thus are tetravalent, whereas conventional bsAbs carry only two antigen binding modules and thus are bivalent.

In conclusion, our results underscore the advantages of using a heavy chain format with soluble nanobodies rather than pairs of VH and VL domains in antibody engineering. Moreover, our study highlights two new strategies for improving the benchmark antibody therapy of multiple myeloma: (1) complementing daratumumab with monospecific hcAbs, and (2) using biparatopic hcAbs as alternative therapeutics, e.g., in combination with other anti-myeloma drugs.

METHODS

Cells

Human cell lines were obtained from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (LP-1, ACC 41; CA-46, ACC 73). The CD38 gene was inactivated in LP-1 cells using CRISPR/Cas9 technology using a commercial double nickase plasmid (Santa Cruz sc-401117-NIC). CD38-negative cells were sorted on a FACS AriaII (Becton Dickinson).

Construction of Monospecific and Biparatopic hcAbs

The coding region of selected nanobodies (WO 2017/081211) was subcloned using NcoI/PciI and NotI upstream of the coding region either for the hinge, CH2 and CH3 domains of human IgG1 (UniProt P01857) or hexahistidine and c-myc tags in pCSE2.5 vectors (46) (kindly provided by Thomas Schirrmann, Braunschweig). The amino acid sequence of the VHH-IgG1 junction is: VTVSSEPKTPKPQP-AAA-SDKTHTCPPCPAP where AAA is encoded by the NotI site. Biparatopic heavy chain antibodies were constructed by gene synthesis, fusing nanobodies WF211 and WF121 via a $G4S_2$ linker, MU1067 and JK36 via a $G4S_3$ linker and MU1068 and MU1067 via a $G4S_7$ linker. Each nanobody dimer was flanked by NcoI and NotI and cloned as described above into the hIgG1 pCSE2.5 vector. Similarly, daratumumab scFv was generated by gene synthesis by fusing the VH domain and the VL domain (WO 2011/154453) via a $G4S_3$ linker, flanked by NcoI and NotI sites and cloning into the hIgG1 pCSE2.5 vector.

Construction of E345R HexaBody hcAbs

The E345R mutation was introduced into hcAbs by PCR-mediated mutagenesis. The mutation was verified by sequencing. In order to ensure that no other mutations were introduced into the vector, the human IgG Fc fragment encoding the E345R mutation was recloned into the pCSE2.5 vector using flanking restriction sites (NotI and XbaI).

Production and Purification of hcAbs

HcAbs were expressed in transiently transfected HEK-6E cells cultivated in serum-free medium (26, 47). Six days post

transfection, supernatants were harvested and cleared by centrifugation. Recombinant proteins in cell supernatants were quantified by SDS-PAGE and Coomassie staining relative to marker proteins of known quantities: 10 µl samples of the supernatant were size fractionated side by side with standard proteins: m/M (amount loaded per lane in µg) bovine serum albumin (1/4), IgH (0.5/2), IgL (0.25/1), hen egg lysozyme (0.1/0.4). Yields of recombinant hcAbs typically ranged from 0.5–3 µg/10 µl. HcAbs were purified by affinity chromatography using protein G sepharose (GE healthcare).

Complement-Dependent Cytotoxicity Assays

Cells were incubated for 10–20 min at 4°C with hcAbs or moAbs before addition of human serum (10–15% v/v) and were then further incubated for 30–90 min at 37°C. Cells were washed and resuspended in PBS/0.2% BSA/propidium iodide before FACS analysis.

C1q Binding Assay

Cells were preincubated for 10–20 min at 4°C with hcAbs or moAbs before addition of human serum (10–15% v/v) and further incubation for 30 min at 4°C. Cells were washed and bound C1q was detected with FITC-conjugated rabbit anti-C1q (DAKO F0254) before FACS analysis.

AUTHOR CONTRIBUTIONS

PB and FK-N conceived the project. FK-N wrote the manuscript. All authors established experimental procedures, performed experiments, reviewed, and approved the manuscript.

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REFERENCES

1. Afifi S, Michael A, Lesokhin A. Immunotherapy: A new approach to treating multiple myeloma with Daratumumab and Elotuzumab. *Ann Pharmacother.* (2016) 50:555–68. doi: 10.1177/1060028016642786
2. Rajan AM, Kumar S. New investigational drugs with single-agent activity in multiple myeloma. *Blood Cancer J.* (2016) 6:e451. doi: 10.1038/bcj.2016.53
3. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood* (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
4. van de Donk NW, Janmaat ML, Mutis T, Lammerts van Bueren JJ, Ahmadi T, Sasser AK, et al. Monoclonal antibodies targeting CD38 in hematological malignancies and beyond. *Immunol Rev.* (2016) 270, 95–112. doi: 10.1111/imr.12389
5. Horenstein AL, Chillemi A, Quarona V, Zito A, Roato I, Morandi F, et al. NAD(+)-Metabolizing Ectoenzymes in remodeling tumor-host interactions: the human myeloma model. *Cells* (2015) 4:520–37. doi: 10.3390/cells4030520
6. Horenstein AL, Chillemi A, Zaccarello G, Bruzzone S, Quarona V, Zito A, et al. A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology* (2013) 2:e26246. doi: 10.4161/onci.26246
7. Morandi F, Morandi B, Horenstein AL, Chillemi A, Quarona V, Zaccarello G, et al. A non-canonical adenosinergic pathway led by CD38 in human melanoma cells induces suppression of T cell proliferation. *Oncotarget* (2015) 6:25602–18. doi: 10.18632/oncotarget.4693
8. Chini EN, Chini CCS, Espindola Netto JM, de Oliveira GC, van Schooten W. The pharmacology of CD38/NADase: an emerging target in cancer and diseases of aging. *Trends Pharmacol Sci.* (2018) 39:424–36. doi: 10.1016/j.tips.2018.02.001
9. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol.* (2011) 186:1840–8. doi: 10.4049/jimmunol.1003032
10. Lokhorst HM, Plesner T, Laubach JP, Nahi H, Gimsing P, Hansson M, et al. Targeting CD38 with Daratumumab monotherapy in multiple myeloma. *N Engl J Med.* (2015) 373:1207–19. doi: 10.1056/NEJMoa1506348
11. Melis JP, Strumane K, Ruuls SR, Beurskens FJ, Schuurman J, Parren PW. Complement in therapy and disease: regulating the complement system with antibody-based therapeutics. *Mol Immunol.* (2015) 67(2 Pt. A):117–130. doi: 10.1016/j.molimm.2015.01.028
12. Walport MJ. Complement. First of two parts. *N Engl J Med.* (2001) 344:1058–66. doi: 10.1056/NEJM200104053441406
13. Walport MJ. Complement. Second of two parts. *N Engl J Med.* (2001) 344:1140–4. doi: 10.1056/NEJM200104123441506
14. Borsos T, Rapp HJ. Complement fixation on cell surfaces by 19S and 7S antibodies. *Science* (1965) 150:505–6.
15. Dechant M, Weisner W, Berger S, Peipp M, Beyer T, Schneider-Merck T, et al. Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies. *Cancer Res.* (2008) 68:4998–5003. doi: 10.1158/0008-5472.CAN-07-6226
16. Klausz K, Berger S, Lammerts van Bueren JJ, Derer S, Lohse S, Dechant M, et al. Complement-mediated tumor-specific cell lysis by antibody combinations targeting epidermal growth factor receptor (EGFR) and its variant III (EGFRvIII). *Cancer Sci.* (2011) 102:1761–8. doi: 10.1111/j.1349-7006.2011.02019.x
17. Macor P, Mezzanzanica D, Cossetti C, Alberti P, Figini M, Canevari S, et al. Complement activated by chimeric anti-folate receptor antibodies is an efficient effector system to control ovarian carcinoma. *Cancer Res.* (2006) 66:3876–83. doi: 10.1158/0008-5472.CAN-05-3434
18. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science* (2014) 343:1260–3. doi: 10.1126/science.1248943
19. de Jong RN, Beurskens FJ, Verploegen S, Strumane K, van Kampen MD, Voorhorst M, et al. A novel platform for the potentiation of therapeutic antibodies based on antigen-dependent formation of IgG Hexamers at the cell surface. *PLoS Biol.* (2016) 14:e1002344. doi: 10.1371/journal.pbio.1002344
20. Revets H, De Baetselier P, Muyldermans S. Nanobodies as novel agents for cancer therapy. *Expert Opin Biol Ther.* (2005) 5:111–24. doi: 10.1517/14712598.5.1.111
21. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol.* (2009) 198:157–74. doi: 10.1007/s00430-009-0116-7
22. Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem* (2013) 82:775–97. doi: 10.1146/annurev-biochem-063011-092449
23. Baral TN, MacKenzie R, Arbabi Ghahroudi M. Single-domain antibodies and their utility. *Curr Protoc Immunol.* (2013) 103:1–57. doi: 10.1002/0471142735.im0217s103
24. Ingram JR, Schmidt FI, Ploegh HL. Exploiting nanobodies' singular traits. *Annu Rev Immunol.* (2018) 36:695–715. doi: 10.1146/annurev-immunol-042617-053327
25. Roovers RC, Vosjan MJ, Laeremans T, el Khoulati R, de Bruin RC, Ferguson KM, et al. A biparatopic anti-EGFR nanobody efficiently inhibits solid tumour growth. *Int J Cancer* (2011) 129:2013–24. doi: 10.1002/ijc.26145

26. Danquah W, Meyer-Schwesinger C, Rissiek B, Pinto C, Serracant-Prat A, Amadi M, et al. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. *Sci Transl Med.* (2016) 8:366ra162. doi: 10.1126/scitranslmed.aaf8463
27. Tijink BM, Laeremans T, Budde M, Stigter-van Walsum M, Dreier T, de Haard HJ, et al. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. *Mol Cancer Ther.* (2008) 7:2288–97. doi: 10.1158/1535-7163.MCT-07-2384
28. Farrington GK, Caram-Salas N, Haqqani AS, Brunette E, Eldredge J, Pepinsky B, et al. A novel platform for engineering blood-brain barrier-crossing bispecific biologics. *FASEB J.* (2014) 28:4764–78. doi: 10.1096/fj.14-253369
29. Li T, Qi S, Unger M, Hou YN, Deng QW, Liu J, et al. Immuno-targeting the multifunctional CD38 using nanobody. *Sci Rep.* (2016) 6:27055. doi: 10.1038/srep27055
30. Herce HD, Schumacher D, Schneider AFL, Ludwig AK, Mann FA, Fillies M, et al. Cell-permeable nanobodies for targeted immunolabelling and antigen manipulation in living cells. *Nat Chem.* (2017) 9:762–71. doi: 10.1038/nchem.2811
31. Qasemi M, Behdani M, Shokrgozar MA, Molla-Kazemiha V, Mohseni-Kuchesfahani H, Habibi-Anbouhi M. Construction and expression of an anti-VEGFR2 Nanobody-Fc fusionbody in NS0 host cell. *Protein Expr Purif.* (2016) 123:19–25. doi: 10.1016/j.pep.2016.03.004
32. Bannas P, Hambach J, Koch-Nolte F. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. *Front Immunol.* (2017) 8:1603. doi: 10.3389/fimmu.201701603
33. Ingram JR, Blomberg OS, Rashidian M, Ali L, Garforth S, Fedorov E, et al. Anti-CTLA-4 therapy requires an Fc domain for efficacy. *Proc Natl Acad Sci USA.* (2018) 115:3912–7. doi: 10.1073/pnas.1801524115
34. Peyvandi F, Scully M, Kremer Hovinga JA, Cataland S, Knobl P, Wu H, et al. Caplacizumab for acquired Thrombotic Thrombocytopenic Purpura. *N Engl J Med.* (2016) 374:511–22. doi: 10.1056/NEJMoa1505533
35. Peyvandi F, Scully M, Kremer Hovinga JA, Knobl P, Cataland S, De Beuf K, et al. Caplacizumab reduces the frequency of major thromboembolic events, exacerbations and death in patients with acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost.* (2017) 15:1448–52. doi: 10.1111/jth.13716
36. Van Heeke G, Allosery K, De Brabandere V, De Smedt T, Detalle L, de Fougerolles A. Nanobodies(R) as inhaled biotherapeutics for lung diseases. *Pharmacol Ther.* (2017) 169:47–56. doi: 10.1016/j.pharmthera.2016.06.012
37. European Medicines Agency. *Cablivi (caplacizumab). An Overview of Cablivi and Why It Is Authorized in the EU. EMA/462233/2018* (2018).
38. Fumey W, Koenigsdorf J, Kunick V, Menzel S, Schutze K, Unger M, et al. Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of CD38(+) tumors in mouse models in vivo. *Sci Rep.* (2017) 7:14289. doi: 10.1038/s41598-017-14112-6
39. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. *Nature* (1993) 363:446–8. doi: 10.1038/363446a0
40. Spiess C, Zhai Q, Carter PJ. Alternative molecular formats and therapeutic applications for bispecific antibodies. *Mol Immunol.* (2015) 67(2 Pt. A):95–106. doi: 10.1016/j.molimm.2015.01.003
41. Labrijn AF, Meesters JI, de Goeij BE, van den Bremer ET, Neijssen J, van Kampen MD, et al. Efficient generation of stable bispecific IgG1 by controlled Fab-arm exchange. *Proc Natl Acad Sci USA.* (2013) 110:5145–50. doi: 10.1073/pnas.1220145110
42. Lewis SM, Wu X, Pustilnik A, Sereno A, Huang F, Rick HL, et al. Generation of bispecific IgG antibodies by structure-based design of an orthogonal Fab interface. *Nat Biotechnol.* (2014) 32:191–8. doi: 10.1038/nbt.2797
43. Liu Z, Leng EC, Gunasekaran K, Pentony M, Shen M, Howard M, et al. A novel antibody engineering strategy for making monovalent bispecific heterodimeric IgG antibodies by electrostatic steering mechanism. *J Biol Chem.* (2015) 290:7535–62. doi: 10.1074/jbc.M114.620260
44. Nijhof IS, Casneuf T, van Velzen J, van Kessel B, Axel AE, Syed K, et al. CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma. *Blood* (2016) 128:959–70. doi: 10.1182/blood-2016-03-703439
45. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* (2016) 128:384–94. doi: 10.1182/blood-2015-12-687749
46. Jager V, Bussow K, Wagner A, Weber S, Hust M, Frenzel A, et al. High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. *BMC Biotechnol.* (2013) 13:52. doi: 10.1186/1472-6750-13-52
47. Zhang J, Liu X, Bell A, To R, Baral TN, Azizi A, et al. Transient expression and purification of chimeric heavy chain antibodies. *Protein Expr Purif.* (2009) 65:77–82. doi: 10.1016/j.pep.2008.10.011

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Promises and Pitfalls in the Use of PD-1/PD-L1 Inhibitors in Multiple Myeloma

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In the biology of multiple myeloma (MM), immune dysregulation has emerged as a critical component for novel therapeutic strategies. This dysfunction is due to a reduced antigen presentation, a reduced effector cell ability and a loss of reactive T cells against myeloma, together with a bone marrow microenvironment that favors immune escape. The Programmed Death-1 (PD-1) pathway is associated with the regulation of T cell activation and with the apoptotic pathways of effector memory T cells. Specifically, the binding with PD-1 ligand (PD-L1) on the surface of tumor plasma cells down-regulates T cell-proliferation, thus contributing to the immune escape of tumor cells. In relapsed and/or refractory MM (RRMM) patients, PD-1/PD-L1 blockade was analyzed by using nivolumab, pembrolizumab, and durvalumab. Outcomes with single agents were unsatisfactory, whereas combination strategies with backbone immunomodulatory drugs (IMiDs) suggested a synergistic action in such a complex immunological landscape, even in patients previously refractory to these drugs. Nevertheless, these combinations were also associated with an increased incidence of adverse events. This review aims to analyze the available preclinical and clinical data on the role of PD-1/PD-L1 inhibitors in MM therapy, focusing on available preliminary efficacy and safety data and offering insights for future investigation.

Keywords: multiple myeloma, PD-1, PD-L1, immune dysregulation, T cells

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INTRODUCTION

In the pathogenesis of multiple myeloma (MM), the immortalization of a MM propagating cell is induced by an initiating "hit." The subsequent accumulation of genetic "hits" in a multistep process leads to the typical MM characteristics: the proliferation of monoclonal plasma cells and the consequent overproduction of immunoglobulin or light chains that can cause end-organ damage and specific symptoms (i.e., bone disease, anemia, renal failure, and hypercalcemia) (1, 2). Moreover, an important role is also played by the interactions between the microenvironment—which includes the immune system where the tumor grows—and the MM cells (3). In general, the immune system can potentially recognize a tumor and reject it. Natural killer (NK) cells may detect tumor cells by their typical, although aspecific, tumor characteristics (such as upregulated cell stress ligands and/or downregulated major histocompatibility complex [MHC]) and kill them. Then, dendritic cells (DCs) and macrophages can internalize and process cell products and present derived molecules to B and T cells (4–6). T- and B-cell activation causes the proliferation of cell clones and the production of tumor-specific antibodies, with

the final goals of eliminating the remaining tumor cells and generating immune memory to prevent tumor recurrence (7). Through this process, a strongly immunogenic tumor in a highly immunocompetent subject could potentially eradicate the tumor. In the cases of less immunogenic tumors and/or less immunocompetent individuals, some cancer cells can survive despite remaining under immunosurveillance. Nevertheless, at a certain point, changes in the tumor expression of antigens can allow the tumor to avoid immunosurveillance. Similarly, a weakened immune system can be less efficient in maintaining the tumor under control and, as a consequence, it favors the tumor escape (8). A progressive immune dysregulation strongly characterizes MM, whose plasma cells can easily escape immunosurveillance through many possible mechanisms, such as the deficient B-cell immunity, the expansion of regulatory T cells (Tregs), the DC dysfunction, and the reduction of T-cell cytotoxicity.

The potential role of immunosurveillance on tumor control is the rationale for the use of the immuno-oncology approach in cancer treatment, including MM. Immune checkpoint interactions have emerged as a major mechanism for immunosurveillance and evasion. Immune checkpoint blockade enhances antitumor immunity by blocking cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) or PD-1 ligand (PD-L1). Monoclonal antibodies (mAbs) targeting checkpoint pathway on immune and tumor cells (known as checkpoint inhibitors) proved to be effective in several tumors. Ipilimumab, pembrolizumab, nivolumab, atezolizumab, durvalumab, and avelumab are currently approved by the Food & Drug Administration (FDA) (9). Check-point inhibitors also showed specific side effects, defined as immune-related AEs (irAEs): in fact, they can cause inflammation due to an increased activity of the immune system (9) (see section Immune-Related AEs). Results on solid tumors and on other hematologic cancers provided the basis to evaluate their effectiveness and safety in MM.

RATIONALE FOR CHECKPOINT INHIBITION IN MULTIPLE MYELOMA

PD-1/PD-L1 Pathway in Normal Cells and Myeloma Cells

The immune dysfunction is critical for the genesis of MM and various cells are involved. NK cells show quantitative and functional changes, with a decrease during the advanced disease phase. In this sense, NK cell-mediated cytotoxicity (particularly when enhanced) is a promising target for immunotherapies, mainly for immunomodulatory drugs (IMiDs) and novel mAbs. Also T-cell immunity and the antigen-presenting ability of DCs present some issues: there is a selective loss of myeloma-specific lymphocytes (NKT-cells, γδ T cells) and a coexistent rise in suppressor cells, including regulatory T cells and MDSCs, within the bone marrow microenvironment and in the peripheral blood (10, 11).

In the presence of malignant plasma cells, immune tolerance is fostered by immune checkpoint pathways, which usually help maintain the immune equilibrium. The PD-1 is part of the CD28 receptor family, and is expressed on activated B cells, monocytes, T cells, and NK T cells (12). PD-L1 and PD-L2 are expressed on antigen-presenting cells, including macrophages and DCs (13) (**Figure 1**). PD-L1 is also expressed on non-hematopoietic cells (solid-tumor, endothelial, and epithelial cells) and consequently helps in protecting tissues against immune-mediated injury (14, 15).

PD-1-PD-L1/PD-L2 ligation inhibits Th1 cytokine secretion, T cell proliferation (thus promoting T-cell apoptosis), and cytotoxic T lymphocytes (CTL)-mediated killing. This pathway is fundamental in the physiologic setting, preserving the immunologic balance after the initial T-cell response, which prevents collateral tissue damage, overactivation, and the irregular increase in autoreactive T cells (16). In presence of malignancy, the upregulation of the PD-1/PD-L1 pathway prevents tumor-reactive T cells to be activated and functioning, thus fostering immune escape and tumor growth (17, 18).

For these reasons, the potential benefit of antibody blockade of the PD-1/PD-L1 pathway has been evaluated in patients affected by solid tumors such as renal cancer, melanoma, non-small cell lung cancer, and hematologic malignancies (e.g., Hodgkin Lymphoma and MM).

Preclinical studies showed a higher expression of PD-L1 on MM patients' plasma cells rather than on plasma cells isolated from patients with monoclonal gammopathy of undetermined significance (MGUS) or on normal plasma cells (19). Rosenblatt et al. detected the PD-1 expression on circulating T cells in progressive MM patients, whereas the PD-1 expression on T-cells was reduced in patients with response after high-dose chemotherapy. They also examined PD-1 inhibition on *ex vivo* T-cell response to DC/tumor fusions ("a cancer vaccine in which autologous tumor was fused with dendritic cells, resulting in the presentation of tumor antigens in the context of DC-mediated costimulation"). By using an anti-PD-1 antibody, they promoted the polarization of T cells toward an activated phenotype that expressed Th1 compared with Th2 cytokines and the reduction and the killing of regulatory T cells (16, 20). As a consequence, the PD-1/T cells binding causes anergy (mainly through a blockade of B7-H1 [B7 homolog 1 protein]-PD-1 interaction) and apoptosis (through the inhibition of the anti-apoptotic gene bcl-xL and the activation of the proapoptotic gene Bim) (21, 22).

Moreover, PD-L1 is also expressed on the bone marrow microenvironment accessory cells, such as plasmacytoid DCs and MDSCs. In *in vitro* experiments, PD-1 inhibition restored the ability of plasmacytoid DCs to generate CTL killing of myeloma targets (23–25). PD-L1 on MDSCs may synergize with tumor cells to induce tolerance; therefore, its blockade may contribute to the inhibition of MM cell growth. Finally, PD-1 expression is increased on MM patient-derived NK cells, with an associated loss of effector cell function, which can be subsequently restored by the PD-1 blockade (26).

Abbreviations: MM, multiple myeloma; NK, natural killer; DC, dendritic cells; MDSC, myeloid-derived suppressor cells, TCR, T cell receptor; MHC-Ag, major histocompatibility complex-antigen; PD-1, programmed cell death 1; PDL-1, programmed cell death ligand 1.

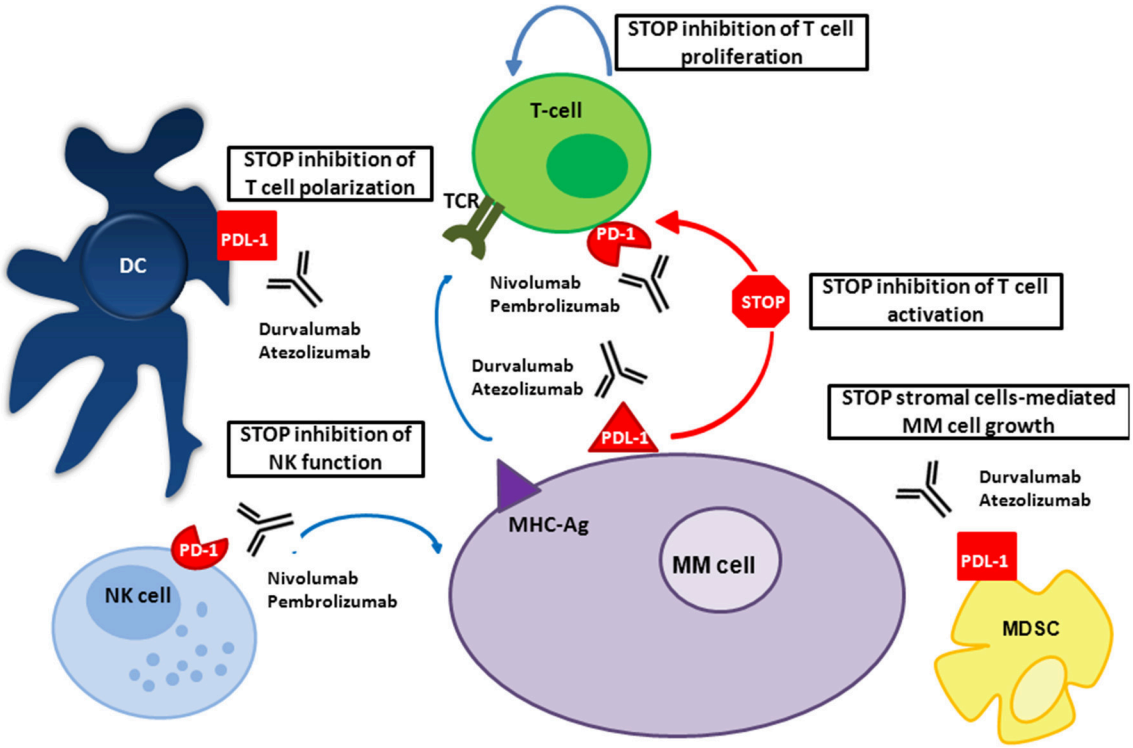

FIGURE 1 | Mechanism of action of PD-1/PD-L1 inhibitors in MM. In patients with MM, PD-L1 is expressed on MM and bone marrow microenvironment accessory cells; PD-1 on NK cells and T cells. PD-1/PD-L1 signaling in patients with MM inhibits the function of these immune cells, allowing MM to escape death. Both anti-PD-1 and anti-PD-L1 mAbs prevent this interaction.

PD-1/PD-L1 Inhibitors in Multiple Myeloma: Preclinical Data and Synergism With Other Compounds and Strategies

PD-1 blockade alone is clinically most effective in tumors (e.g., melanoma and lymphoproliferative diseases) that show high levels of infiltrating effector cells in the tumor background and a high mutational burden, which can result in the production of neo-antigens and non-self epitopes hit by high-affinity T cells. Conversely, MM presents a limited neo-antigen profile, with a less intense infiltration of effector cells and a lower mutational activity than in solid tumors (27). In fact, MM pre-clinical studies showed that checkpoint blockade efficacy could be improved if associated with treatments able to intensify the activity of myeloma-reactive T cells, such as transplantation, cellular therapies, anti-CD38 antibodies, chimeric antigen receptor (CAR) T cells, and IMiDs.

IMiDs enhance T-cell responsiveness to antigen-presenting cells (APC), polarize T cells toward a Th1 phenotype, inhibit MDSC and Tregs, and downregulate PD-L1 expression on tumor cells (28–30). In particular, lenalidomide promotes apoptosis in cancer cells and stimulates NK and T cells, favoring NK-mediated tumor detection and killing (31).

In a preclinical study, NK cells and T cells were sorted by fluorescence-activated cell sorting (FACS) and then separately co-cultured with $CD138^+$ MM cells from relapsed and/or refractory MM (RRMM) patients, plus anti-PD-1, anti-PD-L1, together or alone, and in association with lenalidomide. As a consequence, Görgün et al. demonstrated that the anti-myeloma toxicity deriving from the effector cells is enhanced by the PD-1/PD-L1 inhibition. Compared to T cells, NK cells showed a higher cytotoxicity. Moreover, the cytotoxicity induced by lenalidomide was further increased by checkpoint blockade (30). In another study, isolated $CD4^+$/$CD8^+$ T cells and NK cells from patients with MM were co-cultivated with autologous plasmacytoid DCs, together with the anti-PD-L1. In this way, Ray et al. proved that the use of anti-PD-L1 activated more deeply $CD8^+$ T- and NK-cell cytotoxicity rather than $CD4^+$ T-cell mediated killing (24).

Promising clinical results observed with IMiDs and anti-PD-1 combinations encouraged subsequent studies with agents that induce immune activation in the tumor microenvironment while stimulating myeloma cell killing. The anti-CD38 daratumumab kills malignant PCs through traditional antibody-dependent cellular cytotoxic mechanisms that are potentially able to control myeloma disease. In responding patients, daratumumab depletes subpopulations of Tregs and MDSCs in the myeloma microenvironment, stimulates T-cell expansion and increases T-cell clonality (32). These findings constituted the rationale for daratumumab associated with PD-1/PD-L1 blockade with or without IMiDs (NCT01592370, NCT03000452, and NCT02431208).

The anti-SLAMF7 monoclonal antibody elotuzumab has a dual mechanism of action that directly activates NK cells and causes the induction of NK cell-mediated antibody-dependent cellular cytotoxicity. A study on a mouse tumor model showed that the efficacy of elotuzumab was significantly higher when coadministered with anti-PD-1 antibody, thus promoting tumor-infiltrating NK and $CD8^+$ T-cell activation, as well as augmented intratumoral cytokine and chemokine release. These data provided the rationale for the evaluation of elotuzumab/anti-PD-1 combination in MM patients (33).

It has been shown that cytotoxic therapy depletes suppressor populations and favors the reactivation of myeloma immunity. In a murine model, PD-L1 inhibition was given after stem-cell transplantation and cell vaccination administration, improving the survival of myeloma-bearing mouse models from 0 to 40% (34). One study showed that lymphopoietic reconstitution after stem-cell transplantation resulted in the depletion of regulatory T cells and the concomitant expansion of some MM clones. The inhibition of PD-1 significantly enhanced the proliferation and cytokine production of $CD8^+CD28^{neg}PD\text{-}1^+$ T cells. Nivolumab treatment also increased the secretion of the cytokines IFNγ, IL2, and TNFα. These results suggested that checkpoint blockade can potentially improve or restore T-cell responses in this patient population (35). This provides the rationale to study this drug as maintenance in the post-transplant setting.

In the context of MM, the efficacy of PD-1/PD-L1 blockade may also be favored by the use of tumor vaccines, which can be administered for the expansion of MM-reactive T-cell clones and, as a consequence, for the activation with checkpoint blockade (20, 36).

Very recently, DC vaccination associated with PD-1 blockade and lenalidomide was investigated by Vo et al. in a myeloma-bearing mouse model. This combination inhibited myeloma tumor growth more effectively than other groups of agents, reducing immune suppressor cells (such as MDSCs, M2 macrophages, and Tregs), increasing immune effector cells, and enhancing the activity of NK cells and CTLs. This established a strong two-way anti-myeloma immunity through the inhibition of immunosuppressive cells and the activation of effector cells (37).

Interestingly, the combination of a PD-1 antibody with a CAR T cell showed an improved efficacy, even if the overexcitation of immune effectors could result in potential toxicity. In the study by Cherkassky et al. (38), the effector function of CD28 CAR T cells in a pleural mesothelioma-bearing orthotopic murine model was restored by the use of PD-1 antibody checkpoint blockade. These results allowed an improved understanding of the exhaustion of human CAR T-cell in solid tumors, suggesting that the effectiveness of CAR T-cell therapies may be improved by PD-1/PD-L1 blockade also in the context of hematological malignancies (38). Further studies are needed for the evaluation of the potential synergism of CAR-T therapies and anti-PD-1/PD-L1 checkpoint inhibitors in MM.

PD-1 blockade may also be effective when combined with radiotherapy, resulting in epitope spreading and increased antigen presentation by local APC (39). Temporal PD-L1 upregulation in the irradiated tumor suggested intrinsic mechanisms that inhibit immune responses after radiotherapy, and provided the rationale for blockade of PD-L1 combined with radiotherapy to overcome these mechanisms (40).

PD-1/PD-L1 MONOCLONAL ANTIBODIES: UPDATED CLINICAL RESULTS AND SAFETY CONSIDERATIONS

MAbs targeting both PD-1 (pembrolizumab and nivolumab) and PD-L1 (durvalumab) have been evaluated for MM treatment.

Nivolumab is a human IgG4 mAb that blocks the interaction with PD-L1 and PD-L2 by binding to the PD-1 receptor on activated immune cells (13). Nivolumab has a very high binding affinity to PD-1 with about 80% of saturation reached in <1 day following a single nivolumab infusion at 3 mg/kg; PD-1 occupancy is higher than 70% for almost 60 days, with detectable levels of PD-1 receptor occupancy for more than 3 months (41). Nivolumab clearance is not affected by renal or hepatic impairment (42).

Nivolumab as single agent did not show objective responses in a phase Ib trial enrolling 27 RRMM patients (43). The reasons for the lack of effectiveness in MM are unclear, but they may be related to the immunosuppressive nature of the microenvironment. To be effective, immune checkpoint therapy requires T cells to be able of being activated and, consequently, to have an exhausted phenotype instead of an anergic or senescent one (the key for therapeutic response is considered the reversal of exhausted T cells, rather than the genesis of new ones). In clinical studies on MM, clonal cytotoxic $CD8^+$ T cells are the only T cells that showed to have an impact on survival; however, they did not show the exhausted phenotype. Rather, their phenotype ($CD8^+TCRV\beta^+CD57^+CD28^-$) suggested the presence of terminally differentiated, antigen-specific, senescent cells that were no more able to proliferate after stimulation. Besides, in contrast with solid tumors and tumor-infiltrating lymphocytes, the low expression of PD-1 on clonal bone marrow cytotoxic T cells suggested that, in MM, the local immunosuppressive mechanisms involving PD-1/PD-L1 interactions are less active (44).

Combinations of nivolumab with pomalidomide-dexamethasone (Pd) and other mAbs, such as daratumumab (NCT01592370) and elotuzumab (NCT02726581), have been designed in more recent trials, but data are still not available.

Durvalumab is a human IgG1k antibody targeting PD-L1. Weight-based durvalumab dose (10 mg/kg every 2 weeks) and fixed durvalumab dose (1,500 mg every 4 weeks or 750 mg every 2 weeks) demonstrated similar PK features, with patient and disease characteristics that did not affect drug bioavailability (45). In the MM setting, no clinical data on durvalumab are available and phase I studies investigating durvalumab plus IMiDs are currently on clinical hold on the basis of the results of the KEYNOTE-183 and KEYNOTE-185 trials, which will be described below.

Pembrolizumab is an IgG4k humanized anti-PD-1 mAb. Neither pharmacokinetics nor renal/hepatic impairment are affected by age, thus dose adjustments are not needed (46).

In MM, no data are available on pembrolizumab as single agent. In a phase I study including RRMM patients, pembrolizumab (maximum tolerated dose: 200 mg every 21 days) associated with lenalidomide-dexamethasone (Rd) showed a partial response (PR) rate of 50%. Any-grade treatment-related AEs occurred in 48 (94%) patients, albeit grade ≥3 AEs were observed in 33 (65%) patients. Grade 3 irAEs included increase in transaminases (2%), and renal failure (2%).

Pembrolizumab (200 mg every 2 weeks) was also combined with Pd, showing a PR rate of 60% (47, 48). Thirty-five (73%) patients experienced any-grade treatment-related AEs, albeit ≥3 AEs were observed in 20 (42%) patients. Grade 3–4 irAEs included hypothyroidism (4%), adrenal insufficiency (2%), hepatitis (2%), and pneumonitis (2%).

Based on these studies, two randomized phase-III trials were designed. In the KEYNOTE-185 trial (NCT02579863), pembrolizumab-Rd vs. Rd alone was investigated in transplant-ineligible NDMM patients. On the 3rd of July, 2017, after that interim data had been presented to the Data Monitoring Committee (DMC), the FDA put a hold on the trial because of an increase in deaths in the pembrolizumab arm. Three hundred and one of the planned 640 patients were enrolled (median age 74 years). After a median follow-up of 6.4 vs. 6.9 months, there were 19 (13%) deaths in the pembrolizumab-Rd arm (6 from PD, 13 from AEs) vs. 9 (6%) patients in the Rd arm (1 from PD, 8 from AEs); 6 (4%) treatment-related deaths were observed; 4 (3%) were related to pembrolizumab (1 cardiac arrest, 1 pneumonia; 1 myocarditis, 1 cardiac failure). The other AEs that led to death were: cardiorespiratory arrest and pulmonary embolism (2 patients each), intestinal ischemia, large intestinal perforation, sudden death, suicide, and sepsis (1 patient each). In the Rd arm, the AEs that led to death were myocardial infarction and sudden death (2 patients each), acute cardiac failure, upper intestinal hemorrhage, respiratory failure (1 patient each). This translated into an increased risk of death with pembrolizumab (HR for OS: 2.06; 95% CI 0.93–4.55; $P = 0.97$). The rates of severe (grade 3–5) toxicities were 72% in the experimental arm vs. 50% in the control arm. The rates of serious AEs (SAEs) were 54 vs. 39%, respectively. The rates of discontinuation for AEs were 21 vs. 8%, respectively. AEs (all grades) with more than 5% of difference between arms included: constipation, pyrexia, vomiting, rash, hypothyroidism, oral candidiasis, hyperthyroidism, pruritus, pneumonia, and decrease appetite. In the pembrolizumab-Rd arm, irAEs reported in ≥2% of patients included: hypothyroidism (7%), hyperthyroidism (6%), colitis (2%), and skin reactions (13%). Median progression-free survival (PFS; HR 1.22; 95% CI 0.67–2.22, $P = 0.75$) was not reached in neither arm (49).

The second trial, KEYNOTE-183 (NCT02576977), evaluated pembrolizumab-Pd vs. Pd alone in RRMM patients who received ≥2 lines of treatment including an IMiD and a proteasome inhibitor (PI). Similarly to what happened with KEYNOTE-185, the FDA halted the trial on the 3rd of July, 2017 on the basis of interim data provided to the DMC. The study enrolled 249 of the planned 300 patients (median age: 65 vs. 67 years in pembrolizumab-PD vs. PD arms, respectively, median duration of therapy 4.4 cycles). After a median follow-up of 7.8 vs. 8.6 months, 29 (23%) vs. 21 (17%) patients died (16 from PD, 13 from AEs vs. 18 from PD, 3 from AEs). In the pembrolizumab-Pd arm, 4 (3%) treatment-related deaths occurred: 2 (1.5%) were related to pembrolizumab (1 myocarditis, 1 Steven-Johnson syndrome [SJS]); 1 patient died of neutropenic sepsis. The other AEs that led to death were sepsis (3 patients), pericardial hemorrhage, myocardial infarction, cardiac failure, and respiratory tract infection (1 patient each). In the Pd arm, the AEs that led to death were pneumonia and anemia (1 patient each). The median OS was not reached vs. 15.2 months (HR, 1.61, 95% CI, 0.91–2.85; $P = 0.95$) in the pembrolizumab-Pd vs. Pd arm. The rate of grade 3–4 AEs was 75% in the experimental arm vs. 63% in the control arm. The rates of SAEs were 63 vs. 46%, respectively, 20 vs. 8% discontinued for AEs. AEs (all grades) occurred in ≥20% of patients were: neutropenia, anemia, fatigue, constipation, pyrexia, pneumonia, and thrombocytopenia. No SAEs had more than 5% of difference between arms. In the pembrolizumab-Pd arm, irAEs included: skin reaction (5%), pneumonitis (4%), hyperthyroidism (3%), infusion reaction and myopathy (2% each), SJS, myocarditis, hepatitis, and iridocyclitis (1% each). Median PFS was similar between the two arms (5.6 vs. 8.4 months; HR 1.53, 95% CI 1.05–2.22; $P = 0.98$) (50).

Both trials determined that the risk-benefit profile of adding pembrolizumab to Rd or Pd was unfavorable.

Immune-Related AEs

The precise pathophysiology of irAEs is unknown, although likely related to the ability of immune checkpoints of preserving the normal immunologic homeostasis. These irAEs generally develop within a few weeks or months from the start of treatment, but they may occur at any time, including after stopping therapy (9). Although every organ system may be affected, irAEs usually involve skin, gastrointestinal tract, liver, and endocrine glands (51). Causes of severe irAEs remain unclear. One of the hypotheses was prompted by the association with underline germline genetic factors, since genes may influence the risk of specific autoimmune disorders. Another hypothesis was the association with the patient microbiota (9). It is important to promptly identify the occurrence of irAEs. Most of the times, the diagnosis and the treatment are based on patient-related symptoms, but sometimes blood test and/or imaging can be helpful (e.g., for hepatitis, colitis, or pneumonia). The optimal management is based on clinical experience, mainly in the treatment of solid tumors, since no prospective trials are available. The backbone of irAE therapy is immunosuppression with corticosteroids, with the addition of other immunosuppressive agents if there is no rapid improvement. Most of AEs promptly resolve, and available data do not show a negative impact of immunosuppression on the effectiveness of checkpoint inhibitors (52).

AEs provided evidence of activation of the patient immune system, but irAEs are not required for efficacy. Data from the literature on the correlation between the occurrence of irAEs and treatment efficacy are controversial (9). A *post-hoc* analysis of KEYNOTE-183 and KEYNOTE-185 was performed to examine the correlation between irAEs and efficacy. In the KEYNOTE-185 study, 68 vs. 44% of patients in the pembrolizumab-Rd vs.

Rd arms had an irAE (grade ≥3: 36 vs. 8%). Despite the overall higher rate of irAEs in the pembrolizumab-Rd arm, the overall response rate (ORR) was similar to the one in the Rd arm (64 vs. 62%). Nevertheless, in the pembrolizumab-Rd arm, ORR was higher in patients who experienced an irAE, as compared to patients who did not (73 vs. 45%). Similarly, in the Rd arm, the ORR was 73% in patients with an irAE vs. 53% in those without (53). These results might suggest that anti PD-1 are more effective in patients with activation of the immune system (evidence of which can be considered the development of irAEs). The KEYNOTE-183 study enrolled RRMM patients, who typically have a less effective immune-system as compared to NDMM patients. In this study, both the rate of irAEs and the differences between arms (58 vs. 45% of patients in the pembrolizumab-Pd vs. Pd arms; grade ≥3: 18 vs. 13%) were lower if compared to the NDMM setting (KEYNOTE-185). In the pembrolizumab-Pd arm, the ORR was 37% in patients who developed an irAE, not significantly different than the rate in those without an irAE (31%). In the Pd arm, a trend was noted for improved ORR (49%) in patients who experienced an irAE, as compared to 33% in those who did not. Altogether, these results suggest a higher risk of irAEs in NDMM patients, who probably have a more effective immune system as compared to heavily pretreated patients. A higher effectiveness of PD-1 inhibitors in patients with irAEs still needs to be demonstrated.

CONCLUSION

During the last decade, therapeutic strategies in MM patients have vastly improved thanks to the introduction of mAbs in association with backbone regimens. Based on pre-clinical data, the PD-1/PD-L1 axis may be a good target for mAbs, allowing immune cells to detect and kill neoplastic cells. However, the outcomes of checkpoint blockade alone in MM are inferior to the ones obtained in solid tumors, most likely due to the reduced immune function typical of the immune system of patients affected by MM. In phase II trials, potentially better results have been observed in association with IMiDs, probably due to the possible synergistic effect on the immune system. Nevertheless, despite these promising preliminary data, the toxicity reported in two randomized phase III trials with pembrolizumab associated with lenalidomide and pomalidomide led the FDA to halt trials exploring these combinations. The safety concerns are related to the mechanism of action of both drug classes, as they modify the behavior of immune cells. Moreover, patients treated with mAbs receive continuous therapy with steroids, either as part of treatment or to reduce irAEs. This leads to immunosuppression and may increase the risk of infections, which can ultimately cause drug discontinuation and reduce the efficacy of the treatment itself.

The effective possibility to modulate the immune system would be a great advancement. However, there are still many open issues. We need to ponder how to select and monitor patients for this typology of treatment, and to determine the best and safest drug combination as well as the most suitable time point of administration during the disease course. Moreover, the detection of biomarkers that can potentially predict responses and/or toxicities might help clinicians balance efficacy with safety.

To conclude, more mature safety data and a deeper analysis of the biologic mechanisms will be essential to understand if PD-1/PD-L1 inhibitors may be included in the armamentarium for the treatment of MM patients.

AUTHOR CONTRIBUTIONS

SO, RT, MD, MB, and FG conceptualization and methodology. SO, RT, MD, and FG writing—original draft. SO, MB, and FG supervision. SO, RT, MD, MB, and FG writing—review and editing.

REFERENCES

1. Morgan GJ, Kaiser MF. How to use new biology to guide therapy in multiple myeloma. *Hematol Am Soc Hematol Educ Progr.* (2012) 2012:342–9. doi: 10.1182/asheducation-2012.1.342
2. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
3. Zelle-Rieser C, Thangavadivel S, Biedermann R, Brunner A, Stoitzner P, Willenbacher E, et al. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. *J Hematol Oncol* (2016) 9:116. doi: 10.1186/s13045-016-0345-3
4. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer* (2004) 4:11–22. doi: 10.1038/nrc1252
5. Chang M-H. Cancer prevention by vaccination against hepatitis B. *Cancer Prevention II: Recent Results Cancer Res.* (2009) 181:85–94. doi: 10.1007/978-3-540-69297-3_10
6. Kenter GG, Welters MJP, Valentijn ARPM, Lowik MJG, Berends-van der Meer DMA, Vloon APG, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med.* (2009) 361:1838–47. doi: 10.1056/NEJMoa0810097
7. Finn OJ. Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. *Ann Oncol.* (2012) 23:viii6–viii9. doi: 10.1093/annonc/mds256
8. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* (2011) 331:1565–70. doi: 10.1126/science.1203486
9. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med.* (2018) 378:158–68. doi: 10.1056/NEJMra1703481
10. Luptakova K, Avigan D. Immune therapy in multiple myeloma. *Clin Adv Hematol Oncol.* (2015) 13:767–75. doi: 10.1158/1078-0432.CCR-16-0868
11. Dosani T, Carlsten M, Maric I, Landgren O. The cellular immune system in myelomagenesis: NK cells and T cells in the development of MM and their uses in immunotherapies. *Blood Cancer J.* (2015) 5:e306. doi: 10.1038/bcj.2015.32
12. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
13. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol.* (2001) 2:261–8. doi: 10.1038/85330
14. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med.* (2006) 203:883–95. doi: 10.1084/jem.20051776
15. Menke J, Lucas JA, Zeller GC, Keir ME, Huang XR, Tsuboi N, et al. Programmed death 1 ligand (PD-L) 1 and PD-L2 limit autoimmune

kidney disease: distinct roles. *J Immunol.* (2007) 179:7466–77. doi: 10.4049/JIMMUNOL.179.11.7466
16. Rosenblatt J, Avigan D. Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality? *Blood* (2017) 129:275–9. doi: 10.1182/blood-2016-08-731885
17. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci USA.* (2002) 99:12293–7. doi: 10.1073/pnas.192461099
18. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Erratum: tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* (2002) 8:793–800. doi: 10.1038/nm730
19. Yousef S, Marvin J, Steinbach M, Langemo A, Kovacsovics T, Binder M, et al. Immunomodulatory molecule PD-L1 is expressed on malignant plasma cells and myeloma-propagating pre-plasma cells in the bone marrow of multiple myeloma patients. *Blood Cancer J.* (2015) 5:e285. doi: 10.1038/bcj.2015.7
20. Rosenblatt J, Glotzbecker B, Mills H, Vasir B, Tzachanis D, Levine JD, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances *ex vivo* T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother.* (2011) 34:409–18. doi: 10.1097/CJI.0b013e31821ca6ce
21. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, et al. Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. *Blood* (2007) 110:180–5. doi: 10.1182/blood-2006-11-060087
22. Ostrand-Rosenberg S, Horn LA, Haile ST. The programmed death-1 immune-suppressive pathway: barrier to antitumor immunity. *J Immunol.* (2014) 193:3835–41. doi: 10.4049/jimmunol.1401572
23. Tamura H, Ishibashi M, Yamashita T, Tanosaki S, Okuyama N, Kondo A, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. *Leukemia* (2013) 27:464–72. doi: 10.1038/leu.2012.213
24. Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1–PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. *Leukemia* (2015) 29:1441–4. doi: 10.1038/leu.2015.11
25. Chen D, Tang P, Liu L, Wang F, Xing H, Sun L, et al. Bone marrow-derived mesenchymal stem cells promote cell proliferation of multiple myeloma through inhibiting T cell immune responses via PD-1/PD-L1 pathway. *Cell Cycle* (2018)17:858–67. doi: 10.1080/15384101.2018.1442624
26. Benson DM, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
27. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* (2015) 348:69–74. doi: 10.1126/science.aaa4971
28. Luptakova K, Rosenblatt J, Glotzbecker B, Mills H, Stroopinsky D, Kufe T, et al. Lenalidomide enhances anti-myeloma cellular immunity. *Cancer Immunol Immunother.* (2013) 62:39–49. doi: 10.1007/s00262-012-1308-3
29. Görgün GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood* (2013) 121:2975–87. doi: 10.1182/blood-2012-08-448548
30. Görgün G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. *Clin Cancer Res.* (2015) 21:4607–18. doi: 10.1158/1078-0432.CCR-15-0200
31. Giuliani M, Janji B, Berchem G. Activation of NK cells and disruption of PD-L1/PD-1 axis: two different ways for lenalidomide to block myeloma progression. *Oncotarget* (2017) 8:24031–44. doi: 10.18632/oncotarget.15234
32. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* (2016) 128:384–94. doi: 10.1182/blood-2015-12-687749
33. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K, et al. PD-1 blockade enhances elotuzumab efficacy in mouse tumor models. *Blood Adv.* (2017) 1:753–65. doi: 10.1182/bloodadvances.2017004382
34. Hallett WHD, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. *Biol Blood Marrow Transplant* (2011) 17:1133–45. doi: 10.1016/J.BBMT.2011.03.011
35. Chung DJ, Pronschinske KB, Shyer JA, Sharma S, Leung S, Curran SA, et al. T-cell exhaustion in multiple myeloma relapse after autotransplant: optimal timing of immunotherapy. *Cancer Immunol Res.* (2016) 4:61–71. doi: 10.1158/2326-6066.CIR-15-0055
36. Rosenblatt J, Avivi I, Vasir B, Uhl L, Munshi NC, Katz T, et al. Vaccination with dendritic cell/tumor fusions following autologous stem cell transplant induces immunologic and clinical responses in multiple myeloma patients. *Clin Cancer Res.* (2013) 19:3640–8. doi: 10.1158/1078-0432.CCR-13-0282
37. Vo M-C, Jung S-H, Chu T-H, Lee H-J, Lakshmi TJ, Park H-S, et al. Lenalidomide and programmed death-1 blockade synergistically enhances the effects of dendritic cell vaccination in a model of murine myeloma. *Front Immunol.* (2018) 9:1370. doi: 10.3389/fimmu.2018.01370
38. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, et al. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *J Clin Invest.* (2018) 126:3130–44. doi: 10.1172/JCI83092
39. Formenti SC, Demaria S. Combining radiotherapy and cancer immunotherapy: a paradigm shift. *JNCI J Natl Cancer Inst.* (2013) 105:256–65. doi: 10.1093/jnci/djs629
40. Dovedi SJ, Adlard AL, Lipowska-Bhalla G, McKenna C, Jones S, Cheadle EJ, et al. Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. *Cancer Res.* (2014) 74:5458–68. doi: 10.1158/0008-5472.CAN-14-1258
41. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol.* (2010) 28:3167–75. doi: 10.1200/JCO.2009.26.7609
42. OPDIVO® (nivolumab) *Injection, for Intravenous Use. Full Prescribing Information*. (2018). Available online at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/125554s058lbl.pdf (accessed November 14, 2018).
43. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib study. *J Clin Oncol.* (2016) 34:2698–704. doi: 10.1200/JCO.2015.65.9789
44. Suen H, Brown R, Yang S, Ho PJ, Gibson J, Joshua D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* (2015) 29:1621–2. doi: 10.1038/leu.2015.104
45. Baverel P, Dubois V, Jin C, Song X, Jin X, Mukhopadhyay P, et al. Population pharmacokinetics of durvalumab and fixed dosing regimens in patients with advanced solid tumors. *J Clin Oncol.* (2017) 35:2566. doi: 10.1200/JCO.2017.35.15_suppl.2566
46. Ahamadi M, Freshwater T, Prohn M, Li C, de Alwis D, de Greef R, et al. Model-based characterization of the pharmacokinetics of pembrolizumab: a humanized anti-PD-1 monoclonal antibody in advanced solid tumors. *CPT Pharmacometr Syst Pharmacol.* (2017) 6:49–57. doi: 10.1002/psp4.12139
47. Mateos M-V, Orlowski RZ, Siegel DSD, Reece DE, Moreau P, Ocio EM, et al. Pembrolizumab in combination with lenalidomide and low-dose dexamethasone for relapsed/refractory multiple myeloma (RRMM): final efficacy and safety analysis. *J Clin Oncol.* (2016) 34(15_suppl):Abstract #8010 [ASCO 2016 Annual Meeting]. doi: 10.1200/JCO.2016.34.15_suppl.8010
48. Badros A, Hyjek E, Ma N, Lesokhin A, Dogan A, Rapoport AP, et al. Pembrolizumab, pomalidomide, and low-dose dexamethasone for relapsed/refractory multiple myeloma. *Blood* (2017) 130:1189–97. doi: 10.1182/blood-2017-03-775122
49. Usmani SZ, Schjesvold F, Oriol Rocafiguera A, Karlin L, Rifkin RM, Yimer HA, et al. A phase 3 randomized study of pembrolizumab (pembro) plus lenalidomide (len) and low-dose dexamethasone (Rd) versus Rd for newly diagnosed and treatment-naive multiple myeloma (MM): KEYNOTE-185. *J Clin Oncol* (2018) 36(15_suppl):Abstract #8010 [ASCO 2018 Annual Meeting]. doi: 10.1200/JCO.2018.36.15_suppl.8010

50. Mateos M-V, Blacklock H, Schjesvold F, Rocafiguera AO, Simpson D, George A, et al. A phase 3 randomized study of pembrolizumab (Pembro) plus pomalidomide (Pom) and dexamethasone (Dex) for relapsed/refractory multiple myeloma (RRMM): KEYNOTE-183. *J Clin Oncol* (2018) 36(15_suppl): Abstract #8021 [ASCO 2018 Annual Meeting] doi: 10.1200/JCO.2018.36.15_suppl.8021
51. Weber JS, Hodi FS, Wolchok JD, Topalian SL, Schadendorf D, Larkin J, et al. Safety profile of nivolumab monotherapy: a pooled analysis of patients with advanced melanoma. *J Clin Oncol.* (2017) 35:785–92. doi: 10.1200/JCO.2015.66.1389
52. Friedman CF, Proverbs-Singh TA, Postow MA. Treatment of the immune-related adverse effects of immune checkpoint inhibitors. *JAMA Oncol.* (2016) 2:1346–53. doi: 10.1001/jamaoncol.2016.1051
53. Krauss AC, Mulkey F, Shen Y-L, Rosenberg A, Miller B, Carioti T, et al. FDA analysis of pembrolizumab trials in multiple myeloma: Immune related adverse events (irAEs) and response. *J Clin Oncol.* (2018) 36:Abstract #8008 [ASCO 2018 Annual Meeting]. doi: 10.1200/JCO.2018.36.15_suppl. 8008

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Enhancing the Activation and Releasing the Brakes: A Double Hit Strategy to Improve NK Cell Cytotoxicity Against Multiple Myeloma

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Natural killer (NK) cells are innate lymphocytes with a strong antitumor ability. In tumor patients, such as multiple myeloma (MM) patients, an elevated number of NK cells after stem cell transplantation (SCT) has been reported to be correlated with a higher overall survival rate. With the aim of improving NK cell use for adoptive cell therapy, we also addressed the cytotoxicity of patient-derived, cytokine-stimulated NK cells against MM cells at specific time points: at diagnosis and before and after autologous stem cell transplantation. Remarkably, after cytokine stimulation, the patients' NK cells did not significantly differ from those of healthy donors. In a small cohort of MM patients, we were able to isolate autologous tumor cells, and we could demonstrate that IL-2/15 stimulated autologous NK cells were able to significantly improve their killing capacity of autologous tumor cells. With the aim to further improve the NK cell killing capacity against MM cells, we investigated the potential use of NK specific check point inhibitors with focus on NKG2A because this inhibitory NK cell receptor was upregulated following *ex vivo* cytokine stimulation and MM cells showed HLA-E expression that could even be increased by exposure to IFN-γ. Importantly, blocking of NKG2A resulted in a significant increase in the NK cell-mediated lysis of different MM target cells. Finally, these results let suggest that combining cytokine induced NK cell activation and the specific check point inhibition of the NKG2A-mediated pathways can be an effective strategy to optimize NK cell therapeutic approaches for treatment of multiple myeloma.

Keywords: multiple myeloma, autologous stem cell transplantation, NK cells, adoptive cell therapy, NKG2A blocking, checkpoint inhibition

INTRODUCTION

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells (PCs). The hallmarks of the disease are an excess of monoclonal PCs in combination with monoclonal protein in the blood and/or urine (1). Standard therapy typically involves autologous stem cell transplantation (autoSCT) after induction, followed by high-dose chemotherapy treatment (2).

Importantly, given the risk of the treatment and its side effect, autoSCT is usually recommended only for youngest patients, accounting for approximately 30–40% of the patients with MM. However, the majority of patients will relapse within 2–3 years from the initiation of treatment, and the overall survival (OS) is still limited (3). Nevertheless, significant advances have been made in the treatment of MM by a combination of standard chemotherapy plus novel immunomodulatory drugs (IMiDs) or proteasome inhibitors, such as lenalidomide or bortezomib (4).

Another promising approach is the immunotherapeutic treatment with natural killer (NK) cells, as they present the benefit of enhanced graft-versus-tumor (GvT) effect with a low risk of graft-versus-host disease (GVHD) [for review, see (5–7)]. Furthermore, in MM patients, an elevated number of NK cells directly correlates with a lower tumor burden (8). NK cells, which were first described by Kiessling et al., are part of the innate immune system. NK cells are highly attractive because they are not antigen specific, like T and B cells. Their activity is regulated based on the diverse expression of activating and inhibiting receptors on their surface, by which they also achieve self-tolerance (9–11). After recognition of an infected or malignant cell, NK cells can kill their target by releasing cytoplasmic perforin or granzyme, leading to death receptor-mediated apoptosis or cytokine release (12). Human NK cells are characterized by the expression of CD56 and CD16 and are divided into two distinct subtypes. The more immature subset is characterized by high expression of CD56 and low or no expression of CD16; this subset is mainly situated in the lymph nodes and secondary lymphoid tissues. After maturation, the NK cell population comprises $CD56^{dim}$ $CD16^{bright}$ cells and is mainly found in the bone marrow, blood and spleen (13, 14). This subset has high cytotoxic capabilities and represents approximately 90% of all NK cells in the peripheral blood (15). Furthermore, NK cells have a regulatory effect on other immune cells by secreting soluble factors, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory proteins (MIPs) (16, 17).

For the application in immunotherapy, NK cells can be isolated from either patient or healthy donor derived PBMCs or differentiated from pluripotent stem cells. For most clinical applications, the NK cells are expanded by *ex vivo* culture. To further increase the effect of the therapy, it is important to achieve the optimal NK cell antitumor activity by using the right stimulation protocols. To date, the most common protocols stimulate NK cells with cytokines such as IL-2, IL-15 and IL-21 that induce high cytotoxicity or with IL-12, 15 and 18 to favor NK cell memory (18). Apart from stimulation with interleukins, NK cells can also be co-cultured with so-called accessory or feeder cells such as irradiated, allogeneic PBMCs or different cell lines such as K562 to further enhance NK cell expansion [for review see (18)].

A novel approach toward NK cell therapy is not only to activate them *ex vivo* but also to release the immune system from inhibition by specifically targeting immunologic checkpoints. Inhibitory receptors expressed on the NK cell surface are members of the KIR family and NKG2A. KIR receptors interact with MHC I molecules, and studies have shown that a transfer of KIR-ligand mismatched NK cells led to a lower relapse rate and a greater GvT effect due to their enhanced alloreactivity (19, 20). Moreover, several antibodies that specifically target KIR receptors have been tested or are currently in clinical trials to evaluate their efficacy against different malignancies (21).

However, due to different KIR receptor expression profiles in patients, a therapeutic targeting of selected KIR receptors could lead to a better response in some patients and a worse response in others. Moreover, the results of a clinical phase II trial testing a KIR2D specific antibody showed that treatment with the antibody led to a significant decrease in NK cell activity, directly correlating with loss of KIR2D surface expression (22). In this aspect, NKG2A could be a better therapeutic target, as it is broadly expressed on NK cells and binds specifically to HLA-E that is expressed on most malignant target cells (23). Additionally, overexpression of HLA-E in different tumors has been reported to correlate with shorter disease-free or overall survival (24, 25). In MM, HLA-E is highly expressed by primary cells, and it abolishes the overall response of $NKG2A^{+}$ NK cells (26). Furthermore, Sarkar and colleagues postulated that the most potent NK cell subset for clinical application would be NKG2A-negative and KIR-ligand mismatched. Interestingly, NKG2A is the first inhibitory receptor that is reconstituted after SCT (27, 28). This observation might also highlight the possible relevance of NKG2A as a therapeutic target in the context of allogeneic SCT.

Overall, these findings led us to further investigate the effects of cytokine-induced NK cell activation in combination with the specific checkpoint inhibition of the NKG2A-mediated pathway as a potential strategy to optimize NK cell therapeutic approaches against MM.

RESULTS

Cytokine Stimulation Significantly Increases the NK Killing Ability of Both Patient and Healthy Donor NK Cells Against MM Cell Lines

First, we aimed to test the "natural" ability of NK cells to kill different MM cell lines. Therefore, we isolated peripheral blood (PB) NK cells from healthy donors (HD) or untreated MM patients (Pt) at first diagnosis and co-cultured them with three different MM cell lines (U266, OPM-2, and LP-1) for 24 h (**Figure 1A**). The specific lysis of patient NK cells in resting conditions was approximately 10% against all three cell lines, with a trend toward reduced cytotoxic capacity compared to HD NK cells. To improve NK cell killing capacity against MM cells, we then stimulated both patient and donor NK cells for

Abbreviations: MM, multiple myeloma; CD, cluster of differentiation; CT, chemotherapy; DAMPs, damage associated molecular patterns; DCs, dendritic cells; DNA, deoxyribonucleic acid; GM-CSF, granulocyte macrophage colony-stimulating factor; HD, healthy donors; NK cells, natural killer cells; IFN, Interferon; Il, Interleukin; ns, not significant; Pt, patients; PB, peripheral blood; BM, bone marrow; TP0, treatment point 0; TP1, treatment point 1; TP2, treatment point 2; FACS, fluorescence activated cell sorting; IL15RA, IL-15 receptor alpha.

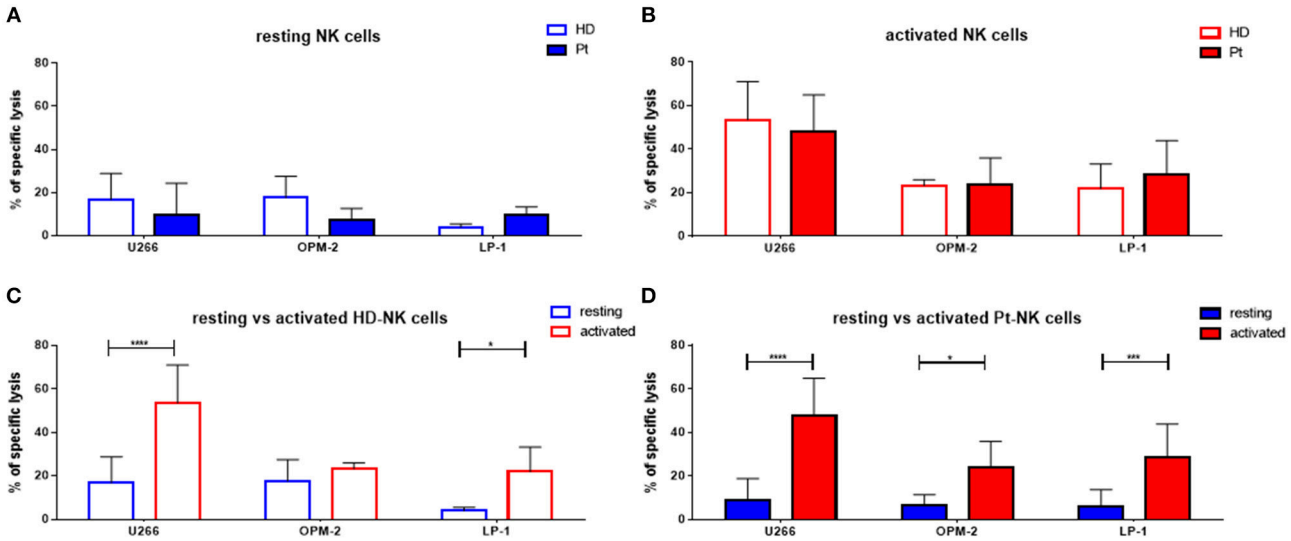

FIGURE 1 | Cytokine stimulation significantly increases the NK killing ability of both patient and healthy donor NK cells against MM cell lines. **(A)** Resting and **(B)** activated (IL-2 + IL-15) NK cells isolated from patients or healthy donors were co-cultured for 24 h with the CFSE-stained multiple myeloma cell lines, LP-1, OPM-2 and U266. The percentage of dead tumor cells was determined with a live-dead stain (DAPI) via flow cytometry after 24 h of co-culture. To further demonstrate the differences in the specific lysis of tumor cells between resting and activated tumor cells, the values for NK cells isolated from **(C)** healthy donors and **(D)** patients were compared. Data on resting NK cells are depicted in blue, whereas data on activated NK cells are shown in red. Data from patient samples are shown with filled-in bars, whereas data from healthy donor samples are shown with empty bars. NK cells and tumor cells were co-cultured with a 2:1 E-T ratio. For healthy donor data, 5–6 individual experiments were performed. For patient samples, 9 individual experiments with resting NK cells and 13 individual experiments with 7 days cytokine activated NK cells were performed. Statistical analysis with two-way ANOVA + Sidak's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

7 days with IL-2/15 cytokine cocktail (IL-2: 100 U/ml; IL-15: 10 ng/ml) prior to performing the killing assay (**Figure 1B**). Of note, both patient and donor cytokine-activated NK cells showed a significantly enhanced killing capacity against the different MM cell lines compared to that of the resting NK cells (**Figures 1C,D**).

Cytokine Stimulation Significantly Increases the Expression of Numerous NK Cell Activation Markers

Given the strong and positive impact of cytokine stimulation on NK cells, we asked whether the expression of NK cell receptors and surface molecules might be modified. Therefore, we analyzed the expression levels of 19 markers by flow cytometry, including activating and inhibitory receptors, markers of activation and maturation, death receptors, homing receptors and exhaustion markers. First, we compared their expression in resting conditions in patients and healthy donors (**Figure 2A**). The only significant difference was the higher expression of TRAIL on patient NK cells. Next, we addressed the expression following *in vitro* cytokine stimulation for 7 days (**Figure 2B**). Interestingly, patient NK cells show a higher expression of activating NKp30, as well as, CD57 and TRAIL receptor. In **Figure 2C** the expression pattern of the 19 surface molecules before and after cytokine activation in healthy donor NK cells is depicted. Interestingly, many activating receptors and markers are strongly and significantly increased after cytokine stimulation, e.g., NKp30, NKp44, CD69, CD25, CD57, and TRAIL. This finding can explain the positive impact on the NK cell mediated killing ability of MM cells. Of note, the inhibitory receptor NKG2A is also highly upregulated after cytokine stimulation. Similar modifications have been observed for NK cells isolated from patients (**Figure 2D**).

BM-Derived NK Cells Show a Similar Phenotype and Killing Behavior to Those of PB-Derived NK Cells in MM Patients

As MM cells reside in the BM, we further asked whether and how patient NK cells derived from the bone marrow would differ from PB NK cells in newly diagnosed and still untreated MM patients (TP0). For that purpose, we performed cytotoxicity assays in resting and activating conditions with NK cell isolated from either BM or PB, as shown in **Figure 3**. These analyses show no significant differences in the killing activity of patient BM and PB NK cells. There is, however, a less significant increase in killing after the activation of BM NK cells, compared with PB NK cells (**Figure 3C**). Similarly, we also investigated the phenotype of BM NK cells. While BM NK cell receptor expression was similar to that of PB NK cells under resting conditions (**Figure 4A**), following cytokine stimulation, the BM NK cells showed significantly lower levels of NKp30 (**Figure 4B**). A comparison of the BM NK cell phenotype before and after activation showed, also in this compartment, a significant increase in the activating markers NKp44, CD69, and CD25 (**Figure 4C**).

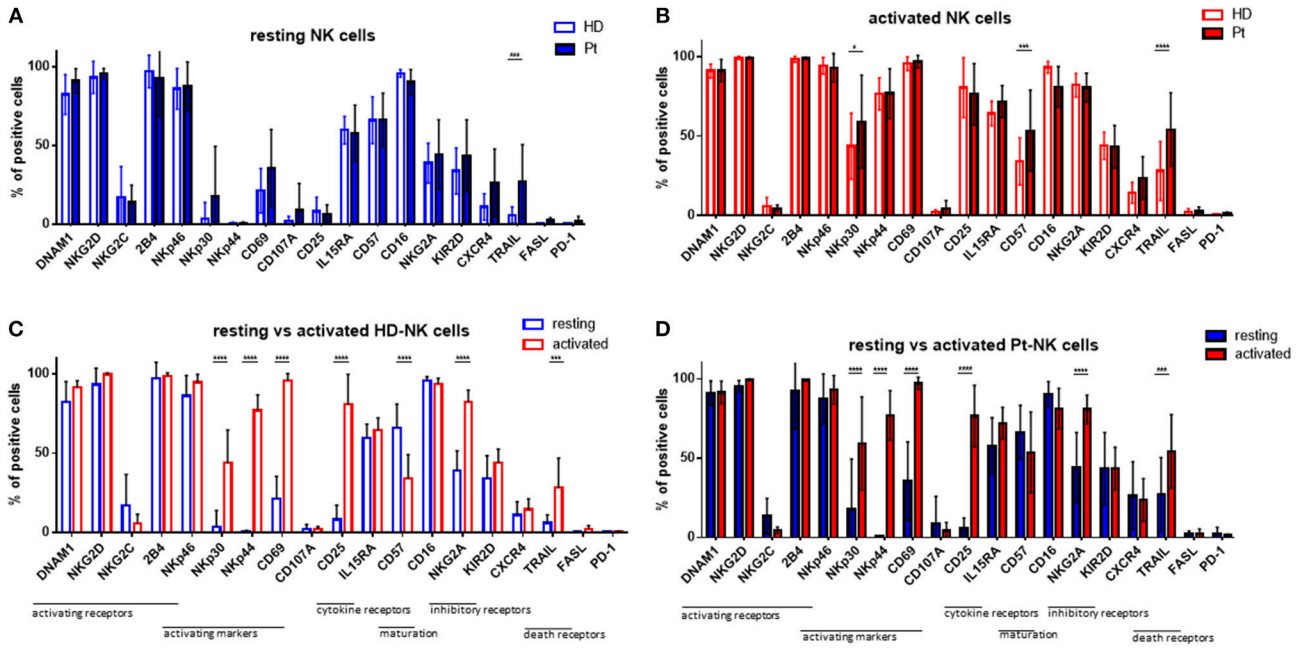

FIGURE 2 | Cytokine stimulation significantly increases the expression of many NK cell activation markers. Isolated NK cells from patients and healthy donors were stained with fluorochrome-conjugated antibodies to determine surface marker expression **(A)** before and **(B)** after activation with IL-2 and Il-15. NK cell phenotypes before and after stimulation were also compared for NK cells isolated from **(C)** healthy donors and **(D)** patients. For healthy donor samples, 17 individual experiments with resting NK cells and 14 individual experiments with activated NK cells were performed ($n = 14–17$). For patient samples, 14 individual experiments with resting NK cells and 15 individual experiments with 7 days cytokine activated NK cells were performed ($n = 14–15$) Statistical analysis with two-way ANOVA + Sidak's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Cytokine Stimulation Significantly Increases the NK Cell Killing Ability of Patient NK Cells at Various Treatment Points

Next, we investigated whether there were differences in the killing ability of MM cells and in the cytokine susceptibility of patient NK cells at different time points (TPs) during the treatment course. Therefore, PB samples from several newly diagnosed MM patients at different treatment stages were collected, at diagnosis (TP0), after induction therapy but before high-dose chemotherapy and autoSCT (TP1) and after hematological reconstitution after autoSCT (TP2) (**Figure 5A**). Patient numbers and characteristics are summarized in **Table 1**.

Cytokine-activation of NK cells significantly improved patient NK cell killing activity, especially toward the cell line U266 and LP1 (**Figure 5B**). There were however no significant differences in the lysis at different TPs.

Moreover, we monitored the expression pattern of 19 surface molecules of interest during the course of therapy and before and after cytokine stimulation, as shown in **Figure 6**. The induction therapy seemed to have a slightly negative impact on the NK cell activation status in resting conditions; the activation marker NKp30 and the BM homing receptor CXCR4 are significantly downregulated at TP1 (**Figure 6A**). The decrease of the activating receptors NKp30 and TRAIL and the increase of NKG2A could partially explain the reduced effect of the cytokine activation on the specific lysis levels (**Figure 5B**). Interestingly, at the end of the therapy (TP2) and more markedly after activation, the expression levels of several markers were restored, as in the case of DNAM1, NKp30, TRAIL, and CXCR4 (**Figure 6A**). However, NK cells at TP2 seem to have an overall more immature phenotype due to the downregulation of CD57, CD16, and KIR2D expression and the upregulation of NKG2A expression (**Figure 6**). These data confirmed the downregulation of CD57, CD16, and KIR2D after therapy and in particular at TP2, as well as, the upregulation of NKG2A, indicating a more immature NK cell phenotype and possibly lower ADCC capacity of NK cells at TP2 (**Figure 6B**).

Cytokine Stimulation Significantly Increases the Killing Ability of MM Patient NK Cells Even Against Autologous MM Cells

To further explore the cytotoxic potential of NK cells, we decided to study patients' killing ability against autologous tumor cells. With that aim, we isolated autologous primary tumor cells from some patients' BM aspirates that were subsequently used as a target for PB and BM NK cells from the same MM patients before and after cytokine stimulation (**Figure 7A**). Remarkably, both PB and BM patient NK cells in resting conditions were unable to kill autologous MM tumor cells. However, after cytokine stimulation, patient NK cells strongly and significantly increased their cytotoxic activity even against autologous MM tumor cells

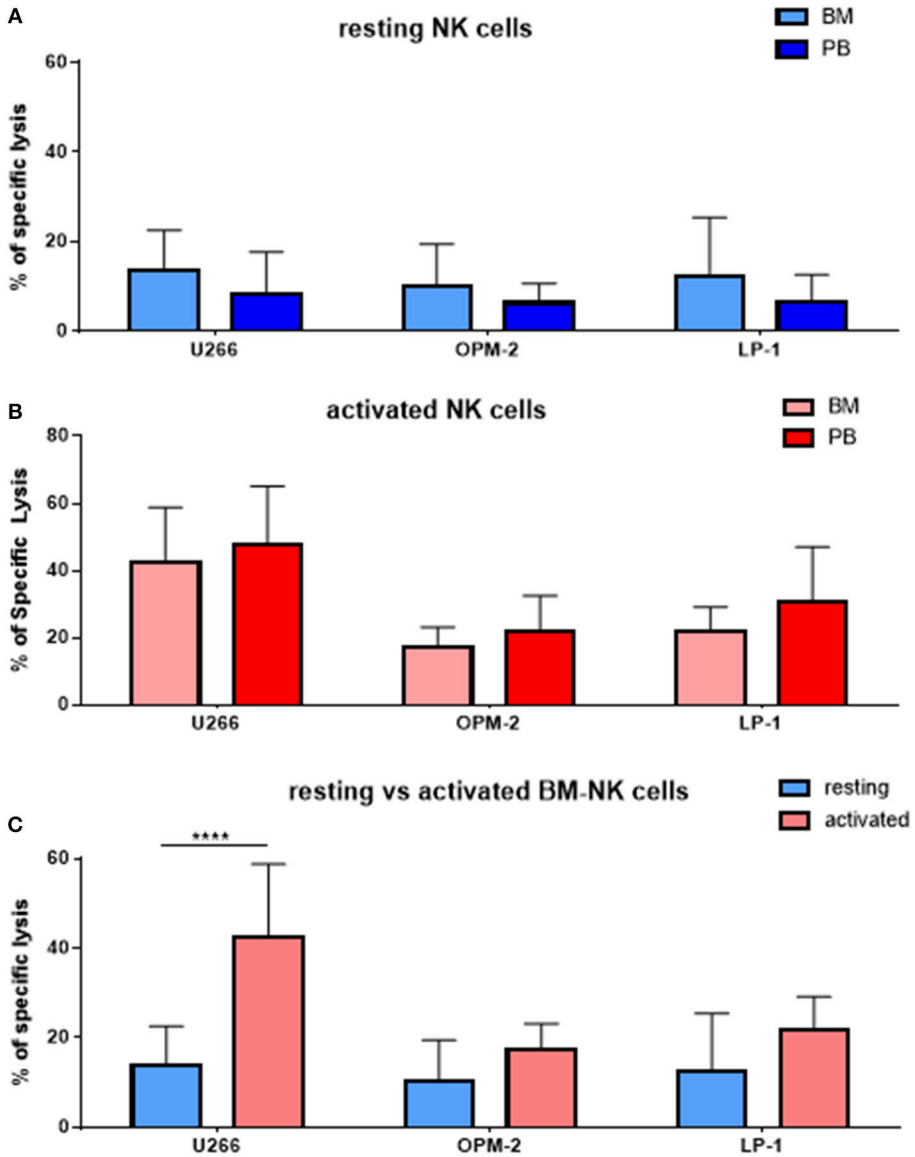

FIGURE 3 | NK cells isolated from peripheral blood or bone marrow exhibit a similar cytotoxic potential and phenotype. **(A)** Resting and **(B)** activated NK cells isolated from peripheral blood and bone marrow of patients were co-cultured for 24 h with the CFSE-stained multiple myeloma cell lines LP-1, OPM-2 and U266 to compare the specific lysis of target cells. **(C)** Comparison of BM NK cytotoxic potential before and after activation. The percentage of dead tumor cells was determined with a live-dead stain (DAPI) via flow cytometry after 24 h of co-culture. For bone marrow samples, 9 individual experiments with resting NK cells and 7 individual experiments with 7 days cytokine activated NK cells were performed ($n = 7$–9). For peripheral blood samples 10 individual experiments were performed ($n = 10$). Statistical analysis with two-way ANOVA + Tukey's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(**Figure 7B**). Of note, there were no significant differences in the killing capacity of PB and BM NK cells.

Expression Levels of NK Cell Receptor Ligands Do Not Correlate With Differences in the Susceptibility of NK Cell Killing

We performed a detailed flow cytometry phenotyping of the various MM target cell lines and MM primary cells to define their expression levels of 12 surface markers, with a special focus on the ligands for NK cell receptors (**Figures 7C**, **8**). The three MM cell lines (**Figure 8A**) revealed differences in the expression levels of several surface markers such as FAS-R, CD56, and CD48. However, the three cell lines only slightly differed in the levels of ligands for the activating and inhibitory receptors. Notably, the phenotype did not correlate with the different levels of susceptibility to NK cell killing. Moreover, we treated the MM cell lines with IFN-γ (**Figure 8B**) to investigate their phenotype under pro-inflammatory conditions, and we observed one major change, namely, the strong increase in HLA-E expression.

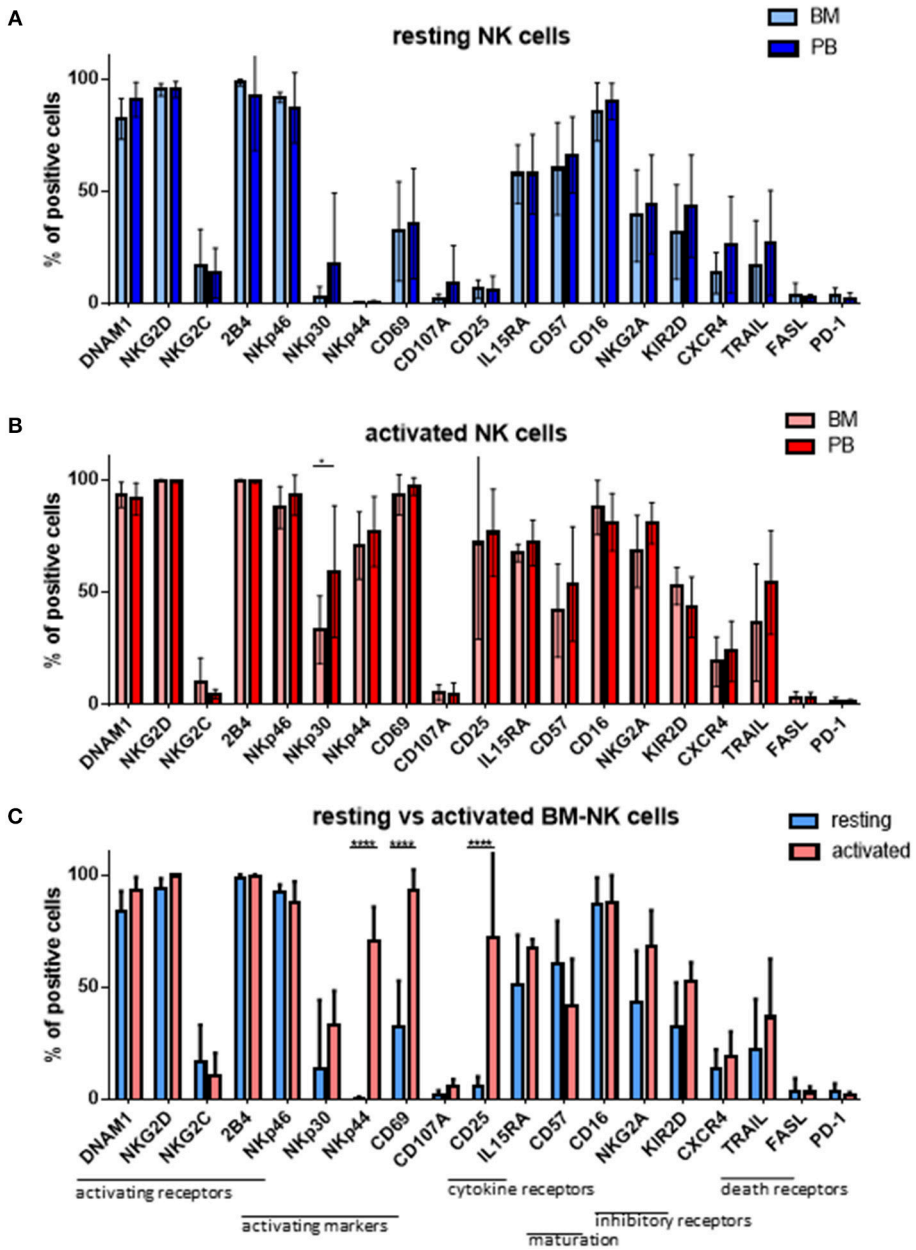

FIGURE 4 | NK cells isolated from peripheral blood or bone marrow exhibit a similar cytotoxic potential and phenotype. The phenotypes of **(A)** resting and **(B)** activated NK cells isolated from peripheral blood and bone marrow of patients at TP0 were compared by staining with fluorochrome-conjugated antibodies. **(C)** Comparison of the BM NK phenotype before and after activation. For bone marrow samples, 7 individual experiments with resting NK cells and 4 individual experiments with 7 days cytokine activated NK cells were performed ($n = 4$–7). For peripheral blood samples, 14 individual experiments with resting NK cells and 15 individual experiments with activated NK cells were performed ($n = 14$–15). Statistical analysis with two-way ANOVA + Sidak's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

The Combination of NKG2A Blocking With Cytokine Stimulation Further Improves NK Cell Killing Activity Against MM Cells

Given that the inhibitory receptor NKG2A was the only inhibitory receptor upregulated following cytokine stimulation, we hypothesized that blocking this inhibitory NK cell checkpoint could further improve the lysis of MM target cells by NK cells (**Figure 9**). Therefore, NK cells from patients were incubated with a blocking antibody or the control isotype prior to the cytotoxicity assay (**Figure 9A**). Importantly, blocking NKG2A resulted in a clear increase in the NK cell-mediated killing of OPM-2 and LP-1 cell lines. Hoping to achieve the possible use of third party or donor NK cells for adoptive therapy in MM, we examined whether the same effect could be seen using HD

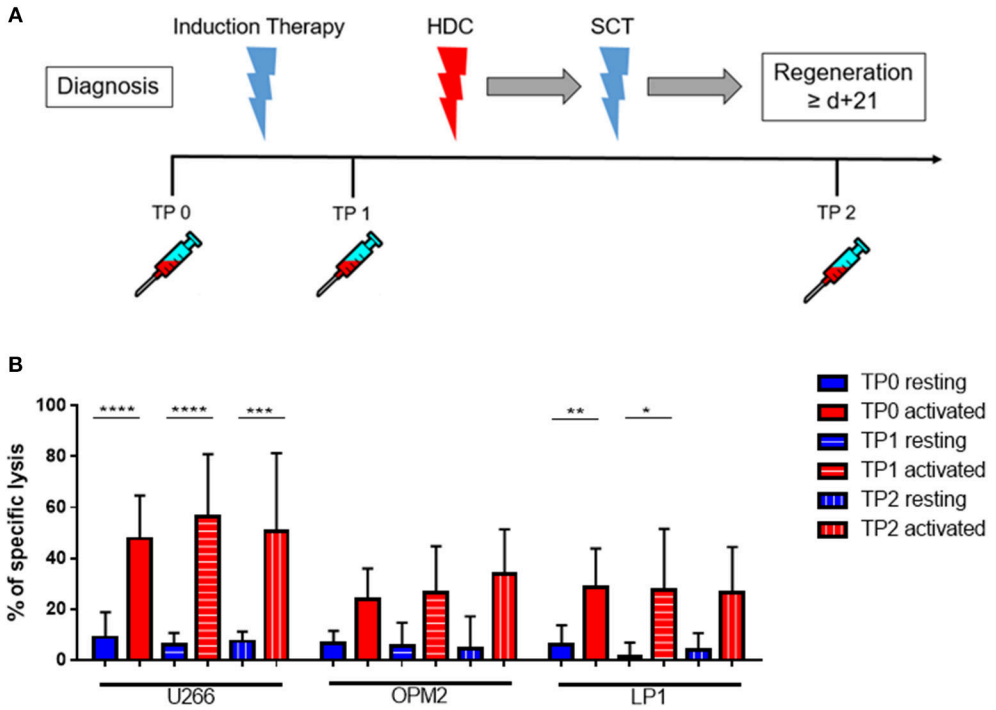

FIGURE 5 | Cytokine stimulation significantly increases the NK killing ability of patient NK cells at various treatment points. **(A)** NK cells from patients were isolated at different treatment points: TP0 after diagnosis, TP1 during high-dose chemotherapy and TP2 more than 21 days after stem cell transplantation. **(B)** The isolated NK cells were co-cultured with the CFSE-stained multiple myeloma cell lines LP-1, OPM-2 and U266 either directly after isolation or after seven days of pre-activation with IL-2 and IL-15. The percentage of dead tumor cells was determined with a live-dead stain (DAPI) via flow cytometry after 24 h of co-culture. NK cells and tumor cells were co-cultured with a 2:1 E-T ratio. For TP0, 7 individual experiments with resting NK cells and 15 individual experiments with 7 days activated NK cells were performed ($n = 7$–15). For TP1, 7 individual experiments with resting NK cells and 5 individual experiments with activated NK cells were performed ($n = 5$–7). For TP2, 3 individual experiments with resting NK cells and 4 individual experiments with activated NK cells were performed ($n = 3$–4). Statistical analysis with two-way ANOVA + Tukey's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

NK cells. HD NK cell show a significant better lysis against the targets U266 and LP-1. Importantly, the improvement of NK cell mediated killing capacity was even stronger when the MM cell lines were previously treated with IFN-γ and were highly HLA-E positive (**Figure 9B**).

DISCUSSION

MM is a highly aggressive plasma cell neoplastic disorder, and despite recent therapeutic advancements, it is still considered an incurable disease. Immunotherapy, including the use of cytokines, checkpoint inhibitors or cellular immunotherapeutics, holds great new promises to expand anti-myeloma treatment options. Because of its given antitumor activity, adoptive NK cell-based immunotherapy represents a potential treatment approach for myeloma patients.

In our study, we demonstrated that NK cells activated with IL-2 and IL-15 were able to efficiently kill multiple myeloma cell lines and autologous myeloma cells. Importantly, their killing activity was independent of the NK cell source from PB or BM or the time point of their isolation during myeloma treatment. Remarkably, their anti-myeloma activity could be further enhanced by an NKG2A checkpoint blockade.

Initially, it has been reported that NK cells from myeloma patients have a defect in their cytotoxic activity (29–31) and that myeloma cells are becoming more resistant to NK cell killing during disease progression (32). As an example of tumor immune evasion strategies it has been reported that high TGF-β levels in the tumor environment decrease the ability of NK cells to respond to IL-12 and IL-15 (33). On the other side, expression of IL-15 receptor and autocrine production of IL-15 has been suggested as mechanism of tumor propagation in MM (34). Moreover, the expression of activating NK cell receptors (e.g., NCR, NKG2D, 2B4, DNAM1) is known to be decreased in MM patients (35, 36). Although our data only demonstrated a difference in membrane bound TRAIL expression on resting NK cells of healthy donors and MM patients, the NK cell mediated cytotoxic activity against myeloma cell lines was only marginal.

However, upon *ex vivo* cytokine stimulation, the cytotoxicity of NK cells against multiple myeloma cell lines significantly improved. Furthermore, autologous inhibition was overcome and led to increased anti-myeloma activity even against autologous, primary myeloma cells. This result is in line with other studies investigating *ex vivo* NK cell expansion/activation protocols for anti-myeloma immunotherapy.

TABLE 1 | Patients characteristics.

Pt. Nr	MM type	%PC–BM	ISS stage	Risk	Response t1	Response t2
#1	IgA kappa	30	I	SR	PR	VGPR
#2	kappa	20	III	n.a.	n.a.	n.a.
#3	IgG kappa	80	III	SR	MR	VGPR
#4	IgA lambda	25	III	HR	VGPR	PR
#5	IgG kappa	40	I	SR	n.a.	n.a.
#6	IgA kappa	20	I	SR	PR	VGPR
#7	IgG kappa	20	I	SR	VGPR	CR
#8	IgG kappa	90	II	SR	PR	VGPR
#9	IgG kappa	25	I	SR	VGPR	VGPR
#10	IgG kappa	20	II	SR	VGPR	CR
#11	IgG lambda	15	I	SR	VGPR	CR
#12	IgG lambda	50	I	SR	VGPR	CR
#13	IgG kappa	30	I	HR	VGPR	n.a.
#14	IgG kappa	n.a.	III	n.a.	n.a.	n.a.
#15	IgG kappa	40	III	n.a.	n.a.	n.a.
#16	kappa	n.a.	I	n.a.	n.a.	n.a.

Sixteen patients with newly diagnosed multiple myeloma have been included in this study. Fifteen patients had symptomatic myeloma and needed an immediate treatment. Of these 15 patients, 11 patients were treated with a bortezomib-based triplet, followed by stem cell mobilization and high-dose chemotherapy with 200 mg/m2 melphalan. The remaining 4 patients were treated with conventional dose bortezomib/ dexamethasone or lenalidomide/ dexamethasone. Responses (according to IMWG) were evaluated after induction treatment (t1), and after minimum 60 days at hematological reconstitution after high-dose chemotherapy (t2). Risk stratification delineates the following cytogenetic risk factors by FISH: high risk (HR) with presence of $t_{(4;14)}$, or $t_{(14;16)}$, or del(17p); standard risk (SR) with Absence of above named. PC, plasma cell; BM, bone marrow; MR, minimal response; CR, complete remission; PR, partial remission; VGPR, very good partial remission; n.a., not available.

One study established a GMP-compliant protocol to expand NK cells from MM patients using IL-2 and anti-CD3 for 20 days, leading to a sufficient NK cell expansion of an average 511-fold (37). Importantly, only activated and expanded NK cells were able to kill autologous myeloma cells *in vitro*, without any cytotoxicity against autologous $CD34^+$ cells. This effect was time dependent since the NK cells' anti-myeloma activity was only marginal after a 5-day stimulation period.

Another approach was to use a genetically modified K562 cell line expressing the 41BB-ligand and IL-15. NK cells from healthy donors and myeloma patients were successfully expanded and able to kill allogeneic and autologous primary myeloma cells *in vitro* and in an *in vivo* mouse model (38). Again, only expanded NK cells demonstrated a significant killing activity against myeloma cells, whereas non-expanded NK cells did not. In accordance with our data, expanded NK cells from HD and MM patients demonstrated similar cytotoxic activities against allogeneic myeloma targets, indicating that *ex vivo* cytokine stimulation is able to overcome the NK cell cytotoxicity defects of myeloma patients.

The mechanism behind their improved cytotoxicity has been attributed to the increased expression of activating NK cell receptors (e.g., NKG2D, DNAM1, and NCRs) and cytotoxic effector molecules (e.g., granzyme B and perforin), as well as, membrane bound death receptor ligands (e.g., TRAIL). Their contributions have been demonstrated by performing blocking experiments upon NK cell cytokine stimulation (32, 38, 39), which is of particular importance since anti-myeloma drugs are known to increase activating or decrease inhibitory NK cell receptor ligands on myeloma cells (40–42). We observed an increase in the surface expression of activating receptors upon cytokine stimulation. In addition, TRAIL expression was significantly increased upon cytokine stimulation on myeloma patients' NK cells, which is in concordance with the results of previous reports demonstrating TRAIL upregulation and improved NK cell cytotoxicity upon IL-2 and/or IL-15 stimulation (43, 44). Based on this knowledge, the first clinical trials using adoptive NK cell transfer to treat myeloma patients have been completed. While one trial used the allogeneic NK cell line, NK-92, within a phase-I dose-escalating trial for treating refractory hematological malignancies in a non-transplantation setting (45), two other trials were performed within an autoSCT setting. The first used haploidentical, KIR-ligand mismatched NK cells expanded with IL-2 and anti-CD3, demonstrating the safe engraftment of autologous stem cells with no signs of GVHD in treated myeloma patients (46). Similar safety results were obtained in a second trial expanding NK cells using irradiated K562 cells expressing membrane bound IL-21 in combination with IL-2 (47).

Based on these first in-human trials, we investigated the best time point for harvesting NK cells to expand them for adoptive NK cell transfer within an autoSCT setting. Interestingly, in this study independent of prior treatments with the proteasome-inhibitor bortezomib, immune modulating substances (IMiDs) such as lenalidomide or chemotherapeutics including melphalan, NK cells isolated at all chosen TPs demonstrated similar cytotoxic activity against myeloma cell lines. This result was surprising as an NK cell activating effect of IMiDs and a more inhibitory effect of proteasome inhibitors have been previously reported (for review see (48)). Furthermore, despite the differential expression of activating and inhibitory receptors in resting NK cells, especially the upregulation of NKG2A expression and the downregulation of CD57 expression at TP2, indicate a more immature NK cell phenotype, in line with earlier reports (28). In addition, lower TRAIL expression upon cytokine expression on NK cell from TP1 did not seem to have a negative influence on their anti-myeloma activity.

Therefore, we propose that NK cells can be freshly isolated before the start of high-dose chemotherapy (HD) and autoSCT in order to be activated, expanded and re-infused before or after autoSCT. Using freshly isolated NK cells is of importance since adoptive NK cell transfer studies have demonstrated that the use of fresh NK cells may be more beneficial than using cryopreserved cells (49, 50).

Although we observed increased anti-myeloma activity and expression of activating NK cell receptors, there was a significant upregulation of the inhibitory receptor NKG2A, while other receptors such as KIR2D or PD1 were not upregulated. This result is in contrast to other reports, which have demonstrated the strong upregulation of PD1 upon cytokine stimulation (51). In addition, CD57 expression was downregulated on our

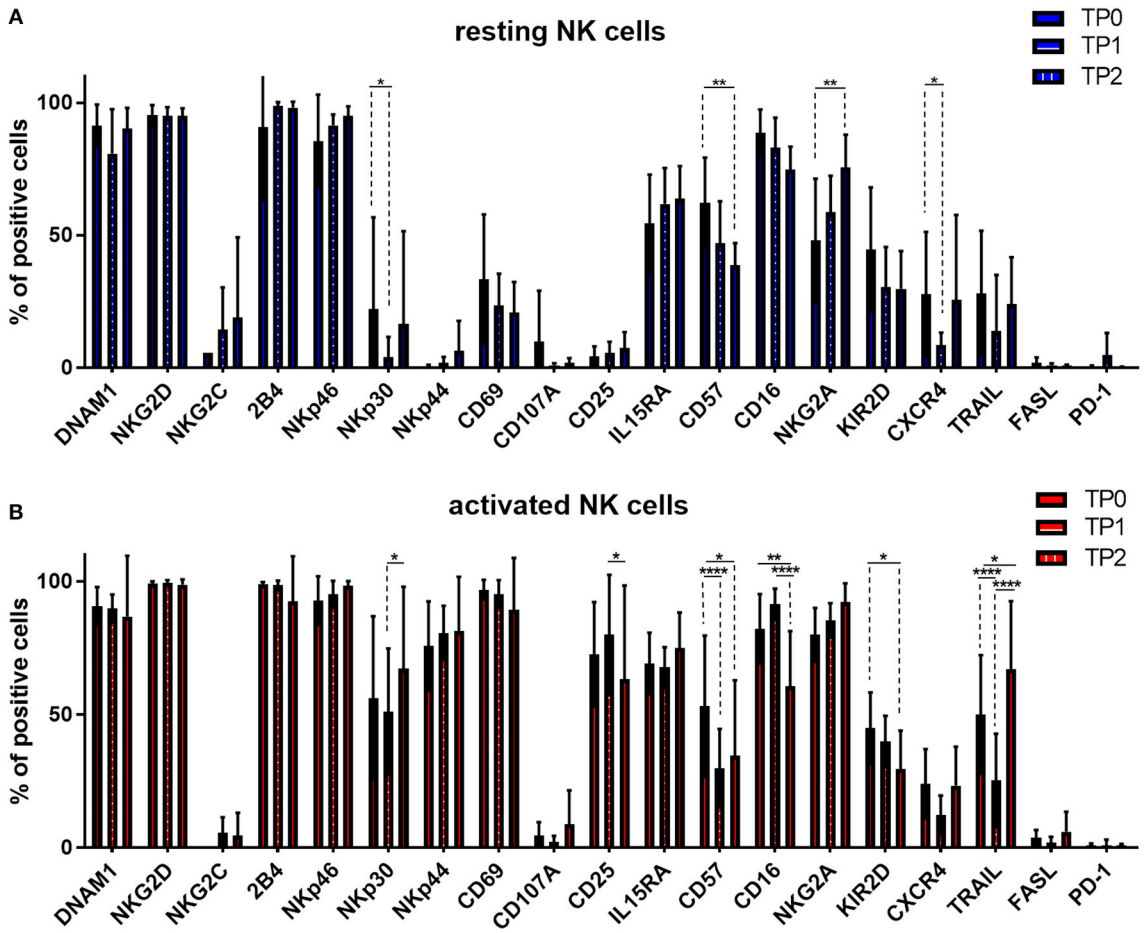

FIGURE 6 | Expression of several NK cell markers significantly changed between different treatment points. NK cells from patients were isolated at different treatment points: TP0 after diagnosis, TP1 during high-dose chemotherapy and TP2 more than 21 days after stem cell transplantation. After isolation, NK cells were stained with fluorochrome-conjugated antibodies to determine surface marker expression **(A)** before and **(B)** after activation with IL-2 and Il-15. For TP0, 11 individual experiments with resting NK cells and 15 individual experiments with activated NK cells were performed ($n = 11$–15). For TP1, 10 individual experiments with resting NK cells and 16 individual experiments with 7 days activated NK cells were performed ($n = 10$–16). For TP2, 5 individual experiments with resting NK cells and 7 individual experiments with activated NK cells were performed ($n = 5$–7). Statistical analysis with two-way ANOVA + Sidak's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

activated NK cells, corresponding to a more immature phenotype (52) as has been described by others. In addition, also CD16 was downregulated which might reduce the NK cell ADCC capacity. Despite their more immature phenotype, those NK cells were multifunctional since they demonstrated increased cytokine production, proliferation capacity and degranulation upon target cell recognition. In addition, cytotoxic molecules were strongly upregulated (51). These data indicate that the classical view of NKG2A^{+} NK cells as being more immature and the main cytokine producers does not hold true when the cells are stimulated with cytokines.

NKG2A is known to interact with HLA-E and is known to be increased on myeloma cell lines. Although HLA-E was low on our myeloma cell lines, its expression was increased on primary myeloma cells. This finding is in line with other reports demonstrating higher levels of HLA-E on primary myeloma cells than on cell lines, which can be upregulated upon *in vivo* transfer (26). In addition, HLA-E expression was upregulated upon IFN-γ treatment, diminishing their susceptibility toward NK cell treatment, which was reverted upon blocking the NKG2A-/HLA-E interaction. The importance of the NKG2A/HLA-E interaction has been demonstrated for other diseases, e.g., within a humanized, post-transplantation model, which demonstrated that re-constituted NKG2A^{+} NK cells were able to kill human primary leukemia cells when mice were injected with an anti-human NKG2A antibody (53). In addition, blocking the NKG2A/HLA-E interaction was able to restore NK cell dysfunction against CLL cells (54). This finding has led to different clinical phase 1 trials to evaluate the efficacy and safety of the NKG2A blockade in patients with CLL disease (NCT02557516) or in a post-allogeneic SCT setting (NCT02921685).

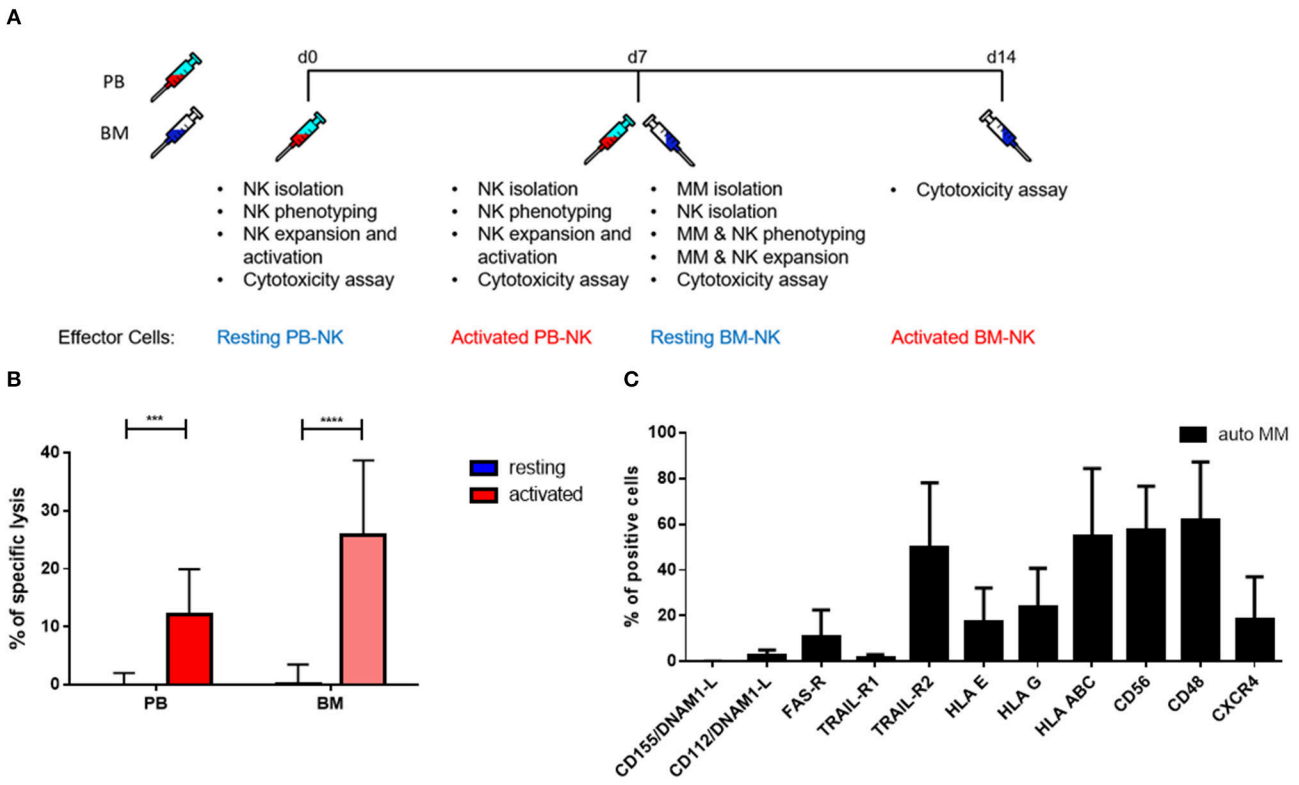

FIGURE 7 | Pre-activation of NK cells significantly increases autologous tumor cell lysis. **(A)** Schematic of the experimental procedure. On d0, NK cells from peripheral blood were isolated and used for cytotoxicity assays at d0, before activation, and d7, after activation. On d7, MM cells and NK cells were isolated from BM aspirates. Those cells were also used for cytotoxicity assays at d7, before activation, and d14, after activation. **(B)** Resting and 7 days cytokine activated NK cells isolated from peripheral blood and bone marrow of patients were co-cultured for 24 h with CFSE-stained autologous multiple myeloma cells to compare the specific lysis of target cells. The percentage of dead tumor cells was determined with a live-dead stain (DAPI) via flow cytometry after 24 h of co-culture. NK cells and tumor cells were co-cultured with a 2:1 E-T ratio. **(C)** From patients, isolated primary multiple myeloma cells were stained with fluorochrome-conjugated antibodies to determine marker expression. For the bone marrow samples, 9 individual experiments with resting NK cells and 5 individual experiments with activated NK cells were performed ($n = 5$–9). For the peripheral blood samples, 9 individual experiments were performed ($n = 9$). For the phenotyping of autologous MM cells, 5 individual experiments were performed. Statistical analysis with two-way ANOVA + Tukey's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In summary, our data provide evidence for the use of *ex vivo* cytokine-activated NK cells as an immunotherapy to treat myeloma patients within an autoSCT setting in combination with NKG2A blockade.

MATERIALS AND METHODS

NK Cell Isolation and Culture

Blood samples were obtained from healthy donors or from diagnosed MM patients in accordance with the Declaration of Helsinki. All subjects provided written, informed consent. This study was carried out in accordance with the recommendations of the Ethics Committee of the University Hospital Frankfurt. The protocol of this study (SHN-02-2015) was approved by the above mentioned committee. Patient numbers and characteristics are summarized in **Table 1**. Patient selection was randomly at the onset of MM diagnosis prior to any treatment. The age range for patients was from 45 to 70 years, but only from 29 to 64 years for healthy donors.

Blood from patients was collected at three different treatment points (TP0 after diagnosis, TP1 in the pause before high-dose chemotherapy and autoSCT, TP2 >21 d after SCT). Peripheral and bone marrow blood mononuclear cells were isolated by Ficoll density gradient centrifugation (Biochrom, #L6115). NK cells were enriched by negative selection of NK cells with an EasySep NK Cell Enrichment Kit (StemCell Technologies, #19055) according to the manufacturer's protocol. NK cell purity was assessed by flow cytometry and was >90% with the used isolation method. Cells were cultured in X-VIVO 10 (Lonza, #BE04-743Q) medium supplemented with 5% heat-inactivated human plasma (DRK Blutspendedienst) and 1% penicillin with streptomycin (Invitrogen, #15140-122). NK cells were expanded *ex vivo* by additionally supplementing the medium with IL-2 (100 U/ml) (Peprotech, #200-02) and IL-15 (10 ng/ml) (Peprotech, #200-15). Every third day 80 µl of old medium were removed and100 µl of fresh medium with cytokines were added to the wells. All cells were maintained at 37°C with 5% CO_2 atmospheric conditions. Unless otherwise stated, NK cells were used in the experiments after 7 days of culture with IL-2/15.

Culture and Treatment of MM Cells

Autologous MM cells were isolated from bone marrow aspirates using CD138 MicroBeads and a MidiMACS Separator (Miltenyi

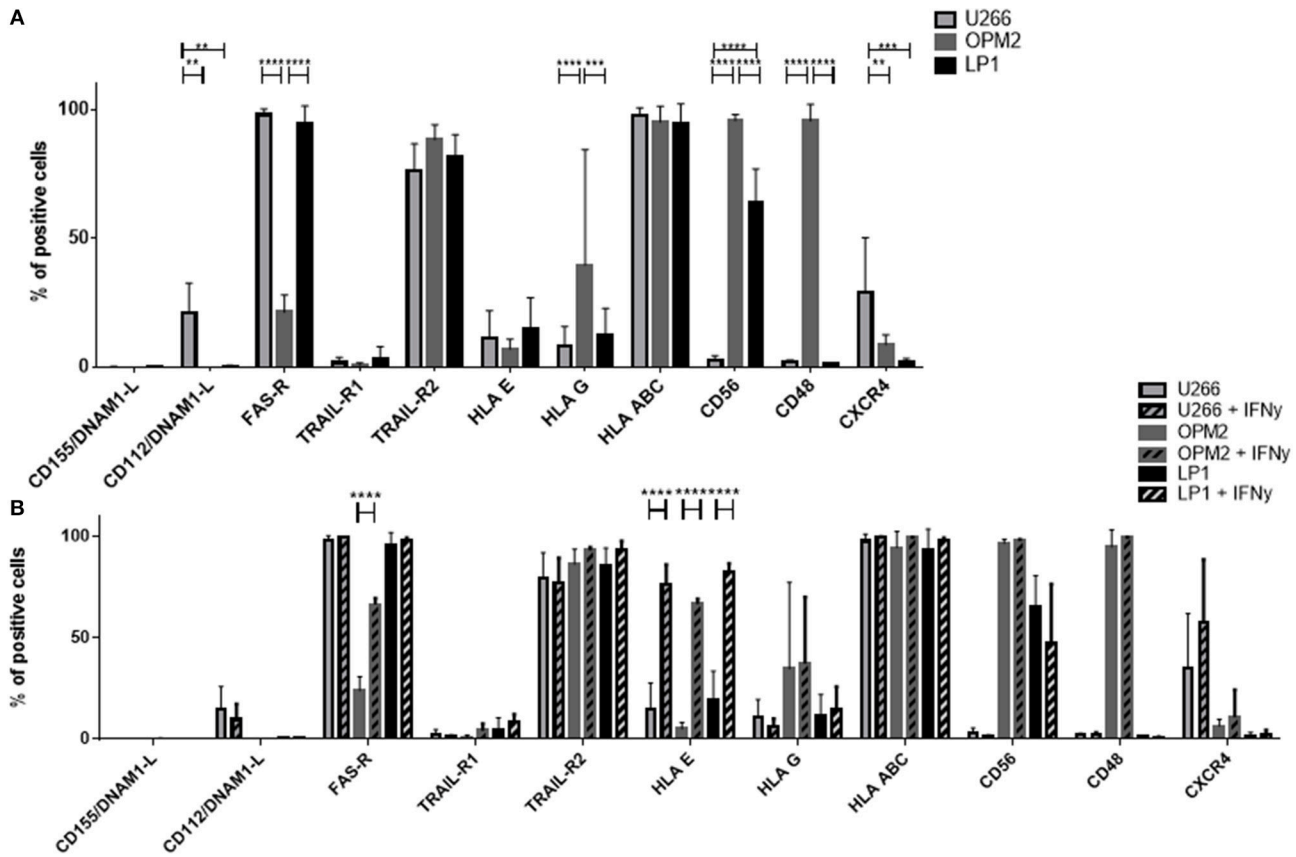

FIGURE 8 | Determination of the expression of surface molecules on multiple myeloma cells. **(A)** The marker expression of different multiple myeloma cell lines was measured via FACS analysis by staining the cell lines LP-1 (black), OPM-2 (dark gray) and U266 (light gray) with fluorochrome-conjugated antibodies. **(B)** To further increase the expression of HLA-E, the multiple myeloma cell lines were incubated for 24 h with 25 nM IFN-γ before measuring surface marker expression via flow cytometry. For phenotyping the MM cells, five individual experiments were performed ($n = 5$). For phenotyping experiments after stimulation with IFN-γ, 3 individual experiments were performed ($n = 3$). Statistical analysis with two-way ANOVA + Sidak's/Tukey's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Biotech, #130-042-30, #130-097-614). Cells were cultured in X-VIVO 10 (Lonza, #BE04-743Q) medium supplemented with 5% heat-inactivated human plasma (DRK Blutspendedienst) and 1% penicillin with streptomycin. Multiple Myeloma cell line originally were received from the DSMZ (Leibniz-Institut DSMZ-German Collection of Micro-organisms and Cell Cultures GmbH). To further confirm the identity of the cell lines we performed phenotypic flow cytometry analysis (**Supplemental Figures 4, 6**), HLA-ABC genotyping (**Supplemental Table 1**) and and short tandem repeat (STR) analysis of the cell line U266 (**Supplemental Table 2**). Taken all the data together, we could confirm the identity of our MM cell lines by different orthogonal methods, demonstrating therefore absence of cross-contamination. The MM cell line LP-1 was cultured in IMDM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin and glutamine. The cell lines OPM-2 and U266 were cultured in RPMI 1640 + Glutamax (Life Technologies, #31870-025) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin with streptomycin. All cells were maintained at 37°C with 5% CO_2 atmospheric conditions.

FACS Phenotyping and Purity Check

For the flow cytometric measurements, 1×10^5-10^6 cells were used per reaction tube. Cells were stained for 20 min at 4°C. For NK cell phenotyping experiments, cells were stained with the following antibodies: 7AAD PerCP (#559925), CD107a APC-H7 (#561343), CD184 PE-Cy7 (#560669), CD226 FITC (#559788), CD25 BV605 (#562660), CD335 PE (#331908), CD56 BV421 (#562751), CD69 BV605 (#562989) (all from BD Biosciences), CD16 APC ALEXA700 (#302025), CD19 PerCP (#302228), CD215 APC (# 330209), CD253 PE (# 308206), CD3 PerCP (# 300428), CD336 PE (# 558563), CD57 APC (# 322314) (all from Biolegend), CD138 BV510 (# 130-101-169), CD14 PerCP (# 130-094-969), CD244 PE-Cy7 (# 130-099-074), CD279 PE (# 130-096-164), KIR2D FITC (# 130-098-689) (all from Miltenyi Biotech), CD159a PE (PNIM3291U), CD314 APC (# A22329) (both from Beckman Coulter) and CD159c ALEXA488 (# FAB138G-100), CD337 ALEXA488 (#

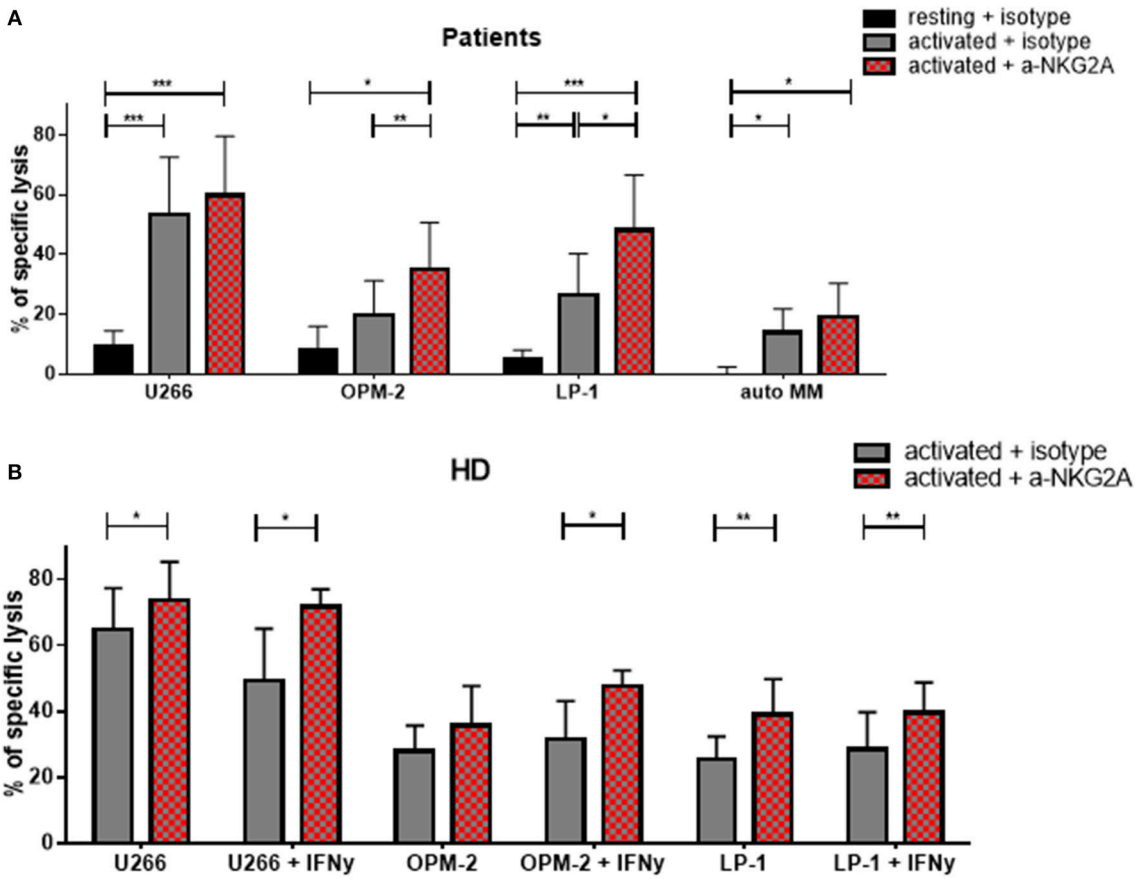

FIGURE 9 | Functional blockade of NKG2A significantly increases NK cell cytotoxic potential. Isolated NK cells from **(A)** patients and **(B)** healthy donors were pre-activated by stimulating them with IL-2 and IL-15 for seven days and then co-cultured with different multiple myeloma cell lines, as well as, autologous, primary cells for 24 h. Prior to co-culture, the NK cells were treated with either an anti-NKG2A or isotype antibody to functionally block NKG2A on the NK cell surface. The percentage of dead tumor cells was determined with a live-dead stain (DAPI) via flow cytometry after 24 h of co-culture. NK cells and tumor cells were co-cultured with a 2:1 E-T ratio. For patient samples, 8 individual experiments for resting + isotype, 9 individual experiments for activated + isotype and activated + a-NKG2A were performed ($n = 8$–9). For healthy donor samples, 5 individual experiments were performed ($n = 5$). Statistical analysis with two-way ANOVA + Sidak's multiple comparison: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

FAB1849G) (both from R&D). Purity of the isolated NK cells was checked with the following: DAPI (AppliChem), CD56 FITC (# 345811) (BD Biosciences), CD16 APC ALEXA700 (# 302025), CD19 PerCP, CD3 APC (# 300412) (all from Biolegend), CD45 PE (# MHCD4504) (Invitrogen) and CD138 BV510 (Miltenyi Biotech). The NK cell gating strategy for all 19 surface markers that have been analyzed by flow cytometry is depicted in **Supplemental Figures 1, 2** performed on a representative healthy donor sample. Dead NK cells have been excluded by 7AAD staining, and gates defining the positive percentage of NK cell populations were set using positive, negative and internal controls (**Supplemental Figure 5**).

For MM cell phenotyping cells were stained with the following antibodies: CD184 PE-Cy7, CD56 BV421 (BD Biosciences), CD112 PE, CD261 PE, CD262 APC, CD95 BV412, HLA-ABC BV605, HLA-E PE-Cy7, HLA-G APC (Biolegend), CD138 BV510, CD48 APC-H7 (Miltenyi), and CD155 FITC (R&D). Dead cells have been excluded by DAPI-expression. The gating strategy for all 11 surface markers that have been analyzed by flow cytometry is depicted in **Supplemental Figure 4**. Gates defining the positive percentage of MM cells expressing a specific surface marker were set using positive, negative and internal controls (**Supplemental Figure 6**).

The purity of the isolated, autologous MM cells was determined with the following antibodies: CD184 PE-Cy7, (BD Biosciences), CD45 FITC (#6603838), CD56 APC (#IM2474) (both from Beckmann Coulter), CD19 PerCP (Biolegend), CD38 PE (DakoCytomation), and CD138 BV510 (Miltenyi Biotech). MM cells were defined as DAPI-, CD45-/low, CD19-, $CD138^{+}$ and $CD38^{+}$ as shown in **Supplemental Figure 3**. Of note, CD138 staining following MM cell isolation was often low due to the rapid internalization and the possible competition with the CD138-beads used for the positive selection procedure.

Analysis of Tumor Cell Death

MM tumor cells were stained with the Cell Trace^{TM} Cell Proliferation Kit (Invitrogen, #C34554), resuspended in X-VIVO

10 media supplemented with 5% heat-inactivated human plasma and 1% penicillin with streptomycin and seeded in a 96 V-bottom well plate. NK cells were seeded into the corresponding wells. In experiments where activated NK cells were used, the medium was additionally supplemented with IL-2 (100 U/ml) and IL-15 (10 ng/ml). All experiments were performed with an E:T ratio of 2:1. The 96-well plates were incubated for 24 h at 37°C with 5% CO_2 atmospheric conditions. After incubation, the cells were resuspended and transferred to FACS tubes. Directly before measurement, 250 µl DAPI solution (DAPI 1:6000 in PBS) (AppliChem, #A4099,0010) was added to the tubes and incubated for 3 min. For each combination, two wells were filled, representing technical replicates. In addition, control wells for all tumor targets used in the experiment were added, containing target cells only in order to determine the spontaneous lysis. In the final evaluation of the experiment, the specific lysis was calculated as the percentage of dead tumor cells in the wells containing target and effector cells minus the spontaneous lysis of the respective tumor cell condition. Through this calculation of the specific lysis as the percentage of killed tumor cells was attributed completely to the NK cell effector function. Dead target cells were calculated as Cell Trace+ DAPI+ cells.

Blocking Experiments

For blocking experiments NK cells were incubated for at least 30 min with 30 µg/ml blocking antibody prior to co-culture. Blocking experiments were performed with an anti-NKG2A antibody (Beckman Coulter, #IM2750). As control a Purified Mouse IgG2b isotype (Biolegend, #400302) was used.

AUTHOR CONTRIBUTIONS

ST, IvM, and EU designed the project. ST, SW, and BR performed the experiments. ST and SW analyzed the data. ST, SW, HS, PB, IvM, BJ, and EU discussed the data. ST, SW, and EU wrote the manuscript with the contribution of all other co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02743/full#supplementary-material

REFERENCES

1. Landgren O, Rajkumar SV. New developments in diagnosis, prognosis, and assessment of response in multiple myeloma. *Clin Cancer Res.* (2016) 22:5428–33. doi: 10.1158/1078-0432.CCR-16-0866
2. Giralt S. Advances in stem cell transplantation: making it better and safer. *Clin Lymphoma Myeloma* (2009) 9 (Suppl. 3):S293–5. doi: 10.3816/CLM.2009.s.026
3. Lehners N, Becker N, Benner A, Pritsch M, Löpprich M, Mai EK, et al. Analysis of long-term survival in multiple myeloma after first-line autologous stem cell transplantation: impact of clinical risk factors and sustained response. *Cancer Med.* (2018) 7:307–16. doi: 10.1002/cam4.1283
4. Cook G, Zweegman S, Mateos MV, Suzan F, Moreau P. A question of class: treatment options for patients with relapsed and/or refractory multiple myeloma. *Crit Rev Oncol Hematol.* (2018) 121:74–89. doi: 10.1016/j.critrevonc.2017.11.016
5. Rezvani K, Rouce RH. The application of natural killer cell immunotherapy for the treatment of cancer. *Front Immunol.* (2015) 6:578. doi: 10.3389/fimmu.2015.00578
6. Simonetta F, Alvarez M, Negrin RS. Natural killer cells in graft-versus-host-disease after allogeneic hematopoietic cell transplantation. *Front Immunol.* (2017) 8:465. doi: 10.3389/fimmu.2017.00465
7. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat Immunol.* (2008) 9:486–94. doi: 10.1038/ni1580
8. Osterborg A, Nilsson B, Björkholm M, Holm G, Mellstedt H. Natural killer cell activity in monoclonal gammopathies: relation to disease activity. *Eur J Haematol.* (1990) 45:153–7.
9. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* (1986) 319:675–8.
10. Ullrich E, Koch J, Cerwenka A, Steinle A. New prospects on the NKG2D/NKG2DL system for oncology. Oncoimmunology (2013) 2:e26097. doi: 10.4161/onci.26097
11. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol.* (1975) 5:117–21. doi: 10.1002/eji.1830050209
12. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol.* (2012) 12:239–52. doi: 10.1038/nri3174
13. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* (2003) 101:3052–7. doi: 10.1182/blood-2002-09-2876
14. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol.* (2007) 178:4947–55. doi: 10.4049/jimmunol.178.8.4947
15. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol.* (1989) 143:3183–91.
16. Paya CV, Kenmotsu N, Schoon RA, Leibson PJ. Tumor necrosis factor and lymphotoxin secretion by human natural killer cells leads to antiviral cytotoxicity. *J Immunol.* (1988) 141:1989–95.

17. Wendt K, Wilk E, Buyny S, Buer J, Schmidt RE, Jacobs R. Gene and protein characteristics reflect functional diversity of CD56dim and CD56bright NK cells. *J Leukoc Biol.* (2006) 80:1529–41. doi: 10.1189/jlb.0306191
18. Granzin M, Wagner J, Köhl U, Cerwenka A, Huppert V, Ullrich E. Shaping of natural killer cell antitumor activity by *ex vivo* cultivation. *Front Immunol.* (2017) 8:458. doi: 10.3389/fimmu.2017.00458
19. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol.* (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
20. Pende D, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, et al. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* (2009) 113:3119–29. doi: 10.1182/blood-2008-06-164103
21. Benson DM Jr, Cohen AD, Jagannath S, Munshi NC, Spitzer G, Hofmeister CC, et al. A phase I trial of the anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. *Clin Cancer Res.* (2015) 21:4055–61. doi: 10.1158/1078-0432.CCR-15-0304
22. Carlsten M, Korde N, Kotecha R, Reger R, Bor S, Kazandjian D, et al. Checkpoint inhibition of KIR2D with the monoclonal antibody IPH2101 induces contraction and hyporesponsiveness of NK cells in patients with myeloma. *Clin Cancer Res.* (2016) 22:5211–22. doi: 10.1158/1078-0432.CCR-16-1108
23. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, López-Botet M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA.* (1998) 95:5199–204.
24. de Kruijf EM, Sajet A, van Nes JG, Natanov R, Putter H, Smit VT, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol.* (2010) 185:7452–9. doi: 10.4049/jimmunol.1002629
25. Levy EM, Sycz G, Arriaga JM, Barrio MM, von Euw EM, Morales SB, et al. Cetuximab-mediated cellular cytotoxicity is inhibited by HLA-E membrane expression in colon cancer cells. *Innate Immun.* (2009) 15:91–100. doi: 10.1177/1753425908101404
26. Sarkar S, van Gelder M, Noort W, Xu Y, Rouschop KM, Groen R, et al. Optimal selection of natural killer cells to kill myeloma: the role of HLA-E and NKG2A. *Cancer Immunol Immunother.* (2015) 64:951–63. doi: 10.1007/s00262-015-1694-4
27. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood* (2005) 106:4370–6. doi: 10.1182/blood-2005-04-1644
28. Jacobs B, Tognarelli S, Poller K, Bader P, Mackensen A, Ullrich E. NK cell subgroups, phenotype, and functions after autologous stem cell transplantation. *Front Immunol.* (2015) 6:583. doi: 10.3389/fimmu.2015.00583
29. Uchida A, Yagita M, Sugiyama H, Hoshino T, Moore M. Strong natural killer (NK) cell activity in bone marrow of myeloma patients: accelerated maturation of bone marrow NK cells and their interaction with other bone marrow cells. *Int J Cancer* (1984) 34:375–81. doi: 10.1002/ijc.2910340314
30. Matsuzaki H, Kagimoto T, Oda T, Kawano F, Takatsuki K. Natural killer activity and antibody-dependent cell-mediated cytotoxicity in multiple myeloma. *Jpn J Clin Oncol.* (1985) 15:611–7.
31. Frassanito MA, Silvestris F, Cafforio P, Silvestris N, Dammacco F. IgG M-components in active myeloma patients induce a down-regulation of natural killer cell activity. *Int J Clin Lab Res.* (1997) 27:48–54. doi: 10.1007/BF02827242
32. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* (2005) 105:251–8. doi: 10.1182/blood-2004-04-1422
33. Yu J, Wei M, Becknell B, Trotta R, Liu S, Boyd Z, et al. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity* (2006) 24:575–90.s doi: 10.1016/j.immuni.2006.03.016
34. Tinhofer I, Marschitz I, Henn T, Egle A, Greil R. Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma. *Blood* (2000) 95:610–8.
35. Fauriat C, Just-Landi S, Mallet F, Arnoulet C, Sainty D, Olive D, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. *Blood* (2007) 109:323–30. doi: 10.1182/blood-2005-08-027979
36. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, et al. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res.* (2007) 67:8444–9. doi: 10.1158/0008-5472.CAN-06-4230
37. Alici E, Sutlu T, Björkstrand B, Gilljam M, Stellan B, Nahi H, et al. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. *Blood* (2008) 111:3155–62. doi: 10.1182/blood-2007-09-110312
38. Garg TK, Szmania SM, Khan JA, Hoering A, Malbrough PA, Moreno-Bost A, et al. Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. *Haematologica* (2012) 97:1348–56. doi: 10.3324/haematol.2011.056747
39. Feng X, Yan J, Wang Y, Zierath JR, Nordenskjöld M, Henter JI, et al. The proteasome inhibitor bortezomib disrupts tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and natural killer (NK) cell killing of TRAIL receptor-positive multiple myeloma cells. *Mol Immunol.* (2010) 47:2388–96. doi: 10.1016/j.molimm.2010.05.003
40. Fionda C, Abruzzese MP, Zingoni A, Cecere F, Vulpis E, Peruzzi G, et al. The IMiDs targets IKZF-1/3 and IRF4 as novel negative regulators of NK cell-activating ligands expression in multiple myeloma. *Oncotarget* (2015) 6:23609–30. doi: 10.18632/oncotarget.4603
41. Shi J, Tricot GJ, Garg TK, Malaviarachchi PA, Szmania SM, Kellum RE, et al. Bortezomib down-regulates the cell-surface expression of HLA class I and enhances natural killer cell-mediated lysis of myeloma. *Blood* (2008) 111:1309–17. doi: 10.1182/blood-2007-03-078535
42. Yang G, Gao M, Zhang Y, Kong Y, Gao L, Tao Y, et al. Carfilzomib enhances natural killer cell-mediated lysis of myeloma linked with decreasing expression of HLA class I. *Oncotarget* (2015) 6:26982–94. doi: 10.18632/oncotarget.4831
43. Mirandola P, Ponti C, Gobbi G, Sponzilli I, Vaccarezza M, Cocco L, et al. Activated human NK and $CD8^+$ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. *Blood* (2004) 104:2418–24. doi: 10.1182/blood-2004-04-1294
44. Sheard MA, Asgharzadeh S, Liu Y, Lin TY, Wu HW, Ji L, et al. Membrane-bound TRAIL supplements natural killer cell cytotoxicity against neuroblastoma cells. *J Immunother.* (2013) 36:319–29. doi: 10.1097/CJI.0b013e31829b4493
45. Williams BA, Law AD, Routy B, denHollander N, Gupta V, Wang XH, et al. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. *Oncotarget* (2017) 8:89256–68. doi: 10.18632/oncotarget.19204
46. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br J Haematol.* (2008) 143:641–53. doi: 10.1111/j.1365-2141.2008.07340.x
47. Shah N, Li L, McCarty J, Kaur I, Yvon E, Shaim H, et al. Phase I study of cord blood-derived natural killer cells combined with autologous stem cell transplantation in multiple myeloma. *Br J Haematol.* (2017) 177:457–66. doi: 10.1111/bjh.14570
48. Krieg S, Ullrich E. Novel immune modulators used in hematology: impact on NK cells. *Front Immunol.* (2012) 3:388. doi: 10.3389/fimmu.2012.00388
49. Szmania S, Lapteva N, Garg T, Greenway A, Lingo J, Nair B, et al. *Ex vivo*-expanded natural killer cells demonstrate robust proliferation *in vivo* in high-risk relapsed multiple myeloma patients. *J Immunother.* (2015) 38:24–36. doi: 10.1097/CJI.0000000000000059
50. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. *Blood* (1996) 87:1196–8.
51. Lieberman NAP, DeGolier K, Haberthur K, Chinn H, Moyes KW, Bouchlaka MN, et al. An uncoupling of canonical phenotypic markers and functional

potency of *ex vivo*-expanded natural killer cells. *Front Immunol.* (2018) 9:150. doi: 10.3389/fimmu.2018.00150

52. Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* (2010) 116:3853–64. doi: 10.1182/blood-2010-04-281675
53. Ruggeri L, Urbani E, André P, Mancusi A, Tosti A, Topini F, et al. Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. *Haematologica* (2016) 101:626–33. doi: 10.3324/haematol.2015.135301
54. McWilliams EM, Mele JM, Cheney C, Timmerman EA, Fiazuddin F, Strattan EJ, et al. Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. *Oncoimmunology* (2016) 5:e1226720. doi: 10.1080/2162402X.2016.1226720

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REVIEW
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CD38: A Target for Immunotherapeutic Approaches in Multiple Myeloma

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Multiple Myeloma (MM) is a hematological cancer characterized by proliferation of malignant plasma cells in the bone marrow (BM). MM represents the second most frequent hematological malignancy, accounting 1% of all cancer and 13% of hematological tumors, with ∼9,000 new cases per year. Patients with monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic smoldering MM (SMM) usually evolve to active MM in the presence of increased tumor burden, symptoms and organ damage. Despite the role of high dose chemotherapy in combination with autologous stem cell transplantation and the introduction of new treatments, the prognosis of MM patients is still poor, and novel therapeutic approaches have been tested in the last years, including new immunomodulatory drugs, proteasome inhibitors and monoclonal antibodies (mAbs). CD38 is a glycoprotein with ectoenzymatic functions, which is expressed on plasma cells and other lymphoid and myeloid cell populations. Since its expression is very high and uniform on myeloma cells, CD38 is a good target for novel therapeutic strategies. Among them, immunotherapy represents a promising approach. Here, we summarized recent findings regarding CD38-targeted immunotherapy of MM in pre-clinical models and clinical trials, including (i) mAbs (daratumumab and isatuximab), (ii) radioimmunotherapy, and (iii) adoptive cell therapy, using chimeric antigen receptor (CAR)-transfected T cells specific for CD38. Finally, we discussed the efficacy and possible limitations of these therapeutic approaches for MM patients.

Keywords: CD38, multiple myeloma, immunotherapy, preclinical models, clinical trials

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MULTIPLE MYELOMA AND CD38: BACKGROUND

Multiple myeloma (MM) is a neoplasm characterized by a clonal expansion of malignant plasma cells (PC) in the bone marrow (BM). MM arises from pre-malignant asymptomatic proliferation of PC, that are classified as monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM) (1). Patients with MGUS are characterized by low levels of serum M-protein (<3 g/dL) and monoclonal PC in BM (<10%), whereas patients with SMM display higher levels of serum M-protein (≥3 g/dL) and/or PC in the BM (≥10%). In contrast, diagnosis of MM includes the presence of end-organ damage associated with the presence of serum M-spike and/or

monoclonal PC in the BM (2, 3). Malignant transformation of PC, that are derived from post-germinal center B cells, is usually driven by multiple genetic and environmental changes. Indeed, different genetic abnormalities have been detected in MM and play a role in the pathogenesis of MM, including (i) translocation of chromosome 14 (t[14;16] and t[14;4]), (ii) MYC amplification, (iii) activation of NRAS and KRAS, (iv) mutations in FGFR3 and TP53, and (v) inactivation of cyclin-dependent kinase inhibitors CDKN2A and CDKN2C (4, 5). MM accounts 1% of all cancer, and represents the second most common hematological malignancy, with 25,000–30,000 new cases per year and an incidence of 5 cases per 100,000 (6, 7). The median age of MM patients at diagnosis ranged from 66 to 70 years, and only 37% of patients display an age below 65 years (7). The median survival of relapsed MM patients has increased from 12 months (before 2000) to 24 months after 2000, due to the availability of effective treatments (8). Modern therapies, such as immunomodulatory drugs and proteasome inhibitors, have further prolonged the 5- and 10-years survival rates of MM patients, and a doubling of the median survival time has been observed in patients diagnosed in the last decade (8). However, prognosis of relapsed MM patients is still poor, and novel therapeutic approaches are urgently needed. In this context, CD38 represents a promising therapeutic target, since its expression is high and uniform on malignant PC, whereas it is relatively low on normal lymphoid and myeloid cells and on non-hematopoietic tissues. CD38 is a 45 KDa surface glycoprotein, firstly identified as an activation marker (9): successively the molecule was reported as an adhesion molecule, able to interact with endothelial CD31 (10). These finding highlighted the possibility that CD38 may act as a receptor, notwithstanding a structural ineptitude to do so. It was shown indeed that CD38 act as an accessory component of the synapse complex (11). CD38 was then identified as an ectoenzyme involved in the metabolism of extracellular nicotinamide adenine dinucleotide (NAD^+) and cytoplasmic nicotinamide adenine dinucleotide phosphate (NADP) (12). The results is the production of Ca^{2+}-mobilizing compounds, such as cyclic adenosine diphosphate [ADP] ribose, ADP ribose (ADPR) and nicotinic acid adenine dinucleotide phosphate. CD38 enzymatic activities were shown as able to rule the NAD^+ levels and improve the function of proteasome inhibitors (13). Further, ADPR produced by CD38 can be further metabolized by the concerted action of CD203a/PC-1 and CD73, to produce the immunosuppressive molecule adenosine (ADO). This feature points out the role of CD38 in the escape of tumor cells from the control of the immune system (14).

CD38-TARGETED IMMUNOTHERAPEUTIC STRATEGIES: RATIONALE, APPLICATIONS AND LIMITATIONS

It has been demonstrated that conventional therapies, such as vincristine and doxorubicin, induce the expression of multidrug resistance genes and p-glycoprotein in tumor cells, that become resistant to different drugs (15). Thus, conventional therapies may be combined with immunotherapeutic strategies targeting CD38 to improve their efficacy. Indeed, it has been already demonstrated that combined therapies simultaneously target multiple pathways and prevent escape/resistance mechanisms of tumor cells. Moreover, combination of tumor-specific mAbs and standard chemotherapy is already a standard-of-care in several hematologic (Hodgkin's lymphoma and CLL) and solid (breast cancer and colon carcinoma) tumors (16).

In the context of MM, we have recently demonstrated that, within the bone niche, only PCs express CD38 at high levels. Moreover, CD38 expression can be detected on monocytes and early osteoclast progenitors but not on osteoblasts and mature osteoclasts, thus suggesting that CD38 expression was lost during *in vitro* osteoclastogenesis. Accordingly, we found that Daratumumab inhibited *in vitro* osteoclastogenesis and bone resorption activity from BM total mononuclear cells of MM patients, targeting CD38 expressed on monocytes and early osteoclast progenitors (17). In addition, several studies reported that anti-CD38 mAbs are able to deplete $CD38^+$ immunosuppressive cells, such as myeloid-derived suppressor cells, regulatory T cells and regulatory B cells, leading to an increased anti-tumor activity of immune effector cells (18, 19).Thus, these data provide a rationale for the use of an anti-CD38 antibody-based approach as treatment for MM patients.

However, CD38 is known to be also detectable on other normal cell subsets, such as NK cells, B cells and activated T cells and the use of anti CD38 abs could thus affect the activity of normal cells. NK cells specifically play a pivotal role for the therapeutic effects of anti-CD38 mAbs, since they mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP).This issue can be addressed by using anti-CD38 F(ab')2 fragments to protect normal cells from subsequent anti-CD38 mAb-mediated lysis, or by infusion of *ex-vivo* expanded NK cells (20).

Another possible limitation of CD38-targeted therapy may be represented by the variable expression of CD38 on malignant PC. In particular, CD38 expression may be downregulated following the first infusions of anti-CD38 mAbs, favoring immune escape and disease progression (21). On this regard, combined therapy has been proposed to increase CD38 expression on malignant cells, using a pan–histone deacetylase inhibitor (Panobinostat) (22) or all-trans reticnoic acid (ATRA) (23). These studies have demonstrated that anti-CD38 mAb-mediated ADCC dramatically increased *in vitro* after the treatment, following the up-regulation of CD38 expression on MM cells (22, 23).

Anti-CD38 treatment may also generate resistance and induce tumor immune escape, through the up-regulation of two complement inhibitor proteins, CD55 and CD59 on MM cells. However, Nijhof and coworkers have demonstrated that ATRA treatment is also able to reduce CD55 and CD59 expression on anti-CD38-resistant MM cells, thus supporting the use of a combined therapy to improve complement-mediated cytotoxicity (CDC) against malignant cells (21).

In the last years, several novel immunotherapeutic approaches have been tested for MM patients, using CD38 as target, both in preclinical models and in clinical trials. These strategies include (i) mAbs specific for CD38, (ii) radioimmunotherapy, using

radionuclides targeted to CD38 molecule, and (iii) adoptive cell therapy, using T cells transfected with a chimeric antigen receptor (CAR) specific for CD38.

Anti-CD38 mAbs

Development of mAbs against CD38 started in 1990 and anti-CD38 mAbs have been tested as immunotherapeutic strategy for MM patients, so far with limited beneficial effects. The anti-tumor effect of anti-CD38 mAbs is related to their ability to induce ADCC, CDC and ADCP of opsonized $CD38^+$ cells. Moreover, anti-CD38 mAbs can induce a direct apoptosis of $CD38^+$ MM cells via Fc-γ receptor-mediated crosslinking (24). Crosslinking of anti-CD38 mAbs on MM cells leads to clustering of cells, phosphatidylserine translocation, loss of mitochondrial membrane potential, and loss of membrane integrity. This effect is called homotypic aggregation, and may be related or not to caspase-3 cleavage (25). The mechanism(s) of action of anti-CD38 mAbs on MM cells are represented in **Figure 1**.

Here, we summarized novel findings obtained using anti-CD38 mAbs as therapeutic strategy for MM *in vitro*, in preclinical studies and, finally, in clinical trials.

Daratumumab

Daratumumab is a human anti-CD38 mAb, which is able to trigger ADCC and CDC *in vitro* against $CD38^+$ tumor cells, using either autologous or allogeneic effector cells. Daratumumab-mediated ADCC and CDC *in vivo* is not affected by the presence of BM stromal cells, thus suggesting that this mAb can kill MM tumor cells in a tumor-preserving BM microenvironment. Moreover, Daratumumab is able to inhibit tumor growth in xenograft models at low doses (26). Another study demonstrated that Daratumumab is able to trigged programmed cell death (PCD) of MM $CD38^+$ cells when cross-linked *in vitro* by secondary mAbs or via an FcγR. Moreover, in a syngeneic *in vivo* tumor model, Daratumumab is able to induce PCD of MM cells, through the cross-linking mediated by both inhibitory FcγRIIb and activating FcγRs. These data suggested that the therapeutic effect of Daratumumab may be at least in part related to the induction of PCD of MM cells through cross-linking (25). The interaction between soluble Daratumumab and FcRs appears critical for the action of the antibody. The marked polar aggregation is followed by a significant release of microvesicles (MV) (27). Generation of MV is a physiological event: the difference with the same MV after antibody treatment is the fact that they are covered with

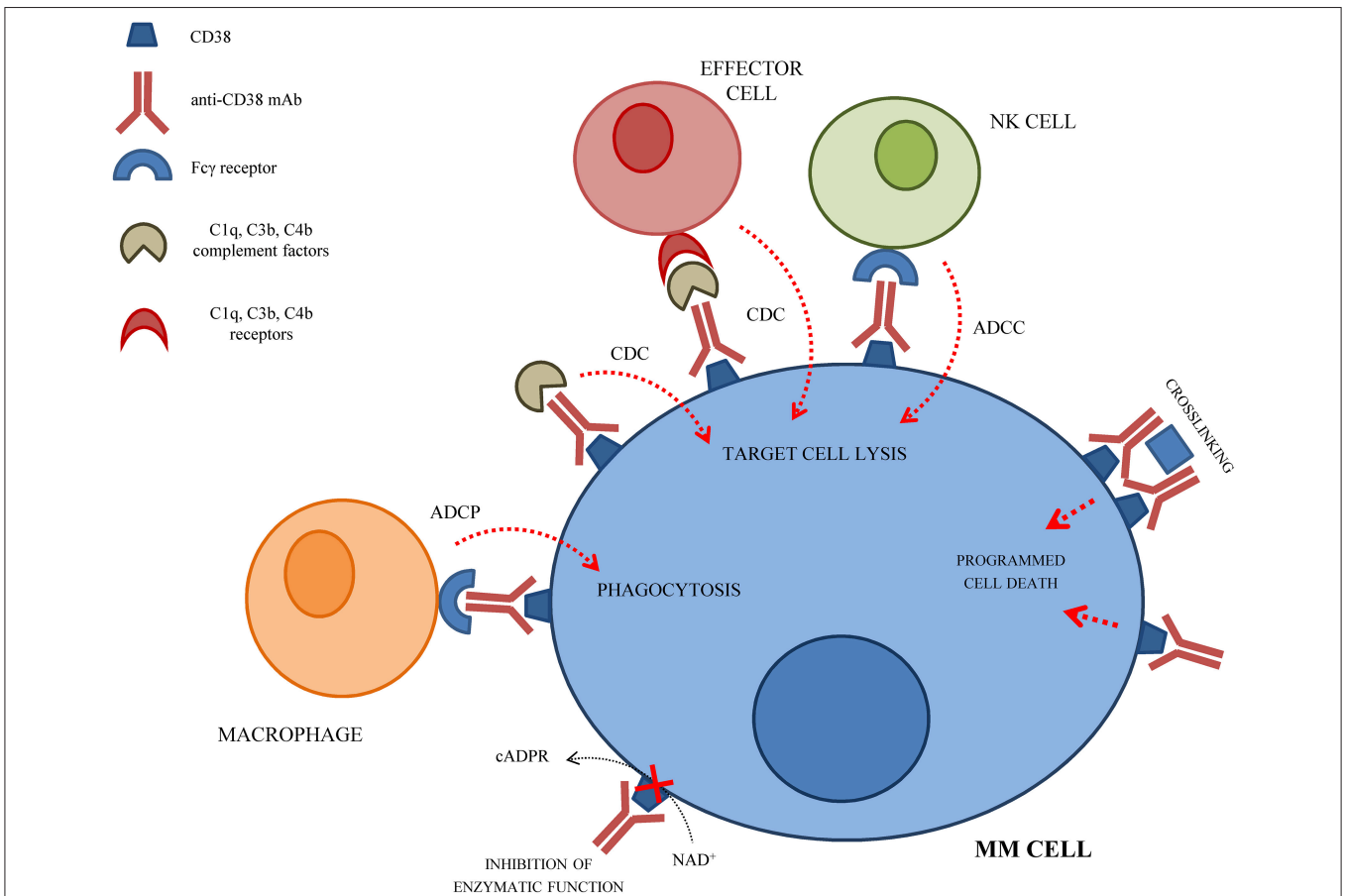

FIGURE 1 | Schematic representation of the mechanism(s) of action of anti-CD38 mAbs on MM cells.

the therapeutic IgG. This makes their destination mandatory to FcR-expressing cells and tissues (28). CD38 is expressed at high levels in BM niche only by PC. However, its expression can be detected at lower levels also on monocytes and early osteoclast progenitors, but not on mature osteoblasts and osteoclasts, since CD38 expression is downregulated during *in vitro* osteoclastogenesis (17). Consistently, it has been demonstrated that Daratumumab reacts with CD38 expressed on monocytes and inhibited *in vitro* osteoclastogenesis and bone resorption activity from BM total mononuclear cells (MNC) of MM patients, by targeting $CD38^+$ osteoclast progenitors. Thus, Daratumumab may be effective also to prevent osteoclastogenesis induced by MM (17). The anti-tumor efficacy of Daratumumab may be increased by the combination with immunomodulatory drugs. One study analyzed the combined effect of human anti-CD38 mAb Daratumumab and lenalidomide, a drug that is able stimulate the immune system and to induce apoptosis of tumor cells and inhibition of angiogenesis. They have demonstrated that effector cells derived from peripheral blood (PB) MNC from healthy individuals pretreated with lenalidomide displayed *in vitro* an increased ADCC mediated by Daratumumab against primary $CD38^+$ MM cells and UM-9 MM cell line. Same results were obtained using BM MNC of MM patients, thus indicating that lenalidomide can increase Daratumumab-mediated lysis of MM cells by activating autologous effector cells within the natural environment of malignant cells. Finally, they have demonstrated an increased Daratumumab-dependent ADCC against MM cells using PB derived from lenalidomide-treated MM patients as effector cells. These data suggested that the combination of lenalidomide and Daratumumab may represent an effective novel therapeutic strategy for MM patients (29). This conclusion was confirmed by another study, where Daratumumab was combined with lenalidomide and bortezomib (30). Daratumumab induced lysis of (i) MM cells that were resistant to lenalidomide and bortezomib and (ii) primary MM cells using BM MNC derived from MM patients that were refractory to lenalidomide and/or bortezomib treatment. This study confirmed that lenalidomide (but not bortezomib) synergistically enhanced Daratumumab-mediated lysis of MM cells through activation of NK cells. Moreover, the combination of daratumumab with lenalidomide effectively reduced the growth of primary MM cells from a lenalidomide- and bortezomib-refractory patient *in vivo* using a xenograft model (30). We summarized the clinical results obtained with Daratumumab in a recent Review article (31).

Isatuximab

Isatuximab (formerly known as SAR650984) is a humanized anti-CD38 mAb that exerts a strong pro-apoptotic activity independent of cross-linking agents, and potent anti-tumor activity related to CDC, ADCC and ADCP. These functions are equivalent *in vitro* to those observed for rituximab in $CD20^+$ and $CD38^+$ models. Moreover, isatuximab is able to partially inhibit ADP-ribosyl cyclase activity of CD38, through an allosteric antagonism (32). Additional mechanism of action have been characterized by Jiang et al., who have demonstrated that isatuximab is able to induce homotypic aggregation-associated cell death in MM cells, that is related to the level of CD38 expression on cell surface and depends on actin cytoskeleton and membrane lipid raft (33). Isatuximab and its F(ab)'2 fragments also induce (i) apoptosis of MM cells highly expressing CD38, through the activation of caspase 3 and 7, (ii) lysosome-dependent cell death by enlarging lysosomes and increasing permeabilization of lysosomal membrane, and (ii) upregulation of reactive oxygen species. It has been also demonstrated that SAR650984-mediated killing of MM cells is enhanced by the antitumoral drug pomalidomide, even in MM cells resistant to pomalidomide/lenalidomide (33). Feng and coworkers have demonstrated that isatuximab is able to decrease the frequency of $CD38^{hi}$ Treg and to increase the frequency of $CD4^+CD25^-$ T cells. Treatment with isatuximab downmodulate Foxp3 and IL10 in Tregs and restores proliferation and function of T cells. Furthermore, isatuximab increases MM cell lysis by $CD8^+$ T and NK cells *in vitro* (34). MM cells are able to induce the expansion of $CD38^{hi}$ Tregs *in vitro* when cultured with $CD4^+CD25^-$ T cells. In this context, isatuximab is able to inhibit the expansion of inducible Tregs by MM cells and stromal cells, by inhibiting cell-to-cell contact and release of TGFβ/IL10. Thus, this study demonstrated that isatuximab, through CD38 targeting, is able to revert MM-induced immunosuppression and to restore anti-MM immune effector cell functions (34). Finally, it has been demonstrated that isatuximab was effective to eradicate malignant cells *in vivo* in xenograft models of different hematological $CD38^+$ human tumors, including MM. This anti-tumor activity was more potent than that of bortezomib in MM xenograft models set up using NCI-H929 and Molp-8 MM cell lines. More importantly, isatuximab demonstrated a potent pro-apoptotic activity against $CD38^+$ human primary MM cells (32). Taken together, these findings supported the use of isatuximab in phase 1 clinical studies for MM patients, alone or in combination with other drugs such as pomalidomide or lenalidomide.

CD38-Specific Chimeric mAbs and Nanobodies

In the past, several anti-CD38 mAb have been developed and tested for their ability to induce ADCC and CDC against $CD38^+$ MM cells. Stevenson and coworkers have developed a chimeric anti-CD38 mAb, composed by the Fab portion of OKT10 murine mAb linked to a human IgG1 Fc fragment. This chimeric mAb, but not the parental mAb, mediated ADCC using human mononuclear effector cells, and displayed limited side effects on other $CD38^+$ cell populations (i.e., NK cells and granulocyte/macrophage or erythroid progenitor cells). Chimeric mAb induced ADCC using cells isolated from 14 MM patients subjected to various chemotherapeutic regimes, and such function was similar to that observed in normal individuals, thus suggesting that treatment with anti-CD38 chimeric antibody may be effective in these patients (35). Similarly, Ellis and coworkers developed a humanized IgG1 mAb and a chimeric mAb (composed by mouse Fab cross-linked to two human gamma 1 Fc fragments) against CD38. Both mAbs efficiently directed ADCC against $CD38^+$ cell lines, without down-modulating CD38 expression or enzymatic activity, thus representing a promising therapeutic strategy against MM and other diseases involving $CD38^+$ cells (36).

More recently, different studies have been aimed at the generation of novel mAbs targeting CD38. In one of these studies, a series of nanobodies against CD38 with high affinities have been generated. The authors identified the epitopes that bind these nanobodies on the carboxyl domain of CD38 molecule. Next, they binded these nanobodies to fluorescent proteins to quantify CD38 expression then confirming the higher CD38 expression on MM cells as compared to normal leukocytes. More importantly, they have generated an immunotoxin, binding nanobodies with a bacterial toxin, that displayed a highly selective cytotoxicity against patient-derived MM cells and MM cell lines, even at very low concentrations. Such effect can be further enhanced by stimulating CD38 expression using retinoid acid. These results suggested that these anti-CD38 nanobodies may represent a novel diagnostic and therapeutic tool for MM patients (37). The development of anti-CD38 nanobodies has been carried out also by Fumey and coworkers. They have identified 22 nanobody families specific for CD38 molecule from llamas immunized with recombinant non-glycosylated CD38 ecto-domain, using a phage display technology (38). They performed cross-blockade analyses by flow cytometry using CD38-transfected cells, and an in-tandem epitope binding using CD38 molecule immobilized on biosensors, demonstrating that these nanobody families recognize three different non-overlapping epitopes, with four nanobody families showing a complementary binding to Daratumumab. Three nanobody families inhibit the enzymatic activity of CD38 *in vitro*, while two other families act as enhancers. All nanobodies also recognized native CD38 on tumor cells and lymphoid cells (T, B, and NK cells), and some of them still recognized tumor cells after opsonization with daratumumab, thus suggesting that these nanobodies recognized a different epitope. Finally, fluorochrome-conjugated CD38 nanobodies efficiently reach $CD38^+$ tumors in a rodent model within 2 h after intravenous injection, thus allowing *in vivo* tumor imaging. This study suggested that anti-CD38 nanobodies may be effective for the modulation of CD38 enzymatic activity and for the diagnosis of CD38-expressing tumors, also in patients treated with daratumumab (38). Barabas and colleagues have developed novel anti-CD38 mAbs by injecting an immune complex, composed by CD38 antigen and homologous anti-CD38 lytic IgG mAbs, in rabbits. Recipient rabbits produced mAbs with the same specificity against CD38 antigen. Such mAbs demonstrated *in vitro* a potent agglutinating, precipitating and lytic function. Moreover, in the presence of complement, donor and recipient rabbits' immune sera lysed $CD38^+$ MM cells *in vitro*. Thus, they demonstrated that this "third vaccination" method has good potential for MM therapy (39). Moreover, they have demonstrated that passive immunization of SCID mice injected subcutaneously with human MM cells with heterologous anti-CD38 IgG antibody containing serum significantly decreased cancer growth in the presence of complement, thus confirming the efficacy of this methods also in preclinical models (40).

Radioimmunotherapy

Since malignant PC are very radiosensitive, CD38 has been used as target for radioimmunotherapy (RIT) in preclinical models of MM. Green and coworkers investigated both conventional RIT (directly radiolabeled antibody) and streptavidin-biotin pretargeted RIT (PRIT) directed against CD38 as therapeutic approach to deliver radiation doses sufficient for MM cell eradication. They demonstrated that the biodistribution was increased using PRIT as compared to conventional RIT. They achieved a tumor/blood ratio of 638:1 24 h after PRIT, whereas ratios never exceeded 1:1 with conventional RIT. (90)Yttrium absorbed dose displayed an excellent target/normal organ ratios (6:1 for kidney, lung and liver; 10:1 for whole body). Moreover, they observed an objective remission of MM in 100% of mice treated with doses ranging from 800 to 1,200 μCi of anti-CD38 pre-targeted (90)Y-DOTA-biotin 7 days after the treatment, with a complete remission at day 23, with undetectable tumor masses. Moreover, 100% of mice bearing MM xenografts treated with 800 μCi of anti-CD38 pre-targeted (90)Y-DOTA-biotin achieved a long-term tumor-free survival (more than 70 days) compared with 0% in the control group (41). Since immunogenicity and endogenous biotin blockade may limit the clinical translation of PRIT, the authors developed a new approach based on the use of an anti-CD38 bispecific fusion protein conjugated with 90Y. This protein eliminates the interference due to biotin and is less immunogenic, and demonstrated an excellent blood clearance and targeting of MM cells in xenograft models. Indeed, they demonstrated a high tumor-absorbed dose and, more importantly, a high tumor-to-normal organ dose ratios (7:1 for liver and 15:1 for lung and kidney), thus demonstrating that fusion protein targets tumor cells but not normal tissues. They obtained a 100% of complete remissions at day 12 and 80% of mice cured at optimal doses (1,200 μCi), thus demonstrating an efficacy of the fusion protein equal to streptavidin-biotin-based PRIT. Furthermore, bispecific proteins display a superior efficacy as compared to the latter method, in terms of overall survival, using lower radiation doses (600–1,000 μCi). Thus, bispecific PRIT represents an attractive candidate for clinical translation, especially for MM patients with refractory disease, which typically retained sensitivity to radiation (42).Teiluf and coworkers tested radioimmunoconjugates, consisting of the α-emitter ^{213}Bi conjugated to anti-CD38 mAb in preclinical models of MM. ^{213}Bi-anti-CD38 mAb was effective in the induction of DNA double-strand breaks in different MM cell lines, inducing apoptosis, cell cycle arrest and mitotic arrest, with subsequent mitotic catastrophe. The anti-tumor effect of therapeutic strategy correlated with the expression level of CD38 on MM cell lines. More importantly, they demonstrated that mice bearing MM xenografts treated with ^{213}Bi-anti-CD38 mAb display a limited tumor growth via induction of apoptosis in tumor tissue, and a significantly prolonged survival compared to controls. Moreover, no signs of ^{213}Bi-induced toxicity was observed in the major organ systems (43). These studies suggest that CD38-targeted RIT may represent a promising therapeutic tool for MM patients.

Cellular Therapy

Recent findings suggest that CD38 may represent a good target for antigen-specific adoptive cell therapy. Indeed, T cells expressing CAR have been successfully used in several clinical trials for solid and hematological tumors (44). Moreover, CAR T cells specific for different MM associated antigens, such as

CS1 (45), B-cell maturation antigen (46), SLAMF7 (47), and CD19 (48) proved to be effective in preclinical models and/or in clinical trials. Mihara and coworkers developed anti-CD38 CAR T cells through retroviral vector-mediated transduction of the transmembrane domain of CD8α, the intracellular domains of 4–1BB and CD3ζ and anti-CD38 single-chain variable domain (scFv). Anti-CD38 CAR T cells displayed cytotoxic activity *in vitro* against either MM cell lines or primary MM cells isolated from patients. Thus, these cells may represent a powerful therapeutic tool in preclinical models of MM (49). This issue was addressed by Drent et al., who tested anti-CD38 CAR T cells *in vivo* using a xenotransplant model (using UM9 MM cell line), in which MM cells were grown in a humanized BM microenvironment. Anti-CD38 CAR T cells demonstrated a potent anti-tumor effect when administered intravenously or intratumorally, thus suggesting that these cells efficiently migrate,

TABLE 1 | CD38-targeted ongoing clinical trials (www.clinicaltrials.gov).

Study title	Interventions	Status
Study to evaluate the safety and efficacy of anti-CD38 CAR-T in relapsed or refractory multiple myeloma patients	Biological: Anti-CD38 A2 CAR-T cells	Recruiting
Daratumumab (HuMax®-CD38) safety study in multiple myeloma	Drug: Daratumumab plus Methylprednisolone and Dexamethasone	Completed
Monoclonal antibodies for treatment of multiple myeloma. emphasis on the CD38 antibody Daratumumab	Drug: Daratumumab plus Lenalidomide and Dexamethasone	Completed
A Phase I/IIa study of human anti-CD38 antibody MOR03087 (MOR202) in relapsed/refractory multiple myeloma	Drug: MOR03087 phase 1 dose escalation plus Dexamethasone and others	Active, not recruiting
A study of JNJ-54767414 (HuMax CD38) (Anti-CD38 monoclonal antibody) in combination with backbone treatments for the treatment of patients with multiple myeloma	Drug: Daratumumab plusVelcade, Pomalidomide and others	Active, not recruiting
Phase II study of the CD38 antibody Daratumumab in patients with high-risk MGUS and low-risk smoldering multiple myeloma	Drug: Daratumumab	Recruiting
CAR-T cells therapy in relapsed/refractory multiple myeloma	Biological: Anti-CD38 CAR-T Cells	Recruiting
Isatuximab single agent study in japanese relapsed and refractory multiple myeloma patients	Drug: Isatuximab SAR650984	Active, not recruiting
SAR650984 in combination with Carfilzomib for treatment of relapsed or refractory multiple myeloma	Drug: SAR650984 plus Carfilzomib	Recruiting
Study of GBR 1342, a CD38/CD3 Bispecific antibody, in subjects with previously treated multiple myeloma	Biological: GBR 1342	Recruiting
Efficacy and safety study of Pembrolizumab (MK-3475) in combination with Daratumumab in participants with relapsed refractory multiple myeloma	Biological: Pembrolizumab plus Daratumumab	Not yet recruiting
SAR650984 (Isatuximab), Lenalidomide, and Dexamethasone IN Combination in RRMM patients	Drug: isatuximab SAR650984 plus lenalidomide and dexamethasone	Active, not recruiting
2015-12: a study exploring the use of early and late consolidation/maintenance therapy	Drug: Daratumumab plus carfilzomib, thalidomide, dexamethasone and others	Recruiting
Daratumumab in combination With ATRA	Drug: Daratumumab plus all-trans retinoic acid (ATRA)	Recruiting
Daratumumab in combination with Bortezomib and Dexamethasone in subjects with relapsed or relapsed and refractory multiple myeloma and severe renal impairment	Drug: Daratumumab plus bortezomib and dexamethasone	Recruiting
Study of Isatuximab Combined With Bortezomib + Cyclophosphamide + Dexamethasone (VCD) and Bortezomib + Lenalidomide + Dexamethasone (VRD) in newly diagnosed multiple myeloma (MM) non-eligible for transplant	Drug: Daratumumab plus lenalidomide, bortezomib, cyclophosphamide and others	Recruiting
SAR650984, Pomalidomide and Dexamethasone in combination in RRMM patients	Drug: Isatuximab SAR650984 plus pomalidomide and dexamethasone	Active, not recruiting
Daratumumab in Treating Patients With multiple myeloma	Biological: Daratumumab	Active, not recruiting
Daratumumab, Thalidomide and Dexamethasone in Relapse and/or refractory myeloma	Drug: Daratumumab plus thalidomide and dexamethasone	Not yet recruiting
Copper 64Cu-DOTA-Daratumumab positron emission tomography in diagnosing patients with relapsed multiple myeloma	Biological: Daratumumab plus imaging agent using positron emission tomography	Recruiting
Daratumumab in treating transplant-eligible participants with multiple myeloma	Drug: Daratumumab plus autologous hematopoietic stem cell transplantation	Recruiting
Daratumumab after stem cell transplant in treating patients with multiple myeloma	Drug: Daratumumab plus autologous hematopoietic stem cell transplantation and melphalan	Not yet recruiting
Multi-CAR T cell therapy in the treatment of multiple myeloma	Biological: Anti-CD38 CAR-T cells	Recruiting

TABLE 2 | Response rates in CD38-targeted ongoing clinical trials.

Study	Response rate		
	ORR	**PR**	**CR**
Daratumumab Plus Lenalidomide and Dexamethasone (DRd) vs. Lenalidomide and Dexamethasone (Rd) (56)	DRd: 93% Rd: 76%	DRd: 78% Rd: 45%	DRd: 46% Rd: 20%
Daratumumab plus Pomalidomide and Dexamethasone (D,pom/dex) vs. Pomalidomide and Dexamethasone (pom/dex) (57, 58)	D, pom/dex: 60% pom/dex: 47%	D, pom/dex: 43% pom/dex: 32%	D, pom/dex: 17% pom/dex: 15%
Daratumumab Plus Bortezomib and Dexamethasone (DVd) vs. Bortezomib and Dexamethasone (Vd) (59)	DVd: 84% Vd: 63%	DVd: 62% Vd: 29%	DVd: 26% Vd: 10%

infiltrate, and eliminate human MM tumors growing in their natural niche. This study demonstrates that CAR mediated targeting of $CD38^+$ MM cells represents a promising therapeutic strategy for MM patients (50). The same authors tested different antibody sequences, and demonstrated that anti-CD38 CART T cells are able to proliferate, to secrete pro-inflammatory cytokines and to lyse malignant cells, irrespective of the donor and antibody sequence. Moreover, they demonstrated that CAR T cells lyse the $CD38^+$ fractions of $CD34^+$ hematopoietic progenitor cells, monocytes, natural killer cells, and to a lesser extent T and B cells. However, they did not inhibit the outgrowth of progenitor cells into myeloid lineages and, furthermore, they were effectively controllable with a caspase-9-based suicide gene, thus guaranteeing the safety of this approach (51). In this line, the same authors recently developed anti-CD38 CAR T cells with a lower affinity for CD38 antigen. They used the "light-chain exchange" technology to combine the heavy chains of two high-affinity CD38 antibodies with 176 different germline light chains, thus generating more than 100 new antibodies with a lower affinity (10- to 1,000- fold) to CD38. Among them, they identified eight antibodies and they isolated the corresponding single-chain variable fragments to generate new anti-CD38 CAR T cells. These cells displayed a 1,000-fold reduced affinity for CD38, and were able to proliferate, produce Th1-like cytokines and, more importantly, to lyse $CD38^{hi}$ MM cells but not $CD38^{low}$ normal cells, either *in vitro* or *in vivo*. Thus, this approach allow to generate CAR T cells highly specific for tumor-associated antigens that are also expressed at low intensity by normal cells (52). These studies confirmed that anti-CD38 CAR T cells may represent a novel and effective therapeutic tool for MM patients. Indeed, three clinical trials based on CD38 CAR T cells are currently recruiting MM patients (www.clinicaltrials.gov).

A limitation on the use of CD38-specific CAR T cells may be represented by a possible toxicity of this approach, due to the presence of CD38 on normal cells, such as NK cells, activated T cells and B cells, as mentioned before. In this line, Drent et al. designed a novel class of doxycycline (DOX)-inducible CD38-specific CAR T cells, that are rapidly inactivated by low doses of DOX, allowing to control off-tumor effects within 24 h. Thus, this strategy adds a second level of safety in CAR T cell-mediated therapy of MM patients, allowing to control the activity of CAR T cells without destroying them permanently (53). Another possible limitation is represented by the variable expression of CD38 on myeloma cells. As mentioned before, ATRA may be administered in combination with CD38-specific CAR T cells to up-regulate CD38 expression on malignant cells and consequently to improve CAR T cell-mediated anti-tumor activity. In this line, Mihara et al. have demonstrated that ATRA increases the cytotoxic activity of anti-CD38 CAR T cells against (i) acute myeloid leukemia (AML) cell lines and (ii) primary AML blasts from patients (54).

On the other hand, the anti-tumor activity of CD38-specific CAR T cells may be enhanced through the combination of these cells with conventional therapies, such as checkpoint inhibitors. Indeed, it has been demonstrated that PD-1 inhibitor pembrolizumab (PEM) increased and/or prolonged detection of circulating anti-CD19 CAR T cells in acute lymphoblastic leukemia (ALL) patients. Consequently, anti-tumor activity of CAR T cells was dramatically improved in PEM-treated patients (55).

CONCLUSIONS

The findings here reported confirmed that CD38 represents a good target for immunotherapeutic approaches for MM patients. Indeed, the efficacy of therapeutic strategies based on the use of mAbs or CAR T cells specific for CD38 has been demonstrated *in vitro* and in preclinical studies. More importantly, some of these therapeutic approaches have already been translated to the clinic, with promising results either as monotherapy or in combination with chemotherapeutic drugs. Currently, 23 clinical trials based on CD38 as target are ongoing (3 not yet recruiting, 12 recruiting, 6 active, and 2 completed, www.clinicaltrials.gov, **Table 1**).

Response rates for ongoing clinical trials with available clinical data are reported in **Table 2**. These studies confirmed that the combination of anti-CD38 mAbs with conventional therapies dramatically improved the clinical outcome of MM patients (56–59).Thus, further studies aimed at the characterization of novel combined therapies that include anti-CD38 immune effectors might be pivotal to design effective clinical strategies to increase progression-free and overall survival of MM patients.

AUTHOR CONTRIBUTIONS

FM analyzed data present in the literature and wrote the manuscript. ALH, FC, NG, FMal and VP contributed to the writing of the final version of the manuscript.

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REFERENCES

1. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. *Nat Rev Cancer* (2002) 2:175–87. doi: 10.1038/nrc746
2. International Myeloma Working Group Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. *Br J Haematol.* (2003) 121:749–57. doi: 10.1046/j.1365-2141.2003.04355.x
3. Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia* (2010) 24:1121–7. doi: 10.1038/leu.2010.60
4. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. *Blood* (2006) 108:2020–8. doi: 10.1182/blood-2005-11-013458
5. Avet-Loiseau H, Attal M, Moreau P, Charbonnel C, Garban F, Hulin C, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. *Blood* (2007) 109:3489–95. doi: 10.1182/blood-2006-08-040410
6. Phekoo KJ, Schey SA, Richards MA, Bevan DH, Bell S, Gillett D, et al. A population study to define the incidence and survival of multiple myeloma in a National Health Service Region in UK. *Br J Haematol.* (2004) 127:299–304. doi: 10.1111/j.1365-2141.2004.05207.x
7. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
8. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, et al. Improved survival in multiple myeloma and the impact of novel therapies. *Blood* (2008) 111:2516–20. doi: 10.1182/blood-2007-10-116129
9. Funaro A, Spagnoli GC, Ausiello CM, Alessio M, Roggero S, Delia D, et al. Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J Immunol.* (1990) 145:2390–6.
10. Deaglio S, Morra M, Mallone R, Ausiello CM, Prager E, Garbarino G, et al. Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J Immunol.* (1998) 160:395–402.
11. Munoz P, Mittelbrunn M, De La Fuente H, Perez-Martinez M, Garcia-Perez A, Ariza-Veguillas A, et al. Antigen-induced clustering of surface CD38 and recruitment of intracellular CD38 to the immunologic synapse. *Blood* (2008) 111:3653–64. doi: 10.1182/blood-2007-07-101600
12. Howard M, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RM, et al. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* (1993) 262:1056–9. doi: 10.1126/science.8235624
13. Cagnetta A, Cea M, Calimeri T, Acharya C, Fulciniti M, Tai YT, et al. Intracellular NAD(+) depletion enhances bortezomib-induced anti-myeloma activity. *Blood* (2013) 122:1243–55. doi: 10.1182/blood-2013-02-483511
14. Chillemi A, Quarona V, Antonioli L, Ferrari D, Horenstein AL, Malavasi F. Roles and modalities of ectonucleotidases in remodeling the multiple myeloma niche. *Front Immunol.* (2017) 8:305. doi: 10.3389/fimmu.2017.00305
15. Yang W-C, Lin S-F. Mechanisms of drug resistance in relapse and refractory multiple myeloma. *BioMed Res Int.* (2015) 2015:17. doi: 10.1155/2015/341430
16. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat Rev Immunol.* (2010) 10:317–27. doi: 10.1038/nri2744
17. Costa F, Toscani D, Chillemi A, Quarona V, Bolzoni M, Marchica V, et al. Expression of CD38 in myeloma bone niche: a rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation. *Oncotarget* (2017) 8:56598–611. doi: 10.18632/oncotarget.17896
18. Krejcik J, Casneuf T, Nijhof IS, Verbist B, Bald J, Plesner T, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* (2016) 128:384–94. doi: 10.1182/blood-2015-12-687749
19. Zhang L, Tai YT, Ho M, Xing L, Chauhan D, Gang A, et al. Regulatory B cell-myeloma cell interaction confers immunosuppression and promotes their survival in the bone marrow milieu. *Blood Cancer J.* (2017) 7:e547. doi: 10.1038/bcj.2017.24
20. Cherkasova E, Espinoza L, Kotecha R, Reger RN, Berg M, Aue G, et al. Treatment of *ex vivo* expanded NK cells with Daratumumab F(ab')2 fragments protects adoptively transferred NK cells from Daratumumab-mediated killing and augments Daratumumab-induced Antibody Dependent Cellular Toxicity (ADCC) of myeloma. *Blood* (2015) 126:4244.
21. Nijhof IS, Casneuf T, Van Velzen J, Van Kessel B, Axel AE, Syed K, et al. CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma. *Blood* (2016) 128:959–70. doi: 10.1182/blood-2016-03-703439
22. Garcia-Guerrero E, Gogishvili T, Danhof S, Schreder M, Pallaud C, Perez-Simon JA, et al. Panobinostat induces CD38 upregulation and augments the antimyeloma efficacy of Daratumumab. *Blood* (2017) 129:3386–8. doi: 10.1182/blood-2017-03-770776
23. Nijhof IS, Lokhorst HM, Van Kessel B, Groen RWJ, Martens AC, Doshi P, et al. Modulation of CD38 expression levels on multiple myeloma tumor cells by all-trans retinoic acid improves the efficacy of the anti-CD38 monoclonal antibody Daratumumab. *Blood* (2014) 124:2096.
24. Jansen JHM, Boross P, Overdijk MB, Van Bueren JJL, Parren PWHI, Leusen JHW. Daratumumab, a human CD38 antibody induces apoptosis of myeloma tumor cells via Fc receptor-mediated crosslinking. *Blood* (2012) 120:2974.
25. Overdijk MB, Jansen JHM, Nederend M, Lammerts Van Bueren JJ, Groen RWJ, Parren PWHI, et al. The therapeutic CD38 monoclonal antibody daratumumab induces programmed cell death via Fcγ receptor–mediated cross-linking. *J Immunol.* (2016) 197:807–13. doi: 10.4049/jimmunol.1501351
26. De Weers M, Tai YT, Van Der Veer MS, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol.* (2011) 186:1840–8. doi: 10.4049/jimmunol.1003032
27. Malavasi F, Chillemi A, Castella B, Schiavoni I, Incarnato D, Oliva S, et al. CD38 and Antibody therapy: what can basic science add? *Blood* (2016) 128:SCI–36.
28. Morandi F, Marimpietri D, Horenstein AL, Bolzoni M, Toscani D, Costa F, et al. Microvesicles released from multiple myeloma cells are equipped with ectoenzymes belonging to canonical and non-canonical adenosinergic pathways and produce adenosine from ATP and NAD+. *Oncoimmunology* (2018) 7:e1458809. doi: 10.1080/2162402X.2018.1458809
29. Van Der Veer MS, De Weers M, Van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. Towards effective immunotherapy of myeloma: enhanced elimination of myeloma cells by combination of lenalidomide with the human CD38 monoclonal antibody daratumumab. *Haematologica* (2011) 96:284–90. doi: 10.3324/haematol.2010.030759
30. Nijhof IS, Groen RW, Noort WA, Van Kessel B, De Jong-Korlaar R, Bakker J, et al. Preclinical evidence for the therapeutic potential of CD38-targeted immuno-chemotherapy in multiple myeloma patients refractory to lenalidomide and bortezomib. *Clin Cancer Res.* (2015) 21:2802–10. doi: 10.1158/1078-0432.CCR-14-1813
31. Van De Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood* (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
32. Deckert J, Wetzel MC, Bartle LM, Skaletskaya A, Goldmacher VS, Vallee F, et al. SAR650984, a novel humanized CD38-targeting antibody, demonstrates

potent antitumor activity in models of multiple myeloma and other CD38+ hematologic malignancies. *Clin Cancer Res.* (2014) 20:4574–83. doi: 10.1158/1078-0432.CCR-14-0695
33. Jiang H, Acharya C, An G, Zhong M, Feng X, Wang L, et al. SAR650984 directly induces multiple myeloma cell death via lysosomal-associated and apoptotic pathways, which is further enhanced by pomalidomide. *Leukemia* (2016) 30:399–408. doi: 10.1038/leu.2015.240
34. Feng X, Zhang L, Acharya C, An G, Wen K, Qiu L, et al. Targeting CD38 suppresses induction and function of T regulatory cells to mitigate immunosuppression in multiple myeloma. *Clin Cancer Res.* (2017) 23:4290–300. doi: 10.1158/1078-0432.CCR-16-3192
35. Stevenson FK, Bell AJ, Cusack R, Hamblin TJ, Slade CJ, Spellerberg MB, et al. Preliminary studies for an immunotherapeutic approach to the treatment of human myeloma using chimeric anti-CD38 antibody. *Blood* (1991) 77:1071–9.
36. Ellis JH, Barber KA, Tutt A, Hale C, Lewis AP, Glennie MJ, et al. Engineered anti-CD38 monoclonal antibodies for immunotherapy of multiple myeloma. *J Immunol.* (1995) 155:925–37.
37. Li T, Qi S, Unger M, Hou YN, Deng QW, Liu J, et al. Immuno-targeting the multifunctional CD38 using nanobody. *Sci Rep.* (2016) 6:27055. doi: 10.1038/srep27055
38. Fumey W, Koenigsdorf J, Kunick V, Menzel S, Schutze K, Unger M, et al. Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of CD38(+) tumors in mouse models *in vivo*. *Sci Rep.* (2017) 7:14289. doi: 10.1038/s41598-017-14112-6
39. Barabas AZ, Cole CD, Graeff RM, Morcol T, Lafreniere R. A novel modified vaccination technique produces IgG antibodies that cause complement-mediated lysis of multiple myeloma cells carrying CD38 antigen. *Hum Antibodies* (2016) 24:45–51. doi: 10.3233/HAB-160294
40. Barabas AZ, Cole CD, Graeff RM, Kovacs ZB, Lafreniere R. Suppression of tumor growth by a heterologous antibody directed against multiple myeloma dominant CD38 antigen in SCID mice injected with multiple myeloma cells. *Hum Antibodies* (2016) 24:53–7. doi: 10.3233/HAB-160295
41. Green DJ, Orgun NN, Jones JC, Hylarides MD, Pagel JM, Hamlin DK, et al. A preclinical model of CD38-pretargeted radioimmunotherapy for plasma cell malignancies. *Cancer Res.* (2014) 74:1179–89. doi: 10.1158/0008-5472.CAN-13-1589
42. Green DJ, O'steen S, Lin Y, Comstock ML, Kenoyer AL, Hamlin DK, et al. CD38-bispecific antibody pretargeted radioimmunotherapy for multiple myeloma and other B-cell malignancies. *Blood* (2018) 131:611–20. doi: 10.1182/blood-2017-09-807610
43. Teiluf K, Seidl C, Blechert B, Gaertner FC, Gilbertz KP, Fernandez V, et al. alpha-Radioimmunotherapy with (2)(1)(3)Bi-anti-CD38 immunoconjugates is effective in a mouse model of human multiple myeloma. *Oncotarget* (2015) 6:4692–703. doi: 10.18632/oncotarget.2986
44. June CH, O'connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science* (2018) 359:1361–5. doi: 10.1126/science.aar6711
45. Chu J, Deng Y, Benson DM, He S, Hughes T, Zhang J, et al. CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance *in vitro* and *in vivo* antitumor activity against human multiple myeloma. *Leukemia* (2014) 28:917–27. doi: 10.1038/leu.2013.279
46. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, et al. T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. *Blood* (2016) 128:1688–700. doi: 10.1182/blood-2016-04-711903
47. Gogishvili T, Danhof S, Prommersberger S, Rydzek J, Schreder M, Brede C, et al. SLAMF7-CAR T cells eliminate myeloma and confer selective fratricide of SLAMF7(+) normal lymphocytes. *Blood* (2017) 130:2838–47. doi: 10.1182/blood-2017-04-778423
48. Garfall AL, Stadtmauer EA, Hwang WT, Lacey SF, Melenhorst JJ, Krevvata M, et al. Anti-CD19 CAR T cells with high-dose melphalan and autologous stem cell transplantation for refractory multiple myeloma. *JCI Insight* (2018) 3:120505. doi: 10.1172/jci.insight.120505
49. Mihara K, Bhattacharyya J, Kitanaka A, Yanagihara K, Kubo T, Takei Y, et al. T-cell immunotherapy with a chimeric receptor against CD38 is effective in eliminating myeloma cells. *Leukemia* (2012) 26:365–7. doi: 10.1038/leu.2011.205
50. Drent E, Groen R, Noort WA, Lammerts Van Bueren J, Parren PWHI, Kuball JH, et al. CD38 chimeric antigen receptor engineered T cells as therapeutic tools for multiple myeloma. *Blood* (2014) 124:4759.
51. Drent E, Groen RW, Noort WA, Themeli M, Lammerts Van Bueren JJ, Parren PW, et al. Pre-clinical evaluation of CD38 chimeric antigen receptor engineered T cells for the treatment of multiple myeloma. *Haematologica* (2016) 101:616–25. doi: 10.3324/haematol.2015.137620
52. Drent E, Themeli M, Poels R, De Jong-Korlaar R, Yuan H, De Bruijn J, et al. A rational strategy for reducing on-target off-tumor effects of CD38-chimeric antigen receptors by affinity optimization. *Mol Ther.* (2017) 25:1946–58. doi: 10.1016/j.ymthe.2017.04.024
53. Drent E, Poels R, Mulders MJ, Van De Donk NWCJ, Themeli M, Lokhorst HM, et al. Feasibility of controlling CD38-CAR T cell activity with a Tet-on inducible CAR design. *PLoS ONE* (2018) 13:e0197349. doi: 10.1371/journal.pone.0197349
54. Mihara K, Bhattacharyya J, Takihara Y, Kimura A. All-trans retinoic acid enhances the cytotoxic effect of T cells with anti-CD38 chimeric receptor in acute myeloid leukemia. *Blood* (2012) 120:1901. doi: 10.1038/cti.2016.73
55. Maude SL, Hucks GE, Seif AE, Talekar MK, Teachey DT, Baniewicz D, et al. The effect of pembrolizumab in combination with CD19-targeted chimeric antigen receptor (CAR) T cells in relapsed acute lymphoblastic leukemia (ALL). *J Clin Oncol.* (2017) 35:103–103. doi: 10.1200/JCO.2017.35.15_suppl.103
56. San-Miguel J, Dimopoulos MA, Usmani S, Belch AR, Bahlis NJ, White DJ, et al. Depth of response and MRD with Daratumumab plus Lenalidomide and Dexamethasone (DRd) vs Lenalidomide and Dexamethasone (Rd) in RRMM: POLLUX. *Clin Lymphoma Myeloma Leukemia* (2017) 17:e17–8. doi: 10.1016/j.clml.2017.03.029
57. Lacy MQ, Hayman SR, Gertz MA, Short KD, Dispenzieri A, Kumar S, et al. Pomalidomide (CC4047) plus low dose dexamethasone (Pom/dex) is active and well tolerated in lenalidomide refractory multiple myeloma (MM). *Leukemia* (2010) 24:1934–9. doi: 10.1038/leu.2010.190
58. Chari A, Suvannasankha A, Fay JW, Arnulf B, Kaufman JL, Ifthikharuddin JJ, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. *Blood* (2017) 130:974–81. doi: 10.1182/blood-2017-05-785246
59. Spencer A, Mark T, Spicka I, Masszi T, Lauri B, Levin M-D, et al. Depth of response and MRD with Daratumumab plus Bortezomib and Dexamethasone (DVd) vs Bortezomib and Dexamethasone (Vd) in RRMM: CASTOR. *Clin Lymphoma Myeloma Leukemia* (2017) 17:e85. doi: 10.1016/j.clml.2017.03.153

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CD38 in Adenosinergic Pathways and Metabolic Re-programming in Human Multiple Myeloma Cells: In-tandem Insights From Basic Science to Therapy

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Tumor microenvironments are rich in extracellular nucleotides that can be metabolized by ectoenzymes to produce adenosine, a nucleoside involved in controlling immune responses. Multiple myeloma, a plasma cell malignancy developed within a bone marrow niche, exploits adenosinergic pathways to customize the immune homeostasis of the tumor. CD38, a multifunctional protein that acts as both receptor and ectoenzyme, is overexpressed at all stages of myeloma. At neutral and acidic pH, CD38 catalyzes the extracellular conversion of NAD^+ to regulators of calcium signaling. The initial disassembly of NAD^+ is also followed by adenosinergic activity, if CD38 is operating in the presence of CD203a and CD73 nucleotidases. cAMP extruded from tumor cells provides another substrate for metabolizing nucleotidases to signaling adenosine. These pathways flank or bypass the canonical adenosinergic pathway subjected to the conversion of ATP by CD39. All of the adenosinergic networks can be hijacked by the tumor, thus controlling the homeostatic reprogramming of the myeloma in the bone marrow. In this context, adenosine assumes the role of a local hormone: cell metabolism is adjusted via low- or high-affinity purinergic receptors expressed by immune and bone cells as well as by tumor cells. The result is immunosuppression, which contributes to the failure of immune surveillance in cancer. A similar metabolic strategy silences immune effectors during the progression of indolent gammopathies to symptomatic overt multiple myeloma disease. Plasma from myeloma aspirates contains elevated levels of adenosine resulting from interactions between myeloma and other cells lining the niche and adenosine concentrations are known to increase as the disease progresses. This is statistically reflected in the International Staging System for multiple myeloma. Along with the ability to deplete $CD38^+$ malignant plasma cell populations which has led to their widespread therapeutic use, anti-CD38 antibodies are involved in the polarization and release of microvesicles characterized by the expression of multiple adenosine-producing molecules. These adenosinergic pathways provide new immune checkpoints for improving immunotherapy protocols by helping to restore the depressed immune response.

Keywords: CD38, multiple myeloma, metabolic reprogramming, adenosine, immunotherapy

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MULTIPLE MYELOMA

Multiple Myeloma (MM) is the second most common malignancy in hematology (1). MM is seem as the eventual outcome of selective pressures on different cell clones of malignant plasma cells (mPCs) (2) that grow in a hypoxic niche in the bone marrow (BM) (3). When oxygen consumption exceeds its supply from the vascular system, the hypoxic tumor environment favors molecular pathways that fuel tumor aggressiveness (4, 5). Cross talk among the distinct cellular components of the closed BM niche generates extracellular adenosine (ADO), thereby promoting tumor cell survival (6, 7). This occurs through the binding of ADO to purinergic receptors, which leads to the formation of complexes that function as autocrine/paracrine signals with immune regulatory activities.

Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+), and cyclic adenosine monophosphate (cAMP) are, the main intracellular purine molecules serving as leading adenosinergic substrates in the extracellular tumor microenvironment (TME) for generating ADO (8–10). Adenosinergic conversion, which can vary significantly according to the metabolic environment, is exploited by mPCs for migrating and homing to a protected niche and for evading the immune response (1). The expression of multiple specific P1 ADO receptors (ADORs) (11) in the niche completes the profile of a complex regulatory network, whose signals are translated into (i) down-regulation of the functions of most immune effector cells and (ii) enhancement of the activity of cells that suppress anti-tumor immune responses. Both effects facilitate the escape of mPCs from immune surveillance. A translational view of these findings suggests that finely-tuned ADO concentrations in the BM myeloma niche (12) contribute to symptomatic MM among patients with asymptomatic monoclonal gammopathy of undetermined significance (MGUS) and with smoldering multiple myeloma (SMM) (13, 14). Therefore, nucleotide-metabolizing ectoenzymes expressed by BM-resident cells and ADO production may acquire theragnostic relevance in the clinical outcome of MM. The present paper also reviews the contribution of metabolic reprogramming to the development of novel therapy options for MM.

ADENOSINE PRODUCTION WITHIN THE BM NICHE

Purinome and Metabolic Reprogramming

A unique feature shared by the MGUS/SMM/MM stages of myeloma is the dependence of mPCs on BM microenvironmental signals. The BM niche provides a hypoxic habitat for interactions between mPCs and non-tumor immune and non-immune resident cells, yielding functional operating elements, or so-called purinome (e.g., nucleotide channels and transporters, nucleotides byproducts, nucleotide catabolizing ectoenzymes, molecular networks of nucleotide, and nucleoside receptor proteins) (15). Their physical connection is mediated by the plasmatic fluid, which links the different cell components of the purinome (**Figure 1**).

The MM grows in the BM, where mPCs are sheltered in a physically constrained niche containing osteoblasts (OBs), osteoclasts (OCs), stromal cells (SCs), and immune cells (e.g., T and B lymphocytes, NK, MDSC, among others). For their progressive expansion, mPCs overcome the hypoxic niche through a process of metabolic reprogramming based on hijacking the molecular mechanisms of normal cells to create an exclusive immunosuppressive frame. Metabolic adaptation of the cellular component of the BM niche induces a HIF1α-dependent glycolytic program, which increases CD73 and ADOR expression (16). Further, mPCs exploit local metabolic dysregulation, namely a shift from oxidative phosphorylation (OXPHOS) toward a glycolytic metabolism, to demand (i) supplementary sources of energy for a rapid growth; (ii) byproducts (e.g., ribose, glycerol and citrate, and non-essential amino acids) needed for biosynthetic pathways and (iii) an increase in enzymatic activities (e.g., LDH-A to regenerate NAD^+) (17, 18). As shown in **Figure 1**, after consuming glucose at a higher rate than normal cells, MM cells secreted most of the derived byproducts as lactic acid, a phenomenon known as the "Warburg Effect" (19). Simultaneously, the generation of lactic acid and protons (H^+) results in acid accumulation within MM cells. The intracellular metabolic adjustment is neutralized by the overexpression of a monocarboxylate transporter (MCT), resulting in a H^+-linked co-transport of lactic acid across the plasma membrane, with increased extracellular acidity, known as lactic acidosis (**Figure 1**).

Adenosine Production by Canonical and Alternative Ectoenzymatic Pathways

The purinome exploits the metabolically reprogrammed niche to generate extracellular ADO which is locally produced by the multicellular network (**Figure 2**). ADO leads to tumor growth and skews the immune cells toward an immunosuppressive phenotype (20).

Adenosinergic pathways identified on different cell populations (21) confer immunosuppressive properties to the cells in different physiological tissues (e.g., cornea and human placenta) (22, 23) and in pathological environments, such as the BM niche of MM (12). Indeed, BM resident cells constitutively express a complete set of cell surface ectonucleotidases, which scattered on different cells drive the production of ADO under metabolic stress (e.g., hypoxia) (24, 25). ADO is also believed to modulate communication between mPCs and normal cells, contributing to the immunocompromised state of MM patients (26).

ADO is produced from the catabolism of mono- and di-nucleotides of adenine (ATP, NAD^+ and cAMP) (**Figure 1**). The canonical pathway of ADO production starts from extracellular ATP, which is first hydrolyzed to adenosine diphosphate (ADP) and then to adenosine monophosphate (AMP) by nucleoside triphosphate diphosphohydrolase (NTPDase-1/CD39) or directly by the low-affinity nucleotide pyrophosphatase/phosphodiesterase (NPP/CD203a). The final

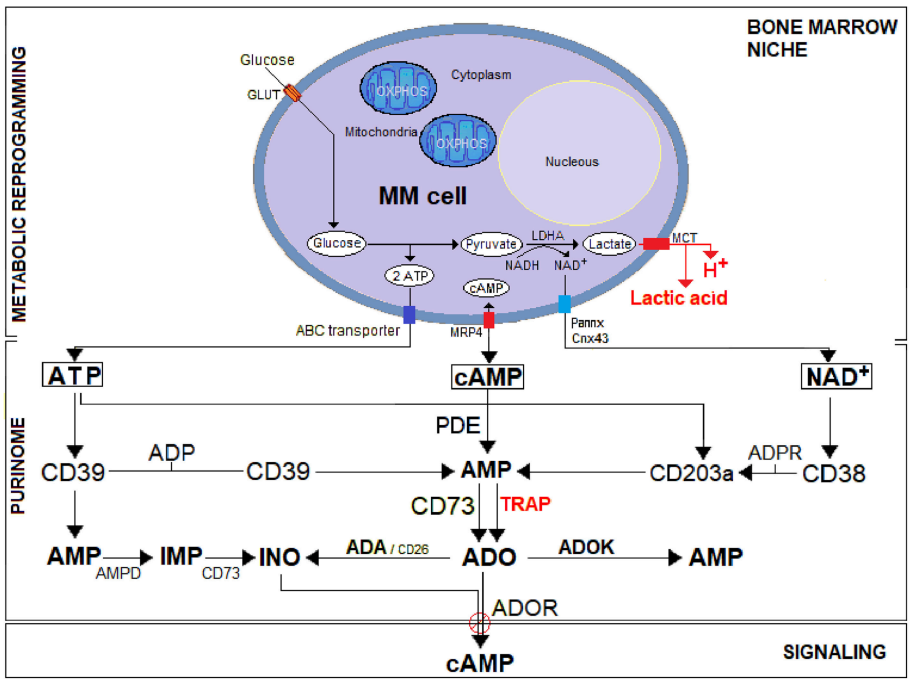

FIGURE 1 | A schematic representation of the purinome in the MM environment under bone marrow niche metabolic reprogramming. Unlike normal cells, tumor cells (i.e., MM) utilize glycolysis instead of OXPHOS for metabolic reprogramming: most of the resulting pyruvate is catalyzed by lactate dehydrogenase A (LDH-A) to lactic acid simultaneously producing protons (H^+). The efflux of lactic acid and H^+ induces lactic acidosis generating an acidic TME (pH < 6.5). Cytoplasmic ATP, cAMP, and NAD^+ actively secreted across nucleotide transporters (e.g., ABC transporter, Pannexin/Connexin channels, MRP4) or passively after cell lysis, are metabolized in the hypoxic acidic BM niche to ADO. The purinome (e.g., complex network of nucleotidic substrates, ectonucleotidases, signaling by-products, and purinergic receptors) operating in the BM niche exploit the classical pathway (CD39/CD73) for ATP substrate and the (CD38/CD203a/CD73) pathway for NAD^+, flanked by an alternative (PDE/CD73) pathway that converts cAMP to AMP for generating the immunosuppressive ADO. Generated ADO binds to P1 purinergic ADO receptors (ADOR) and activates adenylyl cyclase, which catalyzes the formation of the intracellular second messenger cAMP. Eventually, ADO can also be inactivated at the cell surface by an ADA/CD26 complex that converts it into inosine (INO) or internalized by nucleoside transporters. The extracellular ATP breakdown follows under physiological conditions the classical ATP/ADP/AMP/ADO adenosinergic pathway. However, the presence of high ATP concentration in the TME lead AMP to be converted by AMP deaminase (AMPD) into inosine monophosphate (IMP), which in turn is dephosphorylated by 5′-NT/CD73 into INO.

phosphate group from AMP is cleaved by the 5′-nucleotidase (5′-NT/CD73), thus generating ADO (27). As it is the primary substrate for ectonucleotidases to generate immunosuppressive ADO, ATP implicates the canonical CD39/CD73 tandem in the inception of an anergic tumor milieu (28–30). However, there are some doubts about the ability of this classical pathway to function in closed systems (e.g., BM niche) *in vivo*. For example, the optimal pH for the CD39 enzyme is in the alkaline range of 8–8.3 (31). This might preclude the enzymatic activity of CD39 in a hypoxic TME, where an acidic pH is secondary to lactic acidosis (32). Furthermore, the conversion of extracellular ATP to ADO as catalyzed by CD39 is kinetically complex, with the upstream ADP metabolite generated at high ATP levels of the TME, acting as a feed-forward inhibitor of CD73 (33).

Also, the fact that CD203a has a lower affinity for its substrate ATP than CD39 supports the idea that the alternative ectoenzymatic CD38/CD203a tandem using NAD^+ as substrate (**Figure 1**) may become a relevant producer of AMP for ADO production in the BM niche. Therefore, CD39 may not be the only *in vivo* immune system switch that triggers ADO-mediated immunosuppression (34).

Under physiological conditions, the extracellular breakdown of ATP follows the conventional ATP/ADP/AMP/ADO adenosinergic pathway. However, under pathological conditions, the high ATP concentration in the TME causes AMP deaminase (AMPD) to convert AMP into inosine monophosphate (IMP), which in turn is dephosphorylated by 5′-NT/CD73 into inosine (INO) (35) (**Figure 1**). The IMP pathway (ATP/AMP/IMP/INO), originally thought to be found mainly in the cytosolic cell compartment (36), was recently detected by our group in BM plasma from MM and neuroblastoma patients (3). There are other, alternative(s) substrates (i.e., NAD^+, cAMP) for the ADO-generating axis in the MM niche (**Figure 1**). Using T cell leukemia as a model, we confirmed that the canonical CD39/CD73 pathway is flanked by another set of surface molecules leading to the production of ADO, but using NAD^+ as a leading substrate (9). Components of this alternative pathway are NAD^+-glycohydrolase/CD38, the ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1)/CD203a and the 5′-ectonucleotidase (5′NT)/CD73.

CD38, a transmembrane glycoprotein that lacks an internal signaling domain, is a surface molecule expressed by normal T, B, NK and myeloid populations as well as by different

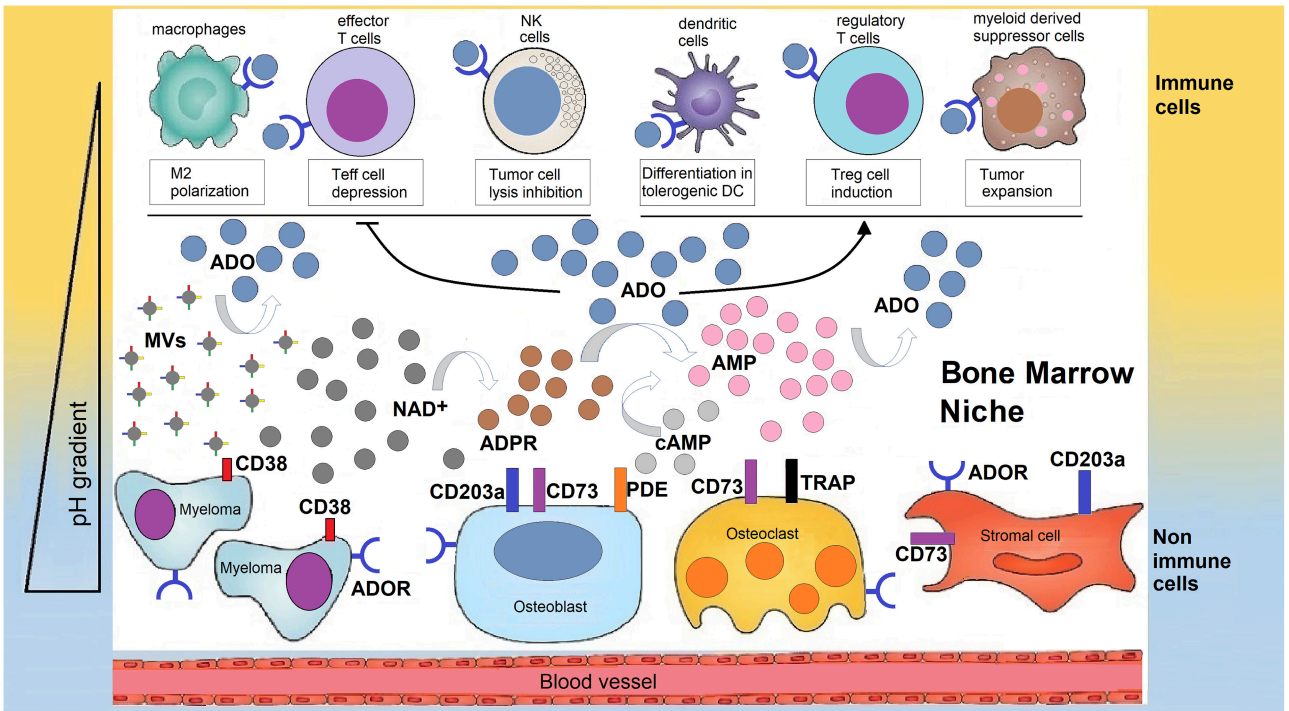

FIGURE 2 | Human bone marrow sheltering malignant plasma cells, bone cells, and immune cell, supports the production of adenosine for the generation of a tolerant niche. In a closed BM niche, ADO is mainly obtained from NAD^+ (and possibly from cAMP) which undergoes reaction through a multicellular chain of ectonucleotidases (CD38, CD203a, and CD73 or TRAP depending on pH status regulated by metabolic reprogramming). According to this view, NAD^+ is disassembled into byproducts that flow in the BM plasma fluid within the myeloma niche, accumulating variable amounts of ADO. Most of ADO is taken-up by purinergic cell receptors (ADOR) expressed by bone cells or immune cells inside the niche. The outcome is either a block of the effectiveness of immune cells (Teff, NK, TAMs) that are capable of destroying tumor cells or that increase the number of regulatory T-cells (Tregs), mesenchymal derived stromal cells (MDSC), or dendritic cells (DC) which suppress immune cells from responding to the tumor.

tumor cells (37). The molecule was initially considered as an adhesion/receptor structure, but a review of the evidence suggests that CD38 is not merely a receptor marker (38, 39). Instead, it possesses a number of enzymatic activities ruling NAD^+ levels inside the BM niche where the mPC grows (25, 40). Indeed, CD38 is located on the mPC surface as well as adjacent non-tumor cells catalyzing the conversion of NAD^+ to cyclic adenosine diphosphate ribose (cADPR) via cyclase activity and cADPR to ADPR via hydrolase activity (37). ADPR is further hydrolyzed by CD203a to produce AMP. CD203a was recently proposed as a key ectoenzyme because of its ability to convert both ADPR and ATP to AMP, which is subsequently metabolized by CD73 into ADO. Alternatively, a CD73-surrogated ectoenzyme, a Tartrate-Resistant Acid Phosphatase (TRAP), is also functionally active according to the environmental pH (7) (**Figure 1**).

As can be seen in **Figure 2**, NAD^+ relies on the CD38/CD203a tandem and CD73 ectonucleotidase to activate a discontinuous multicellular pathway for ADO production, as detected in plasma aspirates from myeloma BM (12). It is not completely clear whether the alternative CD38/CD203a/CD73 and the canonical CD39/CD73 pathways function cooperatively or whether the relative expression of ectonucleotidases determines which pathway is more active in the hypoxic BM niche. What it sure is that metabolic reprogramming in the BM niche leads to an acidic TME. It is therefore reasonable to believe that the CD38-dependent pathway has a compensatory role for CD39 activity in a BM acidic milieu.

The cyclic nucleotide cAMP signaling pathway is a third alternative route to the production of extracellular ADO (**Figure 1**). This axis hinges on the cAMP nucleotide-metabolizing membrane-ectoenzyme phosphodiesterase (PDE) and CD73 (41) and it may flank or synergize the known ATP/NAD^+-catabolic pathways. The cAMP substrate, one of the oldest signaling molecules known, is produced from ATP by membrane-bound adenylyl cyclases (AC) (42, 43). The acidic BM niche improves the egress of cAMP via MRP4 (44) and cAMP efflux might regulate extracellular ADO levels and thus optimize the autocrine and paracrine immunosuppressive effects of ADO. In fact, ADO is rapidly taken up by the red blood cells, which limits its half-life to <1 s in the TME, whereas cAMP is stable in biological fluids, making it possible for it to act at distant sites (45).

ADO levels in the TME are enzymatically balanced by (i) adenosine deaminase (ADA) (which converts ADO into INO) and (ii) ADO kinase (which forms AMP from ADO) (**Figure 1**). Extracellular ADO homeostasis is also maintained

by bidirectional transport through equilibrative nucleoside transporters (ENTs) located in the plasma membrane (46).

The occurrence of an event promoting the extracellular accumulation of nucleotides in MM, their sequential degradation to AMP and the subsequent formation of ADO is followed by a cAMP second messenger pathway coupled to ADO receptors (ADORs) (**Figure 1**). Indeed, the extracellular accumulation of ADO mediates signals by binding to P1-G protein-coupled purinergic (A1, A2A, A2B, and A3) ADORs expressed by different cells, including immune effectors (47).

IS THERE A LINK BETWEEN ADO LEVELS AND DISEASE PROGRESSION?

ADO is produced by interactions between mPCs and other cells lining the BM niche (25) (**Figure 2**). This finding suggested that the expression of ectonucleotidases must somehow be linked to the production of immunosuppressive ADO in the BM plasmatic fluid of MM patients to create a protective TME. The different ADO levels in the BM plasma samples analyzed likely reflect (i) variability in the number of ectoenzymes and their activities according to environmental pH (12) or (ii) their tendency to be shed in the biological fluids (48). Further, (iii) several of these molecules are genetically polymorphic, which influences their function. For instance, the expression of CD38 is regulated by a single nucleotide polymorphism (SNP) located in intron 1 (rs6449182; C>G variation) (49).

The accumulation of ADO (>25 μM) in the BM niche via the CD38/CD203a/CD73 and CD39/CD73 axes works sequentially through mPCs/BMSCs/OCs interactions (26). High levels of ADO as determined by cAMP production have a potent stimulatory action on interleukin-6 (IL-6) secretion by BMSCs (50, 51). Because IL-6 is important for normal OB function, the targeting of IL-6 signal pathways may alter the balance between bone resorption and formation. For this reason, mPC and OB interactions in the BM niche contributes to the development of osteolytic lesions in MM (52). IL-6 is also involved in mPC proliferation, survival and disease progression (52, 53). Accordingly, IL-6 correlates with (i) a decrease in serum albumin secondary to increased albuminuria (54) and (ii) up-regulation of factor HIF-1α (16), which parallels MM progression.

Microvesicles (MVs) isolated from the BM plasma of MM patients represent another source of ADO (26). MVs from MM patients express higher levels of adenosinergic ectonucleotidases than those isolated from MGUS/SMM (26). Similarly, the production of ADO was higher when challenging MVs from MM patients than from asymptomatic MGUS/SMM patient samples. A likely explanation is that MVs contribute to the production of ADO in the BM niche (55) (**Figure 2**).

Results of a recent study in MM patients, evaluated according to the International Staging System (ISS) (56, 57), revealed that ADO levels in BM plasma samples at diagnosis were higher in patients at an advanced stage (ISS=III) with symptomatic MM than in those at the earlier MGUS/SMM stages (pooled ISS=I-II) (12). These findings confirm that ADO production in the BM niche correlates with disease progression and may be useful as a prognostic marker for ISS staging alongside other markers, such as (increasing) serum beta2-microglobulin and (decreasing) serum albumin (58).

These observations support the view that (i) the expression of ectonucleotidases is linked to the production of ADO in the BM plasmatic fluid of MM patients and (ii) that metabolic reprogramming may allow mPCs to construct a microenvironment that favors their survival and protects them from the host immune system. Moreover, the adenosinergic metabolic strategy assists in silencing the immune effectors during progression of MM from indolent monoclonal gammopathy to symptomatic overt disease. It is possible that these observations are only correlative and merely a reflection of tumor burden. Nonetheless, ADO levels in the BM plasma provide a sensitive marker of myeloma progression.

IMPACT OF METABOLIC REPROGRAMMING ON IMMUNE CELL FUNCTIONS

mPCs alter the BM niche, affecting bone cells [e.g., they increase the number and activities of osteoclasts (OCs) and decrease the same on osteoblasts (OBs)], either mediated by soluble factors or by cell-to-cell contacts (6, 59). At the same time, mPCs affect immune events by creating a permissive niche that fosters the colonization of mPCs (60, 61).

Immune cells have regulatory functions originally intended to protect vital organs from inflammatory damage and tumor development (11). However, even activated immune effector T cells that potentially recognize specific tumor-associated antigens have a hard time surviving in the TME while trying to perform their expected functions in harsh metabolic conditions (62). Different kinds of immune cells have developed varied strategies for surviving in the conditions of lactic acidosis (pH $\leq$ 6.5) created by dysregulated metabolism. Lactic acid is reported as modulating proliferation and activation of human T cells. Indeed, T lymphocytes treated with lactic acid show diminished TCR-mediated activation and trafficking to the TME (63). In addition, effector T cell functions in MM patients are blunted, resulting in paresis of cellular and humoral immunities (64, 65). Tumor cells hijack macrophages (TAMs) via lactic acid (66) and natural killer (NK) cells lose almost all of their functions and reach a state of anergy when exposed to an acidic pH (67). In contrast, myeloid-derived suppressor cells (MDSCs) and regulatory T (Treg) lymphocytes boost tumor growth in acidic conditions (68). The concentrations of lactic acid observed in pathology also inhibit the maturation of dendritic cells (DCs) and their antigen presentation (69). All these conditions in the immune compartment correlate *in vivo* with tumor progression and metastatic spread. Finally, lactic acid causes a reduction in LDH-A expression, which is paralleled by diminished tumor growth and a decline in the number of MDSCs (70). An implication of this is that lactic acid is an immuno-modulatory molecule that can strongly repress anti-tumor immunity.

Within such a scenario, metabolic reprogramming has multiple effects, including the extracellular accumulation of

nucleotides (ATP, NAD^+, cAMP) and of ADO, its main catabolic product (25, 71). From the operational point of view, ADO ligation of ADORA2A (dominantly expressed by most immune cells) is followed by decreased proliferation and by inhibition of the cytolytic anti-tumor activities of cytotoxic T lymphocytes (27, 30, 72), and inhibition of cytotoxicity and IFN-γ release by NK cells (73). These effects are followed by suppression of pro-inflammatory activities and by an increased number of immunoregulatory cells. The outcome is the establishment of a long-lasting immunosuppressive environment (74).

The partial block of ADORA2A may increase the concentration of extracellular AMP, favoring internalization and accumulation of the mononucleotide inside the cell. This would lead to activation of the AMP-dependent protein kinase (AMPK) (75), inducing a positive effect on the AMPK/mTOR/p70S6K/rpS6 protein axis, which is reported as inducing suppression of T cell proliferation in human melanoma cells through an adenosinergic pathway led by CD38 (76). The high levels of ADO measured in the culture supernatants of primary melanoma cells and the BM plasma from MM patients (12) were also detected by metabolomic screening using AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide)-treated malignant cells identifying pyrimidine starvation as the mechanism of AICAR-induced apoptosis in mPCs (77). AICAR is a metabolic intermediate in the enzymatic conversion of AMP into inosine monophosphate (IMP), catalyzed by ADO deaminase (3). As an analog of AMP, AICAR activates AMP-dependent protein kinase (AMPK) activity, a signal molecule reported as a potential target in MM that induces G1 arrest in mPCs (78). High extracellular ADO levels, by ligation of low affinity ADORA2B, can influence the antigen-presenting activity of DCs (79, 80) and activate normal infiltrating cells that block the anti-tumor immune response (such as Tregs, MDSCs, and TAMs) (81), leading to peripheral tolerance (**Figure 2**). Although these cell subsets are recruited to the tumor site to fine-tune immune activation, they have the perverse effect of boosting tumor growth (28).

THERAPEUTIC STRATEGIES TO COUNTERACT ADENOSINE SUPPRESSIVE MECHANISMS IN MM

Immune Checkpoint Molecules in MM Bone Marrow

Preclinical and clinical studies revealed that most tumors overexpress immune checkpoint (ICP) molecules, of which the most studied are programmed death-1 (PD-1) and its ligand (PD-L1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) (82, 83). The activation of ICP rules anergy, apoptosis and "exhaustion" to initiate T cell suppression (84–86). In a similar fashion, mPCs can evade immunosurveillance by means of multiple mechanisms of immunosuppression (74), such as the ability of mPCs to hijack inhibitory ICP suppressive mechanisms (87). Therapeutic strategies incorporating inhibition of ICP have shown promising results, although high rates of resistance limit their efficacy (88). For instance, a large subset of patients still remains refractory following PD-1/PD-L1 ICP blockade (89, 90). Recently, the purinergic pathway promoting ADO generation through the CD38/CD203a/CD73 pathway (9) was identified as the main obstacle to the therapeutic benefit of anti–PD-1/PD-L1 blockade (91, 92). The same studies also determined that tumors treated with PD-1/PD-L1-monoclonal antibodies (mAbs) endowed with blocking properties developed resistance through the metabolic upregulation of CD38, akin to that induced by all-trans retinoic acid (ATRA) and interferon-β in the TME (as in non-small cell lung cancer, NSCLC). A pre-existing and inducible high expression of CD38 (as in mPCs) is thought to be the main hindrance to the therapeutic potential of the anti–PD-1/PD-L1 blockade (**Figure 3**). Indeed, it has been reported that (i) CD38 inhibits $CD8^+$ T-cell functions via ADO receptor signaling (76), while either (ii) inhibiting CD38 or blocking ADO receptors was effective in overcoming resistance to combined ICP immunotherapeutic strategies (92). Further, (iii) CD38 KO tumors grow much more slowly than $CD38^+$ wild-type tumors in wild-type mice (92). However, the protective effect of CD38 vanishes in the absence of $CD8^+$ T cells, suggesting that CD38-expressing cells impair $CD8^+$ T cell functions. Together, these data indicate that the NAD^+ adenosinergic pathway helps sustain the production of immunosuppressant ADO in the modified adaptive immune response to anti-PD-1/PD-L1 treatment (**Figure 3**).

The endpoint of ADO signaling is the induction of "anergic" effector cells, which suggests that extracellular ADO functions as a negative ICP molecule (93). This hypothesis is strengthened by evidence of synergic anti-tumor effects elicited by combining anti-PD-1/CTLA-4 and inhibitors of ADO production or signaling (94).

Reducing CD38 Surface Levels

CD38 is expressed at different levels on mPCs from all MM patients (95, 96). A decrease in the level of CD38 in MM cells can occur in several ways: (i) treatment with anti-CD38 mAbs; (ii) generation of MVs followed by their uptake of the CD38-mAb complexes by FcR^+-expressing cells; and (iii) trogocytosis (97). It has been observed that anti-CD38 mAbs (e.g., daratumumab) ligation on mPCs is followed by the aggregation, polarization and release of MVs derived from cell membranes and expressing adenosinergic molecules (CD39, CD203a, CD73) clustered in lipid domains. MVs isolated from the BM plasma of MM patients also contain the target CD38 as well as the specific monoclonal IgG (98). While the exact fate of the MVs is unknown, MV bearing monoclonal IgG exit the BM niche and cluster around cells expressing FcR. Since MVs fuse with the target cells, modulation of immune responses is expected (55).

Transfer of CD38 from the MM cell surface to effector cells either by trogocytic transfer or vesiculation might compromise therapeutic efficacy because of a reduction in mAbs that eliminate MM cells via CDC and ADCC (97, 99). This reduction in surface CD38 could have several beneficial effects. Firstly, CD38 is an immunomodulatory molecule that inhibits T-cell functions via ADOR signaling (100). It is thus possible that a simultaneous down-regulation of CD38 (and associated

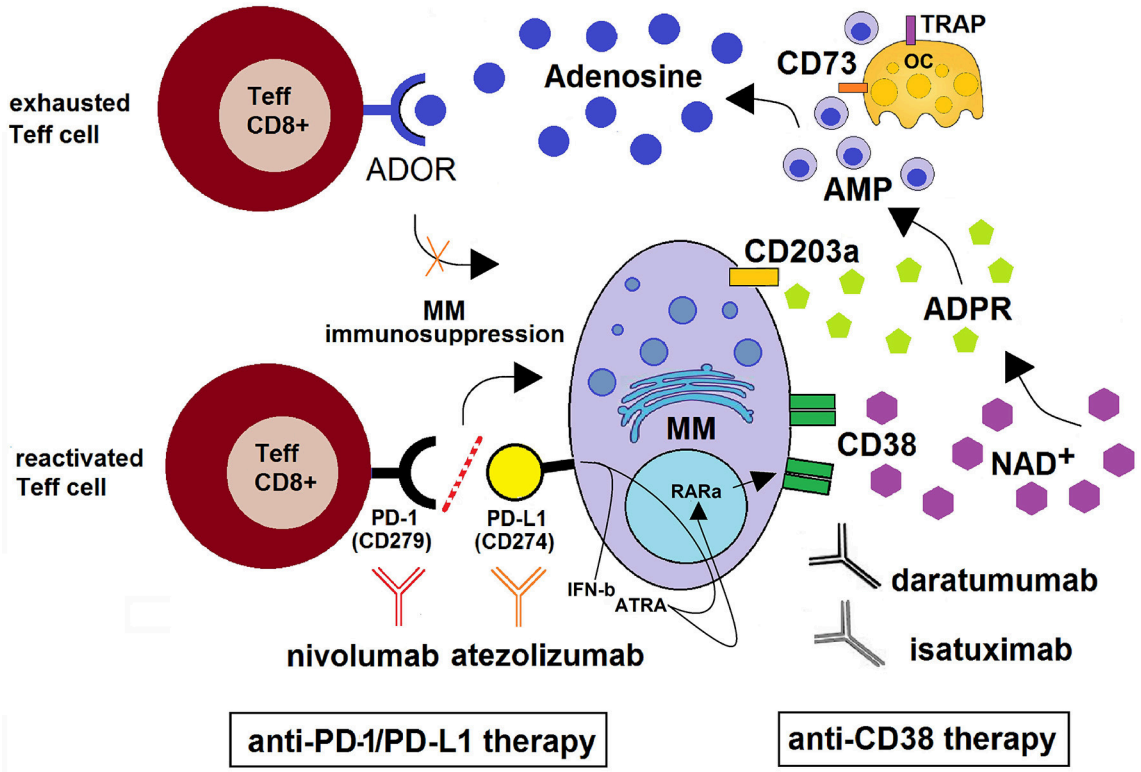

FIGURE 3 | Schematic depiction of the CD38/CD203a/CD73 adenosinergic pathway as a major mechanism of the acquired resistance to anti-PD-1/PD-L1 blockade within the BM niche. CD38^{+high} MM cells catalyze NAD^{+} transformation to ADO via the CD38/CD203a/CD73 ectoenzymatic pathway, discontinuously expressed by BM resident cells (MM, OCs, OBs, BMSC). This step is followed by (i) the activation of the ADOR A2A and A2B on cytotoxic T-lymphocytes, with suppression of their anti-tumor functions and (ii) the induction of an anti-PD-1/PD-L1-mediated resistance to the increase of cytotoxic T-cell infiltration in the BM niche.
Anti–PD-1/PD-L1-resistant MM cells also produce soluble mediators (such as IFN-β and ATRA) leading to increased expression of CD38 on mPCs via RARα. This mechanism support the use of anti-CD38 mAbs (e.g., daratumumab and isatuximab) with the ability to inhibit CD38 cyclase activity. When used in combination with PD-1/PD-L1 immune checkpoint blockade (e.g., nivolumab and atezolizumab), the result may be an improvement of antitumor immune responses with reactivation of CD8^{+} T effector lymphocytes leading to a control of MM cells.

adenosinergic molecules) on MM cells as well as in TME cells by trogocytosis and MV formation may lead to (i) decreased levels of ADO in the BM niche (12) and, consequently, (ii) fewer immune-related adverse events associated with ICP blockade (90). In fact, as shown in **Figure 3**, treatment with anti-CD38 mAbs in combination with anti-PD-1/PD-L1 may induce expansion of BM T effector cells in MM patients.

CD38 can also be transferred by trogocytosis to effector T lymphocytes, along with other molecules located on CD38^{+}mPCs membrane domain. For instance, the expression of adhesion proteins on the surface of (i) MM cells (e.g., CD56, CD49d, and CD138) (101) or of (ii) non-immune and endothelial cells (e.g., CD31/PECAM1) (102) resident in the BM niche are transferred, resulting in diminished expression. It is therefore likely that CD38^{+}mPC interaction with CD31^{+}BMSCs in the protective myeloma niche is hindered, leading to a reduction in pro-survival signals (103).

In contrast with conventional tumor therapies, mAb immunotherapy targets the immune response to provoke a systemic anti-tumor response (104, 105). CD38 is a valuable target for therapeutic mAbs because of (i) its ability to impair tumor growth either by directly targeting cells or by inducing immune modulation (106). Other significant advantages provided by mAbs are (ii) the successful induction of durable responses and increased survival in various types of cancer (107–109).

Pharma companies recognized the value of CD38 as an ideal target for treating human MM with mAbs because of its favorable expression during ontogenesis (37, 110). Indeed, CD38 is not expressed by early hematological precursors (111) and CD38 expression is maintained in spite of the genomic differences marking mPCs.

In the BM niche, NAD^{+} metabolization is mediated by CD38 and elicits rapid functional responses leading to significant accumulation of ADO that induces immune silencing. It is thus reasonable to assume that a mAb-mediated reduction of CD38 on mPCs, mediated by the uptake of CD38^{+}MVs-mAb complexes by FcR^{+}-immune cells, may contribute to an improved host-antitumor immune response (112). Two of the available anti-CD38 therapeutic mAbs, daratumumab

and isatuximab, modulate the enzymatic activity of CD38 *in vitro*, which is able to reduce immunosuppressive ADO levels (3). Daratumumab (human IgG1k), the first therapeutic mAb approved *in vivo* (113, 114), inhibits CD38 cyclase activity, while enhancing hydrolase enzymatic activity (115). Its ligand effect on NAD^+ catalysis was determined on mPCs isolated from the BM plasma of MM patients and, for comparative evaluation, on a continuous human myeloma cell line. It was seen that daratumumab (100 μg/mL) inhibits the cyclase activity of CD38 *in vitro* (45% ± 5 and 32% ± 10, respectively). Furthermore, daratumumab ligation is followed by increased hydrolysis of cADPR (20% ± 5). Thus, daratumumab modulates the enzymatic activities of CD38, partially dampening cyclase activity, while simultaneously enhancing hydrolase activity. These results were measured by HPLC chromatography tests using NAD^+ (or the surrogate NGD^+) as the substrate for cyclase and, cADPR for hydrolase (12, 25). So far, the results indicate that NAD^+ (and NGD^+) are decreased by consumption secondary to CD38 catalysis and cADPR is also reduced. In contrast, ADPR is increased in the presence of daratumumab. Reduced cADPR levels may lead to reduced Ca^{2+} mobilization, which decreases signaling potential. Increased ADPR levels, which contribute to adenosinergic immune suppression, add a further element of complexity to the context of NAD^+ homeostasis and tumor survival in closed systems, (e.g., the BM niche). It is still worthwhile to evaluate the specific contribution of each CD38 cyclase/hydrolase mechanism to the clinical features of MM. In addition, daratumumab-mediated reduction of CD38 on MM cells may also decrease the generation of immunosuppressive ADO molecules (12, 26), which would result in an improved host-anti-tumor immune response (91). Further investigation is needed to determine whether anti-CD38 *in vivo* therapy also modulates the enzymatic activities of the molecule. However, on the basis of *in vitro* experimental observations, we hypothesize that specific monoclonal IgG1 antibodies (e.g., daratumumab and isatuximab) might modulate both the cyclase and hydrolase enzymatic activities of CD38 *in vivo*. This hypothesis is supported by initial experimental evaluation of ADO in paired blood and BM plasma samples from MM patients, obtained before and after treatment with daratumumab (in collaboration with Dr. van de Donk, Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands). Indeed, the experimental adenosinergic trend observed (e.g., decay of ADO contents after daratumumab treatment) seem consistent with the *in vitro* counterpart (Horenstein, personal communication).

MECHANISMS OF RESISTANCE TO IMMUNOTHERAPY: mABs AND IMMUNOMODULATOR DRUGS

Increased understanding of the interactions between malignant cells and the immune system has paved the way to immunotherapy for cancer patients (116). Despite some favorable outcomes, most patients do not respond, likely because of intrinsic tumor resistance mechanisms. These include (i) decreased or absent antigen expression [e.g., tumor antigens, MHC I receptors, MHC I chain-related gene A and B (MICA and MICB)]; (ii) changes in the expression of cell receptors (e.g., tumor-expressed markers, PD-1/PD-L1, CTLA-4, among other ICPs); and (iii) alterations in cellular enzymes and metabolic pathways [e.g., CD38/NAD^+glycohydrolase; indoleamine 2,3-dioxygenase (IDO)] (37, 117) are the most likely involved in changes within the TME, resulting in a lack of response to immunotherapy (109).

Absent of MICA antigen expression has been suggested as a potential predictor of the efficacy of future immunotherapies using cytokine-induced killer (CIK) cells, a T cell population obtained by *in vitro* differentiation of peripheral blood mononuclear cells (PBMC) that represent a promising immunological approach in cancer (118). It has been shown that CIK cells are able (i) to produce extracellular ADO via canonical (CD39/CD73) and/or alternative (CD38/CD203a/CD73 or CD203a/CD73) pathways (119) and (ii) to modulate these ectonucleotidases during PBMC to CIK differentiation. This means that it may be possible to modulate ADO-generating ectoenzymes pharmacologically to improve CIK cell performance. Other treatments for enhancing MICA expression in MM myeloma cell lines and increase cytotoxicity are also being explored (120).

Immunotherapy protocols have also revealed that the initial benefits of mAb therapy can be followed by resistance to anti-tumor immune responses (88). The mechanisms of resistance might be secondary to reprogrammed metabolism, which generates immune privileges in the TME. Such dysregulated metabolic conditions may influence the deterioration of the mAb, reducing its therapeutic efficacy (121–123). There are several possible explanations for such deterioration (e.g., fragmentation, aggregation or denaturation) and potential loss of mAb activity. One is related to the acidic extracellular pH observed in the TME. These effects depend on the properties of the individual mAb as well as on the environmental characteristics where the mAb is expected to operate (124). Furthermore, an acidic pH may induce degradation of the aspartate amino acid in the complementarity-determining regions (CDR): this may reduce or influence the ability of the mAb to bind to its epitope (123). Therefore, the highly acidic nature of the TME in the MM niche is of extreme relevance in determining the therapeutic activity of the anti-CD38 mAb selected.

Although anti-CD38/daratumumab-mediated therapy has single-agent efficacy in MM disease, clinical trials have suggested that outcomes are improved when treatment is combined with immunomodulator drugs (IMiDs: e.g., dexamethasone, thalidomide, doxorubicin, lenalidomide, among others) (125, 126). It must be kept in mind that the positive charge acquired by the weak chemical base (i.e., doxorubicin) in an acidic BM environment inhibits its permeability across biological membranes. Consequently, the efficacy of drug delivery and the resistance mechanisms are now postulated to link an acidic TME with the dynamics of the tumor cell membrane. Importantly, additional mechanisms of resistance continue to be discovered, further elucidating the complex interactions between malignant cells and the immune system (88).

CONCLUSIONS

An accurate depiction of the metabolism of extracellular nucleotides facilitates the design of original strategies for inactivating ADO-dependent immunosuppressive mechanisms. Because ICP therapies, such as anti-PD-1/PD-L1, have acquired resistance to CD38-generated ADO, pathways driven by CD38 that involve ADO production may be considered as a promising therapeutic approach. In line with this, several options to counteract the immunosuppressive effects of ADO are currently under analysis (93, 125). Synergic strategies being evaluated include (i) inhibition of nucleotide-release channels, (ii) use of inhibitors of ADO generation by the CD39/CD73 and CD38/CD203a/CD73 ectoenzymatic pathways, (iii) use of drugs degrading extracellular ADO. Further approaches are (iv) the use of A2A and A2B ADOR antagonists. Still other potential strategies rely on (v) inhibitors of hypoxia-HIF-1α signaling, (vi) activatory mechanisms of ADO hydrolytic deamination to INO [a caveat is that it can mediate immunosuppressive effects long after ADO catabolization (35)], and to (vii) AMP synthesis from ADO by ADO kinase (127). These are the main options under consideration today. Future studies seek additional targets that might amplify the antitumor immune response, with the aim of increasing the rate of lasting response to immunotherapy. For instance, AMPD is a purine metabolic enzyme that converts AMP to IMP (see section Adenosine Production within the BM Niche). The enzyme was analyzed in hematological malignancies to investigate whether it is suitable as a novel target for MM therapy (128). The report raised the possibility that AMPD inhibition might be useful as a novel therapeutic strategy for MM. Moreover, AMPD inhibitors induced cell death in myeloma cell lines.

One of the aims of the present review has been to provide support for the view that the metabolism/immunity tandem can be useful in the development of a new generation of MM therapies. Some of the proposals mentioned are now entering into clinical trials. The results will validate the efficacy of treatment in terms of its impact on disease progression. A precise definition of the mechanisms through which the intricate purinome network operates in MM will facilitate the design of predictive diagnostic procedures as well as the adoption of pharmacological agents able to target adenosinergic pathways. Along with drugs directly targeting mPCs, these results are expected to lead to future theragnostic applications.

AUTHOR CONTRIBUTIONS

AH and FM analyzed data from the literature and wrote the manuscript. CB contributed to unpublished experiments. FMo contributed to analyzed data from the literature.

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REFERENCES

1. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med.* (2011) 364:1046–60. doi: 10.1056/NEJMra1011442
2. Bianchi G, Munshi NC. Pathogenesis beyond the cancer clone(s) in multiple myeloma. *Blood.* (2015) 125:3049–58. doi: 10.1182/blood-2014-11-568881
3. Horenstein AL, Morandi F, Bracci C, Pistoia V, Malavasi F. Functional insights into nucleotide-metabolizing ectoenzymes expressed by bone marrow-resident cells in patients with multiple myeloma. *Immunol Lett.* (2018) 205:40–50. doi: 10.1016/j.imlet.2018.11.007
4. Lukashev D, Ohta A, Sitkovsky M. Hypoxia-dependent anti-inflammatory pathways in protection of cancerous tissues. *Cancer Metastasis Rev.* (2007). 26:273–9. doi: 10.1007/s10555-007-9054-2
5. Pezzolo A, Marimpietri D, Raffaghello L, Cocco C, Pistorio A, Gambini C, et al. Failure of anti tumor-derived endothelial cell immunotherapy depends on augmentation of tumor hypoxia. *Oncotarget.* (2014) 5:10368–81. doi: 10.18632/oncotarget.2015
6. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood.* (2004) 104:2484–91. doi: 10.1182/blood-2003-11-3839
7. Quarona V, Ferri V, Chillemi A, Bolzoni M, Mancini C, Zaccarello G, et al. Unraveling the contribution of ectoenzymes to myeloma life and survival in the bone marrow niche. *Ann N Y Acad Sci.* (2015) 1335:10–22. doi: 10.1111/nyas.12485
8. Apasov S, Koshiba M, Redegeld F, Sitkovsky MV. Role of extracellular ATP and P1 and P2 classes of purinergic receptors in T-cell development and cytotoxic T lymphocyte effector functions. *Immunol Rev.* (1995) 146:5–19. doi: 10.1111/j.1600-065X.1995.tb00680.x
9. Horenstein AL, Chillemi A, Zaccarello G, Bruzzone S, Quarona V, Zito A, et al. A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology.* (2013) 2:e26246. doi: 10.4161/onci.26246
10. Allard D, Turcotte M, Stagg J. Targeting A2 adenosine receptors in cancer. *Immunol Cell Biol.* (2017) 95:333–9. doi: 10.1038/icb.2017.8
11. Sitkovsky MV, Ohta A. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol.* (2005) 26:299–304. doi: 10.1016/j.it.2005.04.004
12. Horenstein AL, Quarona V, Toscani D, Costa F, Chillemi A, Pistoia V, et al. Adenosine generated in the bone marrow niche through a CD38-mediated pathway correlates with progression of human myeloma. *Mol Med.* (2016) 22:694–704. doi: 10.2119/molmed.2016.00198
13. Kuehl WM, Bergsagel PL. Molecular pathogenesis of multiple myeloma and its premalignant precursor. *J Clin Invest.* (2012) 122:3456–63. doi: 10.1172/JCI61188
14. Manier S, Kawano Y, Bianchi G, Roccaro AM, Ghobrial IM. Cell autonomous and microenvironmental regulation of tumor progression in precursor states of multiple myeloma. *Curr Opin Hematol.* (2016) 23:426–33. doi: 10.1097/MOH.0000000000000259
15. Volonte C, D'Ambrosi N. Membrane compartments and purinergic signalling: the purinome, a complex interplay among ligands, degrading enzymes, receptors and transporters. *FEBS J.* (2009) 276:318–29. doi: 10.1111/j.1742-4658.2008.06793.x

16. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest.* (2013) 123:3664–71. doi: 10.1172/JCI67230
17. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
18. Keibler MA, Wasylenko TM, Kelleher JK, Iliopoulos O, Vander Heiden MG, Stephanopoulos G. Metabolic requirements for cancer cell proliferation. *Cancer Metab.* (2016) 4:16. doi: 10.1186/s40170-016-0156-6
19. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell.* (2008) 134:703–7. doi: 10.1016/j.cell.2008.08.021
20. Bolzoni M, Toscani D, Costa F, Vicario E, Aversa F, Giuliani N. The link between bone microenvironment and immune cells in multiple myeloma: Emerging role of CD38. *Immunol Lett.* (2018) 205:65–70. doi: 10.1016/j.imlet.2018.04.007
21. Ferretti E, Horenstein AL, Canzonetta C, Costa F, Morandi F. Canonical and non-canonical adenosinergic pathways. *Immunol Lett.* (2018) 205:25–30. doi: 10.1016/j.imlet.2018.03.007
22. Horenstein AL, Sizzano F, Lusso R, Besso FG, Ferrero E, Deaglio S, et al. CD38 and CD157 ectoenzymes mark cell subsets in the human corneal limbus. *Mol Med.* (2009) 15:76–84. doi: 10.2119/molmed.2008.00108
23. Cecati M, Emanuelli M, Giannubilo SR, Quarona V, Senetta R, Malavasi F, et al. Contribution of adenosine-producing ectoenzymes to the mechanisms underlying the mitigation of maternal-fetal conflicts. *J Biol Regul Homeost Agents.* (2013) 27:519–29.
24. Sitkovsky MV, Kjaergaard J, Lukashev D, Ohta A. Hypoxia-adenosinergic immunosuppression: tumor protection by T regulatory cells and cancerous tissue hypoxia. *Clin Cancer Res.* (2008) 14:5947–52. doi: 10.1158/1078-0432.CCR-08-0229
25. Horenstein AL, Chillemi A, Quarona V, Zito A, Roato I, Morandi F, et al. NAD^+-Metabolizing ectoenzymes in remodeling tumor-host interactions: the human myeloma model. *Cells.* (2015) 4:520–37. doi: 10.3390/cells4030520
26. Chillemi A, Quarona V, Antonioli L, Ferrari D, Horenstein AL, Malavasi F. Roles and modalities of ectonucleotidases in remodeling the multiple myeloma niche. *Front Immunol.* (2017) 8:305. doi: 10.3389/fimmu.2017.00305
27. Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene.* (2010) 29:5346–58. doi: 10.1038/onc.2010.292
28. Stagg J, Johnstone RW, Smyth MJ. From cancer immunosurveillance to cancer immunotherapy. *Immunol Rev.* (2007) 220:82–101. doi: 10.1111/j.1600-065X.2007.00566.x
29. Serra S, Horenstein AL, Vaisitti T, Brusa D, Rossi D, Laurenti L, et al. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. *Blood.* (2011) 118:6141–52. doi: 10.1182/blood-2011-08-374728
30. Sitkovsky MV, Hatfield S, Abbott R, Belikoff B, Lukashev D, Ohta A. Hostile, hypoxia-A2-adenosinergic tumor biology as the next barrier to overcome for tumor immunologists. *Cancer Immunol Res.* (2014) 2:598–605. doi: 10.1158/2326-6066.CIR-14-0075
31. Milosevic M, Petrovic S, Velickovic N, Grkovic I, Ignjatovic M, Horvat A. ATP and ADP hydrolysis in cell membranes from rat myometrium. *Mol Cell Biochem.* (2012) 371:199–208. doi: 10.1007/s11010-012-1436-2
32. Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. *Genes Dis.* (2017) 4:25–7. doi: 10.1016/j.gendis.2017.02.003
33. Gordon EL, Pearson JD, Slakey LL. The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. Feed-forward inhibition of adenosine production at the cell surface. *J Biol Chem.* (1986) 261:15496–507.
34. Zhao H, Bo C, Kang Y, Li H. What else can CD39 tell us? *Front Immunol.* (2017) 8:727. doi: 10.3389/fimmu.2017.00727
35. Yuan S, Jiang X, Zhou X, Zhang Y, Teng T, Xie P. Inosine alleviates depression-like behavior and increases the activity of the ERK-CREB signaling in adolescent male rats. *Neuroreport.* (2018) 29:1223–9. doi: 10.1097/WNR.0000000000001101
36. Barsotti C, Ipata PL. Metabolic regulation of ATP breakdown and of adenosine production in rat brain extracts. *Int J Biochem Cell Biol.* (2004) 36:2214–25. doi: 10.1016/j.biocel.2004.04.015
37. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, et al. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev.* (2008) 88:841–86. doi: 10.1152/physrev.00035.2007
38. Malavasi F, Funaro A, Roggero S, Horenstein A, Calosso L, Mehta K. Human CD38: a glycoprotein in search of a function. *Immunol Today.* (1994) 15:95–7. doi: 10.1016/0167-5699(94)90148-1
39. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood.* (2018) 131:13–29. doi: 10.1182/blood-2017-06-740944
40. Chiarugi A, Dolle C, Felici R, Ziegler M. The NAD metabolome–a key determinant of cancer cell biology. *Nat Rev Cancer.* (2012) 12:741–52. doi: 10.1038/nrc3340
41. Giron MC, Bin A, Brun P, Etteri S, Bolego C, Florio C, et al. Cyclic AMP in rat ileum: evidence for the presence of an extracellular cyclic AMP-adenosine pathway. *Gastroenterology.* (2008) 134:1116–26. doi: 10.1053/j.gastro.2008.01.030
42. Beavo JA, Brunton LL. Cyclic nucleotide research – still expanding after half a century. *Nat Rev Mol Cell Biol.* (2002) 3:710–8. doi: 10.1038/nrm911
43. Pleli T, Mondorf A, Ferreiros N, Thomas D, Dvorak K, Biondi RM, et al. Activation of adenylyl cyclase causes stimulation of adenosine receptors. *Cell Physiol Biochem.* (2018) 45:2516–28. doi: 10.1159/000488270
44. Hofer AM, Lefkimmiatis K. Extracellular calcium and cAMP: second messengers as "third messengers"? *Physiology.* (2007) 22:320–7. doi: 10.1152/physiol.00019.2007
45. Copsel S, Garcia C, Diez F, Vermeulem M, Baldi A, Bianciotti LG, et al. Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J Biol Chem.* (2011) 286:6979–88. doi: 10.1074/jbc.M110.166868
46. Guida L, Bruzzone S, Sturla L, Franco L, Zocchi E, De Flora A. Equilibrative and concentrative nucleoside transporters mediate influx of extracellular cyclic ADP-ribose into 3T3 murine fibroblasts. *J Biol Chem.* (2002) 277:47097–105. doi: 10.1074/jbc.M207793200
47. Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE. International union of basic and clinical pharmacology. LXXXI. Nomenclature and classification of adenosine receptors–an update. *Pharmacol Rev.* (2011) 63:1–34. doi: 10.1124/pr.110.003285
48. Funaro A, Horenstein AL, Calosso L, Morra M, Tarocco RP, Franco L, et al. Identification and characterization of an active soluble form of human CD38 in normal and pathological fluids. *Int Immunol.* (1996) 8:1643–50. doi: 10.1093/intimm/8.11.1643
49. Ferrero E, Saccucci F, Malavasi F. The human CD38 gene: polymorphism, CpG island, and linkage to the CD157 (BST-1) gene. *Immunogenetics.* (1999) 49:597–604. doi: 10.1007/s002510050654
50. Kudo O, Sabokbar A, Pocock A, Itonaga I, Fujikawa Y, Athanasou NA. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone.* (2003) 32:1–7. doi: 10.1016/S8756-3282(02)00915-8
51. Evans BA, Elford C, Pexa A, Francis K, Hughes AC, Deussen A, et al. Human osteoblast precursors produce extracellular adenosine, which modulates their secretion of IL-6 and osteoprotegerin. *J Bone Miner Res.* (2006) 21:228–36. doi: 10.1359/JBMR.051021
52. Hunsucker SA, Magarotto V, Kuhn DJ, Kornblau SM, Wang M, Weber DM, et al. Blockade of interleukin-6 signalling with siltuximab enhances melphalan cytotoxicity in preclinical models of multiple myeloma. *Br J Haematol.* (2011) 152:579–92. doi: 10.1111/j.1365-2141.2010.08533.x
53. Burger R. Impact of interleukin-6 in hematological malignancies. *Transfus Med Hemother.* (2013) 40:336–43. doi: 10.1159/000354194
54. Kasztan M, Piwkowska A, Kreft E, Rogacka D, Audzeyenka I, Szczepanska-Konkel M, et al. Extracellular purines' action on glomerular albumin permeability in isolated rat glomeruli: insights into the pathogenesis of albuminuria. *Am J Physiol Renal Physiol.* (2016) 311:F103–11. doi: 10.1152/ajprenal.00567.2015
55. Morandi F, Marimpietri D, Horenstein AL, Bolzoni M, Toscani D, Costa F, et al. Microvesicles released from multiple myeloma cells are equipped with ectoenzymes belonging to canonical and non-canonical adenosinergic pathways and produce adenosine from ATP and NAD. *Oncoimmunology.* (2018) 7:e1458809. doi: 10.1080/2162402X.2018.1458809

56. Greipp PR, San Miguel J, Durie BG, Crowley JJ, Barlogie B, Blade J, et al. International staging system for multiple myeloma. *J Clin Oncol.* (2005) 23:3412–20. doi: 10.1200/JCO.2005.04.242
57. Mikhael JR, Dingli D, Roy V, Reeder CB, Buadi FK, Hayman SR, et al. Management of newly diagnosed symptomatic multiple myeloma: updated Mayo Stratification of Myeloma and Risk-Adapted Therapy (mSMART) consensus guidelines 2013. *Mayo Clin Proc.* (2013) 88:360–76. doi: 10.1016/j.mayocp.2013.01.019
58. Kyrtsonis MC, Maltezas D, Tzenou T, Koulieris E, Bradwell AR. Staging systems and prognostic factors as a guide to therapeutic decisions in multiple myeloma. *Semin Hematol.* (2009) 46:110–7. doi: 10.1053/j.seminhematol.2009.02.004
59. An G, Acharya C, Feng X, Wen K, Zhong M, Zhang L, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. *Blood.* (2016) 128:1590–603. doi: 10.1182/blood-2016-03-707547
60. Costa F, Toscani D, Chillemi A, Quarona V, Bolzoni M, Marchica V, et al. Expression of CD38 in myeloma bone niche: a rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation. *Oncotarget.* (2017) 8:56598–611. doi: 10.18632/oncotarget.17896
61. Kawano Y, Roccaro AM, Ghobrial IM, Azzi J. Multiple myeloma and the immune microenvironment. *Curr Cancer Drug Targets.* (2017) 17:806–18. doi: 10.2174/1568009617666170214102301
62. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci.* (2012) 125(Pt 23):5591–6. doi: 10.1242/jcs.116392
63. Cascone T, McKenzie JA, Mbofung RM, Punt S, Wang Z, Xu C, et al. Increased tumor glycolysis characterizes immune resistance to adoptive t cell therapy. *Cell Metab.* (2018) 27:977–87.e4. doi: 10.1016/j.cmet.2018.02.024
64. Roodman GD. Pathogenesis of myeloma bone disease. *Leukemia.* (2009) 23:435–41. doi: 10.1038/leu.2008.336
65. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity.* (2013) 39:1–10. doi: 10.1016/j.immuni.2013.07.012
66. Bronte V. Tumor cells hijack macrophages via lactic acid. *Immunol Cell Biol.* (2014) 92:647–9. doi: 10.1038/icb.2014.67
67. Dosani T, Carlsten M, Maric I, Landgren O. The cellular immune system in myelomagenesis: NK cells and T cells in the development of myeloma [corrected] and their uses in immunotherapies. *Blood Cancer J.* (2015) 5:e306. doi: 10.1038/bcj.2015.32
68. Gorgun GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood.* (2013) 121:2975–87. doi: 10.1182/blood-2012-08-448548
69. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood.* (2006) 107:2013–21. doi: 10.1182/blood-2005-05-1795
70. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab.* (2016) 24:657–71. doi: 10.1016/j.cmet.2016.08.011
71. Allard B, Beavis PA, Darcy PK, Stagg J. Immunosuppressive activities of adenosine in cancer. *Curr Opin Pharmacol.* (2016) 29:7–16. doi: 10.1016/j.coph.2016.04.001
72. Ohta A, Kini R, Ohta A, Subramanian M, Madasu M, Sitkovsky M. The development and immunosuppressive functions of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol.* (2012) 3:190. doi: 10.3389/fimmu.2012.00190
73. Morandi F, Horenstein AL, Chillemi A, Quarona V, Chiesa S, Imperatori A, et al. $CD56^{bright}$CD16- NK cells produce adenosine through a CD38-mediated pathway and act as regulatory cells inhibiting autologous $CD4^+$ T cell proliferation. *J Immunol.* (2015) 195:965–72. doi: 10.4049/jimmunol.1500591
74. Ohta A. A metabolic immune checkpoint: adenosine in tumor microenvironment. *Front Immunol.* (2016) 7:109. doi: 10.3389/fimmu.2016.00109
75. Camici M, Allegrini S, Tozzi MG. Interplay between adenylate metabolizing enzymes and AMP-activated protein kinase. *FEBS J.* (2018) 285:3337–52. doi: 10.1111/febs.14508
76. Morandi F, Morandi B, Horenstein AL, Chillemi A, Quarona V, Zaccarello G, et al. A non-canonical adenosinergic pathway led by CD38 in human melanoma cells induces suppression of T cell proliferation. *Oncotarget.* (2015) 6:25602–18. doi: 10.18632/oncotarget.4693
77. Bardeleben C, Sharma S, Reeve JR, Bassilian S, Frost P, Hoang B, et al. Metabolomics identifies pyrimidine starvation as the mechanism of 5-aminoimidazole-4-carboxamide-1-beta-riboside-induced apoptosis in multiple myeloma cells. *Mol Cancer Ther.* (2013) 12:1310–21. doi: 10.1158/1535-7163.MCT-12-1042
78. Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-D- ribofuranoside inhibits cancer cell proliferation *in vitro* and *in vivo* via AMP-activated protein kinase. *J Biol Chem.* (2005) 280:39582–93. doi: 10.1074/jbc.M507443200
79. Fedele G, Di Girolamo M, Recine U, Palazzo R, Urbani F, Horenstein AL, et al. CD38 ligation in peripheral blood mononuclear cells of myeloma patients induces release of protumorigenic IL-6 and impaired secretion of IFNgamma cytokines and proliferation. *Mediators Inflamm.* (2013) 2013:564687. doi: 10.1155/2013/564687
80. Fedele G, Sanseverino I, D'Agostino K, Schiavoni I, Locht C, Horenstein AL, et al. Unconventional, adenosine-producing suppressor T cells induced by dendritic cells exposed to BPZE1 pertussis vaccine. *J Leukoc Biol.* (2015) 98:631–9. doi: 10.1189/jlb.3A0315-101R
81. Sitkovsky M, Lukashev D, Deaglio S, Dwyer K, Robson SC, Ohta A. Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. *Br J Pharmacol.* (2008) 153(Suppl 1):S457–64. doi: 10.1038/bjp.2008.23
82. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734
83. Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol.* (2001) 22:265–8. doi: 10.1016/S1471-4906(01)01888-9
84. Zarek PE, Powell JD. Adenosine and anergy. *Autoimmunity.* (2007) 40:425–32. doi: 10.1080/08916930701464939
85. Borcherding N, Kolb R, Gullicksrud J, Vikas P, Zhu Y, Zhang W. Keeping tumors in check: a mechanistic review of clinical response and resistance to immune checkpoint blockade in cancer. *J Mol Biol.* (2018) 430:2014–29. doi: 10.1016/j.jmb.2018.05.030
86. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* (2018) 359:1350–5. doi: 10.1126/science.aar4060
87. Sharpe AH. Introduction to checkpoint inhibitors and cancer immunotherapy. *Immunol Rev.* (2017) 276:5–8. doi: 10.1111/imr.12531
88. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell.* (2017) 168:707–23. doi: 10.1016/j.cell.2017.01.017
89. O'Donnell JS, Long GV, Scolyer RA, Teng MW, Smyth MJ. Resistance to PD1/PDL1 checkpoint inhibition. *Cancer Treat Rev.* (2017) 52:71–81. doi: 10.1016/j.ctrv.2016.11.007
90. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med.* (2018) 378:158–68. doi: 10.1056/NEJMra1703481
91. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. *Nat Rev Cancer.* (2017) 17:709–24. doi: 10.1038/nrc.2017.86
92. Chen L, Diao L, Yang Y, Yi X, Rodriguez BL, Li Y, et al. CD38-mediated immunosuppression as a mechanism of tumor cell escape from PD-1/PD-L1 blockade. *Cancer Discov.* (2018) 8:1156–75. doi: 10.1158/2159-8290.CD-17-1033
93. Whiteside TL. Targeting adenosine in cancer immunotherapy: a review of recent progress. *Expert Rev Anticancer Ther.* (2017) 17:527–35. doi: 10.1080/14737140.2017.1316197
94. Mittal D, Young A, Stannard K, Yong M, Teng MW, Allard B, et al. Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. *Cancer Res.* (2014) 74:3652–8. doi: 10.1158/0008-5472.CAN-14-0957

95. Arana P, Paiva B, Cedena MT, Puig N, Cordon L, Vidriales MB, et al. Prognostic value of antigen expression in multiple myeloma: a PETHEMA/GEM study on 1265 patients enrolled in four consecutive clinical trials. *Leukemia.* (2018) 32:971–8. doi: 10.1038/leu.2017.320
96. Moreno L, Perez C, Zabaleta A, Manrique I, Alignani D, Ajona D, et al. The mechanism of action of the anti-CD38 monoclonal antibody isatuximab in multiple myeloma. *Clin Cancer Res.* (2019). doi: 10.1158/1078-0432.CCR-18-1597. [Epubh ahead of print].
97. Krejcik J, van de Donk N. Trogocytosis represents a novel mechanism of action of daratumumab in multiple myeloma. *Oncotarget.* (2018) 9:33621–2. doi: 10.18632/oncotarget.26098
98. Chillemi A, Quarona V, Zito A, Morandi F, Marimpietri D, Cuccioloni M, et al. Generation and characterization of microvesicles after daratumumab interaction with myeloma cells. *Blood.* (2015) 126:1849–1849.
99. van de Donk N, Usmani SZ. CD38 antibodies in multiple myeloma: mechanisms of action and modes of resistance. *Front Immunol.* (2018) 9:2134. doi: 10.3389/fimmu.2018.02134
100. Young A, Mittal D, Stagg J, Smyth MJ. Targeting cancer-derived adenosine: new therapeutic approaches. *Cancer Discov.* (2014) 4:879–88. doi: 10.1158/2159-8290.CD-14-0341
101. Krejcik J, Frerichs KA, Nijhof IS, van Kessel B, van Velzen JF, Bloem AC, et al. Monocytes and granulocytes reduce CD38 expression levels on myeloma cells in patients treated with daratumumab. *Clin Cancer Res.* (2017) 23:7498–511. doi: 10.1158/1078-0432.CCR-17-2027
102. Deaglio S, Dianzani U, Horenstein AL, Fernandez JE, van Kooten C, Bragardo M, et al. Human CD38 ligand. A 120-KDA protein predominantly expressed on endothelial cells. *J Immunol.* (1996) 156:727–34.
103. Gallay N, Anani L, Lopez A, Colombat P, Binet C, Domenech J, et al. The role of platelet/endothelial cell adhesion molecule 1 (CD31) and CD38 antigens in marrow microenvironmental retention of acute myelogenous leukemia cells. *Cancer Res.* (2007) 67:8624–32. doi: 10.1158/0008-5472.CAN-07-0402
104. Kumar SK, Anderson KC. Immune therapies in multiple myeloma. *Clin Cancer Res.* (2016) 22:5453–60. doi: 10.1158/1078-0432.CCR-16-0868
105. Kumar S, Saini RV, Mahindroo N. Recent advances in cancer immunology and immunology-based anticancer therapies. *Biomed Pharmacother.* (2017) 96:1491–500. doi: 10.1016/j.biopha.2017.11.126
106. Horenstein AL, Chillemi A, Quarona V, Zito A, Mariani V, Faini AC, et al. Antibody mimicry, receptors and clinical applications. *Hum Antibodies.* (2017) 25:75–85. doi: 10.3233/HAB-160305
107. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat Rev Immunol.* (2010) 10:317–27. doi: 10.1038/nri2744
108. Shepard HM, Phillips GL, D Thanos C, Feldmann M. Developments in therapy with monoclonal antibodies and related proteins. *Clin Med (Lond).* (2017) 17:220–32. doi: 10.7861/clinmedicine.17-3-220
109. Rieth J, and Subramanian, S. Mechanisms of intrinsic tumor resistance to immunotherapy. *Int J Mol Sci.* (2018) 19:E1340. doi: 10.3390/ijms19051340
110. Ferrero E, Lo Buono N, Horenstein AL, Funaro A, Malavasi F. The ADP-ribosyl cyclases– the current evolutionary state of the ARCs. *Front Biosci.* (2014) 19:986–1002. doi: 10.2741/4262
111. Caligaris-Cappio F, Bergui L, Tesio L, Pizzolo G, Malavasi F, Chilosi M, et al. Identification of malignant plasma cell precursors in the bone marrow of multiple myeloma. *J Clin Invest.* (1985) 76:1243–51. doi: 10.1172/JCI112080
112. Chillemi A, Zaccarello G, Quarona V, Ferracin M, Ghimenti C, Massaia M, et al. Anti-CD38 antibody therapy: windows of opportunity yielded by the functional characteristics of the target molecule. *Mol Med.* (2013) 19:99–108. doi: 10.2119/molmed.2013.00009
113. van de Donk NW, Janmaat ML, Mutis T, Lammerts van Bueren JJ, Ahmadi T, Sasser AK, et al. Monoclonal antibodies targeting CD38 in hematological malignancies and beyond. *Immunol Rev.* (2016) 270:95–112. doi: 10.1111/imr.12389
114. Frerichs KA, Nagy NA, Lindenbergh PL, Bosman P, Marin Soto J, Broekmans M, et al. CD38-targeting antibodies in multiple myeloma: mechanisms of action and clinical experience. *Expert Rev Clin Immunol.* (2018) 14:197–206. doi: 10.1080/1744666X.2018.1443809
115. Castella B, Faini A, Yakymiv Y, Morandi F, Larocca A, Oliva S, et al. Induction of structural and functional effects of myeloma cells after daratumumab treatment. *Cancer Res.* 78(13 Suppl):2122. doi: 10.1158/1538-7445.AM2018-2122
116. Franssen LE, Mutis T, Lokhorst HM, van de Donk N. Immunotherapy in myeloma: how far have we come? *Ther Adv Hematol.* (2019) 10:20406207188 22660. doi: 10.1177/2040620718822660
117. Curti A, Aluigi M, Pandolfi S, Ferri E, Isidori A, Salvestrini V, et al. Acute myeloid leukemia cells constitutively express the immunoregulatory enzyme indoleamine 2,3-dioxygenase. *Leukemia.* (2007) 21:353–5. doi: 10.1038/sj.leu.2404485
118. Mesiano G, Zini R, Montagner G, Bianchi N, Manfredini R, Chillemi A, et al. Analytic and dynamic secretory profile of patient-derived cytokine-induced killer cells. *Mol Med.* (2017) 23:235–46. doi: 10.2119/molmed.2017.00084
119. Horenstein AL, Chillemi A, Zini R, Quarona V, Bianchi N, Manfredini R, et al. Cytokine-induced killer cells express CD39, CD38, CD203a, CD73 ectoenzymes and P1 adenosinergic receptors. *Front Pharmacol.* (2018) 9:196. doi: 10.3389/fphar.2018.00196
120. Nwangwu CA, Weiher H, Schmidt-Wolf IGH. Increase of CIK cell efficacy by upregulating cell surface MICA and inhibition of NKG2D ligand shedding in multiple myeloma. *Hematol Oncol.* (2017) 35:719–25. doi: 10.1002/hon.2326
121. Ishikawa T, Ito T, Endo R, Nakagawa K, Sawa E, Wakamatsu K. Influence of pH on heat-induced aggregation and degradation of therapeutic monoclonal antibodies. *Biol Pharm Bull.* (2010) 33:1413–7. doi: 10.1248/bpb.33.1413
122. Latypov RF, Hogan S, Lau H, Gadgil H, Liu D. Elucidation of acid-induced unfolding and aggregation of human immunoglobulin IgG1 and IgG2 Fc. *J Biol Chem.* (2012) 287:1381–96. doi: 10.1074/jbc.M111.297697
123. Wang T, Kumru OS, Yi L, Wang YJ, Zhang J, Kim JH, et al. Effect of ionic strength and pH on the physical and chemical stability of a monoclonal antibody antigen-binding fragment. *J Pharm Sci.* (2013) 102:2520–37. doi: 10.1002/jps.23645
124. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability, and formulation. *J Pharm Sci.* (2007) 96:1–26. doi: 10.1002/jps.20727
125. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:1319–31. doi: 10.1056/NEJMoa1607751
126. Palumbo A, Chanan-Khan A, Weisel K, Nooka AK, Masszi T, Beksac M, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med.* (2016) 375:754–66. doi: 10.1056/NEJMoa1606038
127. Boison D. Adenosine kinase: exploitation for therapeutic gain. *Pharmacol Rev.* (2013) 65:906–43. doi: 10.1124/pr.112.006361
128. Kawano Y, Inada Y, Sasano T, Nishimura N, Hata H, Matsuoka M. The purine metabolic enzyme AMPD1 is a novel therapeutic target for multiple myeloma. *Blood.* (2018) 132:5614. doi: 10.1182/blood-2018-99-118603

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