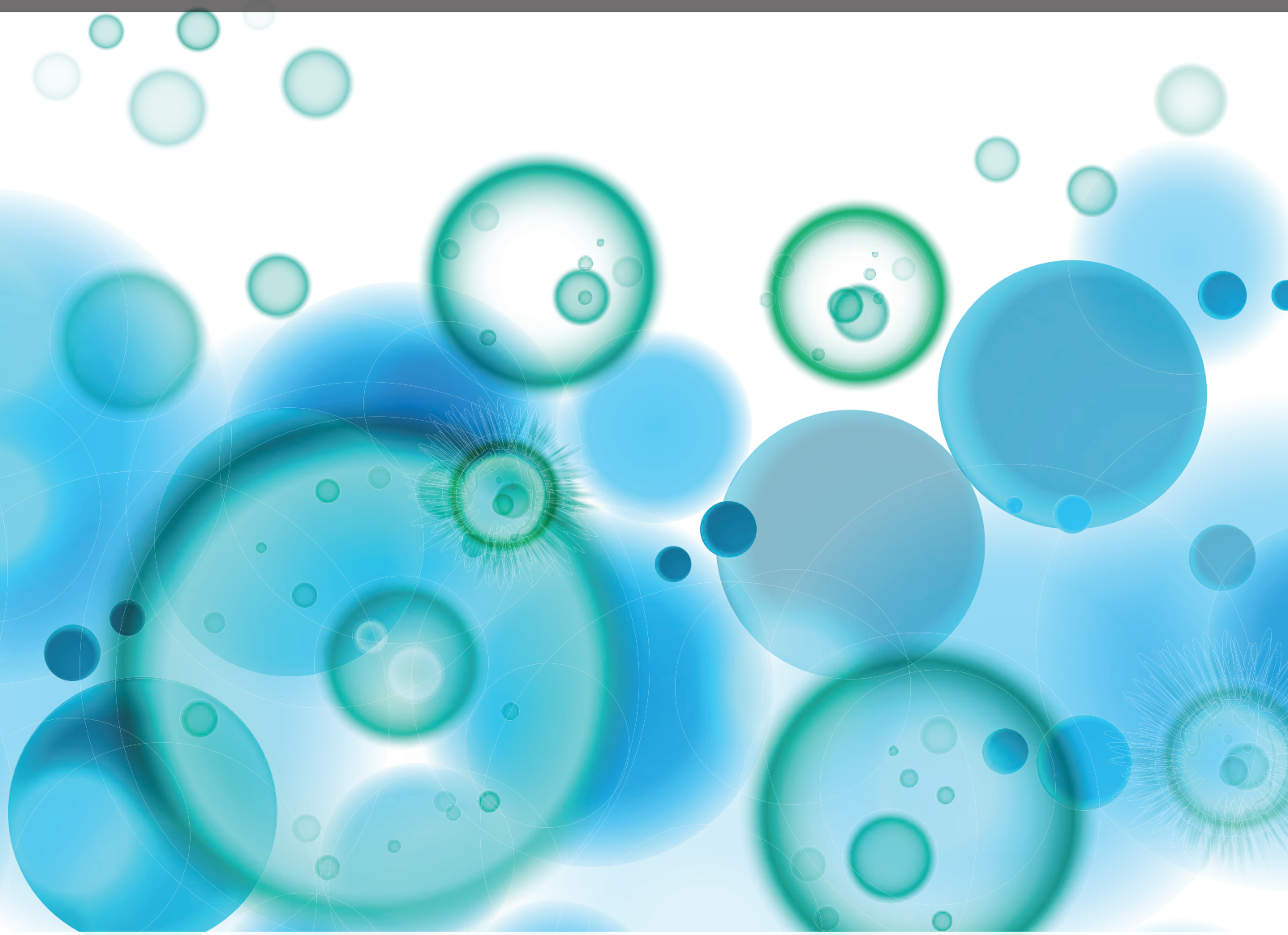
THE ORIGIN OF THE PLASMA CELL HETEROGENEITY

EDITED BY: Catherine Pellat-Deceunynck and Thierry Defrance
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88919-734-7
DOI 10.3389/978-2-88919-734-7

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THE ORIGIN OF THE PLASMA CELL HETEROGENEITY

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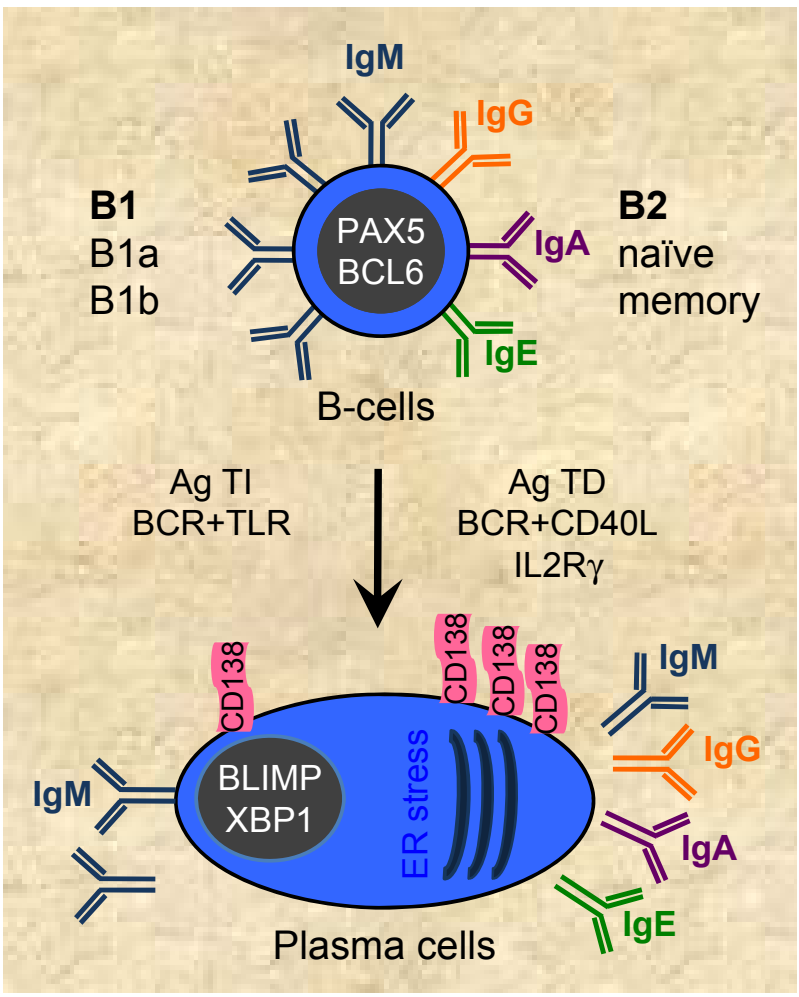

B-cells and PCs express mutually exclusive transcription factors i.e., PAX5 and BCL6 or BLIMP and XBP1. Plasma cells (PCs) differentiate from B-cells of different origins i.e., B1 (B1a or B1b) or B2 (naïve or memory). While T-independent Ags mainly induce secretion of IgM, T-dependent Ags induce secretion of all Ig isotypes. Differentiation induced by T-dependent or T-independent Ags requires signaling through BCR, CD40 and IL2R gamma chain or through BCR and TLRs, respectively. CD138 expression and PC maturity, especially in Humans, are positively correlated. PCs use autophagy to overcome ER stress due to intensive Ig secretion.

Image by Catherine Pellat-Deceunynck

Plasma cells (PCs) are terminally differentiated B-cells producing large amounts of immunoglobulins (Ig). In humans, most of circulating Ig are produced by bone marrow plasma cells. PCs differentiate from activated naïve or memory B-cells usually activated by specific antigens. It is still controversial whether the regulation of PCs numbers and the "active" in vivo Ig diversity depend or not on non-specific reactivation of B-cells during infections. Depending on the stimulus (T-independent/T-dependent antigen, cytokines, partner cells) and B-cell types (naïve or memory, circulating or germinal center, lymph nodes or spleen, B1 or B2…), both the phenotype and isotype of PCs differ suggesting that PC diversity is either linked to B-cell diversity or to the type of stimulus or to both. Knowledge of the mechanisms supporting PC diversity has important consequences for the management of i) plasma cell neoplasia such as Multiple Myeloma and Waldenström's Macroglobulinemia, ii) vaccine protection against pathogens and iii) auto-immune diseases.

Citation: Pellat-Deceunynck, C., Defrance, T., eds. (2015). The Origin of the Plasma Cell Heterogeneity. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-734-7

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frontiers in
IMMUNOLOGY

EDITORIAL
published: 23 January 2015
doi: 10.3389/fimmu.2015.00005

The origin of the plasma-cell heterogeneity

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Keywords: plasma cell, B-cell, cell cycle, IL21, autophagy, B1, autoimmunity, myeloma

Plasma cells (PCs) are terminally differentiated B-cells producing large amounts of immunoglobulins (Igs). In humans, most circulating Igs are produced by bone marrow PCs. PCs differentiate from naïve or memory B-cells usually activated by specific antigens. It is still controversial whether the regulation of PC numbers and the "active" *in vivo* Ig diversity depend or not on non-specific reactivation of B-cells during infections. Depending on the stimulus (T-independent/T-dependent antigen, cytokines, and partner cells) and B-cell types (naïve or memory, circulating or germinal center, lymph nodes or spleen, and B1 or B2), both the phenotype and isotype of PCs differ suggesting that PC diversity is either linked to B-cell diversity or to the type of stimulus or to both. Knowledge of the mechanisms supporting PC diversity has important consequences for the management of: (i) plasma-cell neoplasias such as Multiple Myeloma and Waldenström's Macroglobulinemia, (ii) vaccine protection against pathogens, and (iii) auto-immune diseases.

In this E-book, Drs. Xu and Banchereau review current knowledge on the molecules involved in PC generation induced by either dendritic cells or macrophages in T-dependent or T-independent B-cell activation (1). They further show how dendritic cells or macrophages provide a second signal (membrane-bound or soluble factors) that is required for PC generation. As proposed by the authors, increasing the crosstalk between Ag presenting cells and B-cells may lead to enhanced vaccine-induced Ab responses. On the contrary, interruption of this crosstalk might represent new strategies to treat auto-immune diseases. As splenectomy is the standard of care of patients with auto-immune thrombocytopenia, Dr. Mahévas and collaborators took the opportunity to characterize splenic PCs (2). They show that in patients who did not receive rituximab, spleen PCs had a plasmablast signature. By contrast, spleen PCs from patients who received rituximab had a plasma-cell signature. These *in vivo* findings suggest that short-lived immature PCs differentiated into long-lived PCs *in situ*, raising the question of the existence of splenic niches for long-lived PCs. In the spleens of naïve mice, Dr. Holodick and collaborators assess the origin and function of the small CD138+ B1a cell population. They show that this population is likely responsible for a substantial portion of natural IgM that differs from IgM produced by other B1a cell subsets, underlying the heterogeneity of IgM-secreting cells within B1a cells (3). Dr. Cunningham and collaborators review current knowledge on B1b cells in humans and mice, and discuss how B1 and B2 cells might interplay during the antibody response to proteins like porins from pathogenic microbes, which induce both classical T-dependent and T-independent response (4).

Long-lived PCs are usually found within bone marrow niches, the composition of which is still a matter of debate. Although bone marrow niches are known to provide favorable environments for PC survival, Dr. Tooze explores how a self-renewal model could account for the maintenance of long-lived PCs within niches (5). Indeed, Dr. Tooze describes how regular re-entry into cell cycle could account for PC maintenance. Independently of possible self-renewal, long-life of PCs involves several mechanisms such as autophagy. Indeed, as professional secretory cells dedicated for massive synthesis, assembly, and secretion of Abs, PCs display high ER stress (6). Using mice knocked-out for atg5, Drs. Oliva and Cenci show that both generation and long-life of PCs was decreased in the absence of autophagy, highlighting the role of protein catabolism (6). Generation of long-lived PCs requires cytokines and growth factors. Dr. Moens and collaborators provide an overview of *in vitro* studies (mouse and human origin) that evaluated the role of the different cytokines in inducing differentiation of distinct B cell subsets into PCs. They underline the central role of IL21 in PC generation from naïve B-cells (7). IL21, as other cytokines and growth factors, modulates the expression of Bcl2 molecules, and in that way the survival/death threshold of PCs (7). In myeloma cells, the expression of the pro (BH3-only, Bax, Bak) and anti-apoptotic (Bcl2, Mcl1) molecules of the Bcl2 family is different according to the molecular classification of patients with multiple myeloma, as shown by Drs. Gomez-Bougie and Amiot, who conclude that the apoptosis threshold in myeloma differs greatly between subsets of patients (8). Myeloma cells also display a wide heterogeneity with regard to phenotype. Dr. Robillard and collaborators describe the diversity of myeloma immunophenotypes, which is useful for the

evaluation of minimum residual disease after treatment and represents a source of targets for antibody-based therapy in subsets of patients (9).

The nine contributions in this topic describe the heterogeneity of PCs and discuss mechanisms involved in heterogeneity. Altogether, these contributions provide an overview on mechanisms involved in PC cell diversity in the context of normal and pathologic Ab responses.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 November 2014; accepted: 06 January 2015; published online: 23 January 2015.

*Citation: Pellat-Deceunynck C and Defrance T (2015) The origin of the plasma-cell heterogeneity. Front. Immunol. **6**:5. doi: 10.3389/fimmu.2015.00005*

This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 06 January 2014
doi: 10.3389/fimmu.2013.00504

The antigen presenting cells instruct plasma cell differentiation

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The professional antigen presenting cells (APCs), including many subsets of dendritic cells and macrophages, not only mediate prompt but non-specific response against microbes, but also bridge the antigen-specific adaptive immune response through antigen presentation. In the latter, typically activated B cells acquire cognate signals from T helper cells in the germinal center of lymphoid follicles to differentiate into plasma cells (PCs), which generate protective antibodies. Recent advances have revealed that many APC subsets provide not only "signal 1" (the antigen), but also "signal 2" to directly instruct the differentiation process of PCs in a T-cell-independent manner. Herein, the different signals provided by these APC subsets to direct B cell proliferation, survival, class switching, and terminal differentiation are discussed. We furthermore propose that the next generation of vaccines for boosting antibody response could be designed by targeting APCs.

Keywords: plasma cells, antigen presenting cells, macrophages, dendritic cells, B cells

INTRODUCTION

B cell activation is initiated following engagement of the B cell receptor (BCR) by a specific antigen in either a T-cell-dependent (TD) or T-cell-independent (TI) manner (1). Most long-lived plasma cells (PCs) in the bone marrow are derived from TD responses involving germinal center reactions followed by niches favoring long-term survival. As it usually takes several days for the cognate T cells to help, a prompt TI response provides the first wave of humoral protection by generating short-lived PCs in the extrafollicular foci of the peripheral lymphoid organs such as lymph nodes, spleen, Peyer's patches, and tonsils (2). Indeed, some TI challenges could also induce long-lived antibody responses (3–5).

Professional antigen presenting cells (APCs), including dendritic cells (DCs) and macrophages, present antigens to T cells to initiate adaptive immunity by sequentially delivering signal 1 (antigen), signal 2 (co-stimulation), and signal 3 (polarizing signals mediated by soluble or membrane-bound factors) (6). They can, by similar means, initiate and guide B cell differentiation toward PCs in a TI manner. Precisely, DCs and macrophages efficiently take up large size antigens (such as particulates, immune complexes, and virus that travel through the subcapsular sinus), and present them to naïve B cells in the periphery lymphoid organs (2). Recent advances have revealed that APCs deliver not only signal 1, but also late signals to instruct terminal differentiation of PCs in both a TI and TD manner. In a TD manner, CD40-CD40L interaction between B cells and cognate T cells is instrumental in driving germinal center formation for affinity maturation. Whereas in a TI manner, APC-derived factors and the ligand-receptor signals between APC and B cells combines to deliver signals for PC differentiation (**Figure 1**). This review discusses the signals provided by these APC subsets and shapes a rationale of designing therapeutic vaccines for humoral immunity by targeting APCs.

DC SUBSETS INSTRUCT B CELL DIFFERENTIATION

Back in the 1990s, following the early milestone discovery of DCs in mouse (7) and human (8), DCs have been recognized for their capacity of priming naïve B cells in human *in vitro* settings (9–11). In the presence of CD40 signaling, naïve B cells undergo class switching toward IgA1 and IgA2 isotype by DCs, and class switching (11). These early works using human monocyte-derived DCs provided the first evidence that in addition to their capacity to activate naive T cells in the extrafollicular areas of secondary lymphoid organs, DCs may directly modulate B cell growth and differentiation. Similarly, mouse splenic DCs were able to interact with naïve B cells and induce TI class switching *in vitro* and *in vivo* (12).

Dendritic cells directly induce TI Ab class switching through the upregulation of B lymphocyte stimulator protein (BLyS, also known as BAFF), and a proliferation-inducing ligand (APRIL) (13). BAFF binds to three different receptors, namely transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFF-R) (14–18). On the other hand, APRIL binds to BCMA with high affinity and to TACI with low affinity, but not to BAFF-R (19, 20). Through engagement with its receptors, BAFF activates a CD40-like pathway that enhances B cell survival via upregulation of NF-κB and Bcl-2 (21). APRIIL appears to induce AID expression in B cells through NF-κB-mediated HoxC4 induction (22). The importance of BAFF and APRIL has been documented in animal models where mice deficient for BAFF or APRIL showed a defect in IgA production (23, 24). Interestingly, B cells exposed to BAFF and APRIL do not secrete IgG and IgA

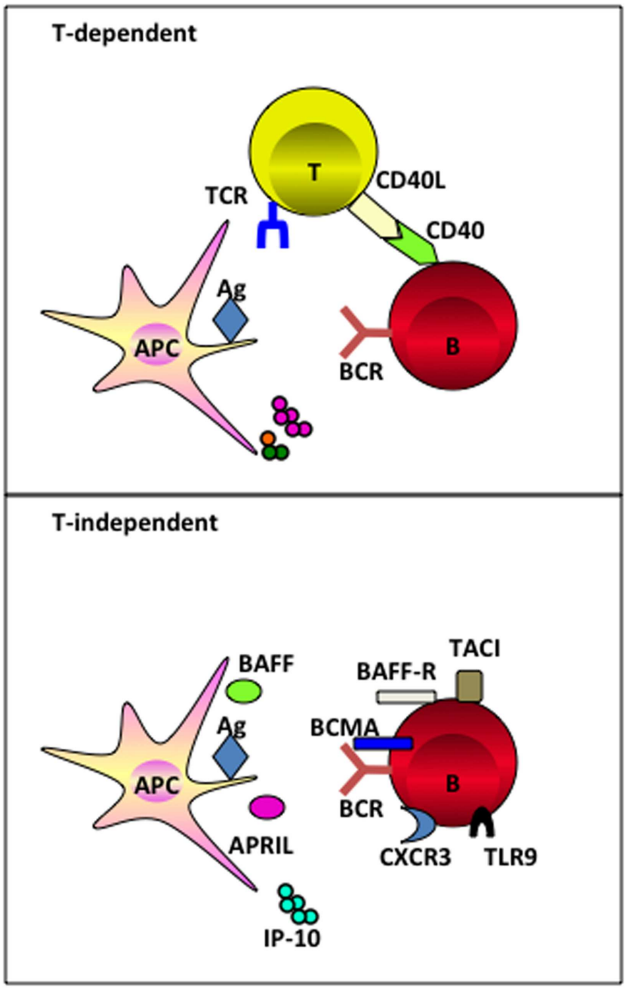

FIGURE 1 | APC subsets induce TI and TD B cell activation. APC subsets including DCs and macrophages, have the capacity to retain and recycle native antigens on their surface and engage with B cell receptor for the signal 1 delivery. Ag-experienced B cells receive cognate T cell signals to form germinal center for the generation of long-lived PCs in a TD manner. Alternatively, APC-derived soluble factors provide late signals to B cells that have the respect receptors during the activation, proliferation, and differentiation, in a TI manner.

unless stimulated through extensive BCR cross-linking. Thus, in a process of DC-mediated B cell differentiation, DCs initially provide TI antigens to engage BCR on B cells for activation. Thereafter, co-signals from other DC-derived factors like BAFF or APRIL or cytokines such as IL-15 cooperatively instruct the terminal differentiation of activated B cells into PCs (13).

Heterogeneous populations of DCs have been discovered in both human and mouse (25). In humans, three subsets have been identified in blood, namely $CD303^+$ plasmacytoid DCs (pDCs), $CD1c^-CD141^+$, and $CD1c^+CD141^-$ circulating DCs (26–28). In the skin, cutaneous DCs express a distinct set of receptors as compared to blood DCs, i.e., $langerin^+$ langerhans cells and $CD14^+$ interstitial dermal DCs (29, 30). Among all subsets, interstitial dermal DCs that represent the *in vivo* counterpart of *in vitro* monocyte-derived DCs, appear to be the ones that preferentially prime B cells for humoral response while poorly triggering $CD8^+$ T cell immunity (31), owing to their capacity to polarize follicular T help cells (Tfh) via DC-derived molecular such as IL-6 (32–34).

Plasmacytoid DCs, the professional type-1 interferon (IFN)-producing cells, promote the differentiation of CD40-stimulated B cells into non-antibody-secreting plasmablasts via IFN-αβ. They sequentially differentiate into antibody-secreting PCs upon additional IL-6 secreted by pDCs (35). Both B cells and pDCs express TLR9. IFN-α production by CpG ligation of the TLR9 on pDCs also generate IgM-producing PCs from both naïve and memory B cells in a TI manner, under the help of other pDC-derived factors such as IL-6, TNF-α, and IL-10 (36). TLR9 ligation of pDCs enhances their CD70 expression to trigger CD27 signaling for B cell survival and differentiation, particularly on memory cells (37). Type-1 IFN can also contribute to PC differentiation indirectly via the upregulation of BAFF and APRIL on myeloid DCs to promote B cell survival, proliferation, and class switching (38), or via promoting Tfh differentiation through myeloid DCs (39). In autoimmune disorders such as systemic lupus erythematosus (SLE), pDCs could be the driver favoring persistence of autoreactive PCs, giving the abnormal signature of type-1 IFN and autologous DNA and DNA-binding proteins (40–42). Indeed, activated pDCs trigger anti-snRNP B cells for enhanced proliferation and antibody production in the mouse (43).

How do B cells acquire antigens from DCs? DCs are found not only in the T cell areas of lymphoid organs where they are ready to prime T cells, but are also interacting with B cells in the follicular areas (44), the red pulp (45), and the marginal zones (46). DCs have a specialized capacity for the retention of antigens (44), enabling delivery of microbes from the intestinal lumen to secondary lymphoid structures (47, 48). Intravital two-photon imaging has revealed that upon lymph node entry, B cells physically survey local antigen-carrying DCs (49). DCs use different receptors to sample antigens that are directed to the degradative compartment for peptide and MHC loading. Interestingly, those antigens or immune complexes internalized by the inhibitory FcγRIIB on DCs were stored in a recycling versical system, largely excluded from the $LAMP\text{-}1^+$ degradative compartment (50). As a consequence, these antigens were trapped in a native form, and recycled to the cell surfaces for the activation of B cells. This strategy for sorting and recycling native antigens through a non-degradative compartment is also used by follicular DCs to access B cells (51). Another inhibitory receptor, dendritic cell immunoreceptor (DCIR), holds the similar property as FcγRIIB for native antigen recycling utilized by marginal zone DCs to initiate B cell activation in a TD manner (52). It has been reported that even in the degradative late endosome, antigens can be released unprocessed by DCs (53). Thus DCs are equipped with an array of machinery to efficiently retain native antigens to BCR engagement on naïve B cells in a TI or TD manner.

MACROPHAGE SUBSETS INSTRUCT B CELL DIFFERENTIATION

Due to the nature of lymphoid structure, it has been conceived for a long time that lymph-born antigens must pass through a zone of macrophages that are beneath the subcapsular sinus *en route*

to reach the follicular B cells (54–56). Macrophages are known to retain antigens for up to 72 h after being exposed to them (57). The very first evidence that macrophages process large size antigens (immune complexes, particulates, and viruses) to present to follicular B cells were found by three impendent groups (58–61). The subcapsular sinus macrophages possibly use CD169 or MAC1 (macrophage receptor 1) to retain antigens on their surface, and consequently B cells acquire antigens from them cumulatively and became the main antigen carriers inside the follicle before polarizing to the B cell-T cell border (58, 59). These studies clearly defined the essential roles of macrophage subsets in the initiation of B cell activation toward lymph-born antigens through dual actions: (1) as innate "flypaper" by preventing the systemic spread of pathogen; (2) as "gatekeepers" at the lymph-tissue interface that facilitate the recognition antigens by B cells and initiate humoral immune responses.

Macrophages residing in the marginal zone have the similar capacity to capture antigen in the spleen (62). Marginal zone macrophages (MZM) express a distinct set of receptors MARCO (macrophage receptor with a collagenous structure) and/or SIGNR1 (a mouse homolog of DC-SIGN), and are therefore different from metallophilic macrophages that express MOMA-1. The first study performed by Ravetch and his colleagues showed that MARCO$^+$ MZM migrate to the red pulp of the spleen and transfer the intact antigens to B cells (63). It seems that SIGNR1 is important for the MZM-mediated B cell response, as MZM that lack expression of SIGNR1 failed to capture the model antigen Ficoll (64), and mice deficient for SIGNR1 failed to mount a humoral response following infection with *Streptococcus pneumoniae* (65).

In humans, the evidence for an exclusive role of macrophages in the induction of humoral response remains scarce. We recently identified that resident tissue macrophages in human tonsils reside closely to the terminally differentiated CD138$^+$ PCs. We went on to unravel that macrophage-derived IP-10 participates in PC development (proliferation, class switching, and terminal differentiation) in the context of an amplification loop where B cell-derived IL-6 induces macrophages to secrete IP-10, which further boosts the B cell autocrine secretion of IL-6 leading to PC differentiation (**Figure 2**) (66). This is the first evidence that a chemokine plays direct role in cell differentiation. In addition, macrophages use VCAM-1 to tether B cells for the delivery of signals (66), supporting the earlier findings that VCAM-1 receptor-ligand interaction promotes membrane-bound antigen recognition and formation of an immune synapse (67).

Like DCs, macrophages promote TI class switching recombination by releasing the essential factors BAFF and APRIL (68–70). Macrophage-derived BAFF and APRIL expression can be enhanced by T cell signals such as IFN-γ and CD40L (68). B cell proliferation and antibody secretion following by BAFF and APRIL stimulation also requires co-stimulatory signals such as IL-6, IL-10, and TGF-β (13, 68, 70). This also implies that there are redundant signaling pathways involved in PC differentiation. For example, in rodents, subcapsular macrophages activate extrafollicular B cells indirectly through presenting CD1d-restricted glycolipid antigens to iNKT cells. PCs homing to the bone marrow require survival niches for long-term residence, and macrophages and their precursors provide such help through APRIL and IL-6 (71–73).

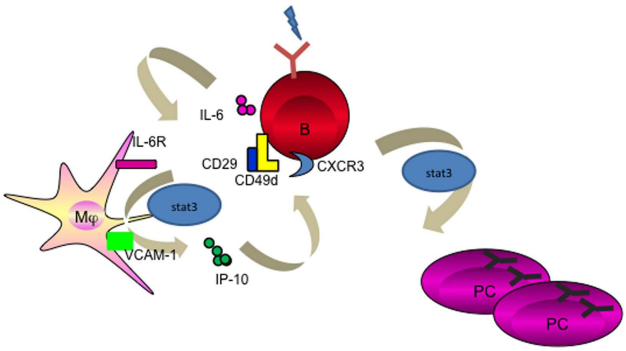

FIGURE 2 | Macrophage mediates PC differentiation. Activated B cells release IL-6 to activate macrophage through STAT3. Macrophage-derived IP-10 sequentially binds to CXCR3 on B cells to trigger amplification loop production of IL-6, leading to STAT3-dependent PC differentiation.

TARGETING APCs FOR A BETTER VACCINE FOR HUMORAL IMMUNITY

Accumulating evidence suggest that APC subsets including DCs and macrophages not only provide "signal 1" for BCR engagement on B cells (74, 75), but further participate in a later stage of cell proliferation and differentiation by providing an additional "signal 2 or 3" such as membrane-bound or soluble factors. While interruption of this pathway might represent an efficient strategy to treat autoimmune diseases, enhancing APC-B cell crosstalk, for example by targeting Ag directly to APCs, may lead to enhanced vaccine-induced Ab responses (**Figure 3**).

Lessons of early pioneering studies *in vivo* targeting DCs through coupling the antigens to a specific receptors such as DEC-205, or DCIR for T cell immunity have paved a solid path toward understanding the efficiency of antigen degradation, and (cross-) presentation (76–78). Indeed, targeting antigens to DC through DCIR (79, 80), DC-SIGN (81), dectin-1 (82), ClEC9A (83), and Langerin (84) generated both humoral and cellular responses. Interestingly, in the absence of adjuvant, targeting antigens to CLEC9A on DCs results in strong antibody response, which is linked to the generation of Tfh cells (85), but no CD8$^+$ T cell immunity despite of the antigen capture and cross-presentation by targeted CD8α$^+$ DCs (83). However, an addition of adjuvant, e.g., poly I:C, skewed a robust CD4$^+$ and CD8$^+$ T cell response (83, 86). Thus, particular DC subsets, antibodies specific for surface receptors, and appropriate adjuvants, combine to define the sequential immune response by DC targeting (**Table 1**) (87).

The strategy of a targeted DC vaccine with an antigen to boost antibody response has met the proof of concept. In two of the studies, targeting DCs through CD11c (N418) showed robust humoral immunity resulting from germinal center formation (88, 89), though mechanistic details about antigen internalization and transfer and the factors involved in PC generation by DCs were lacking. Likely, two principles must be followed to design a better vaccine to boost Ab response by targeting DCs in humans;

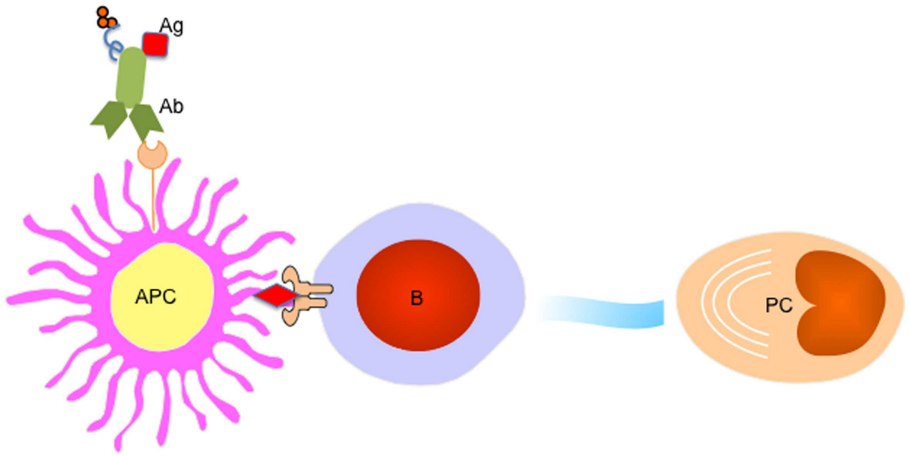

FIGURE 3 | Targeting APC subsets for a better vaccine for humoral immunity. A fusion protein of Ab (recognizing a particular receptor on APC subset) and Ag complex facilitates Ag uptake by targeted APC subset, which processes Ag to B cells to trigger PC differentiation. An adjuvant (for example IP-10) could be linked to the fusion protein to provide additional signals for PC generation and maintenance.

Table 1 | Strategy to design a APC-targeted vaccine.

Selection of APCs	Selection of targeting receptors	Selection of adjuvant
DCs	FcγRIIB	IP-10
	DCIR	IL-6
	DC-SIGN	APRIL
	Dectin-1	BAFF
	CLEC9A	
	Langerin	
	CD11c	
Macrophages	CD163	
	FcγRIIB	

To design the appropriate targeted vaccine, three criteria need to be considered: (1) Select the appropriate DC or macrophage subsets as the targeting APCs; (2) Select appropriate receptor to target, preferentially those receptors with capacity of Ag recycling and retention, such as DCIR or FcγRIIB; (3) Select appropriate adjuvant to provide additional help for PC differentiation.

(1) preferentially target interstitial dermal DCs due to their capacity to activate B cells (31); (2) preferentially deliver antigens through inhibitory receptors such as FcγRIIB (50) or DCIR (52) to enable long-term retention and recycling of native antigens to the cell surfaces. The selection of adjuvant would be based on whether it needs to promote B cell differentiation (such as BAFF and APRIL), or it needs to educate Tfh cells (such as IL-6).

Our study on human macrophages in the induction of PCs (66) suggests that targeting CD163 on resident tissue macrophages would be another approach to potentially trigger preferred antibody response. IP-10 may act as a powerful adjuvant to provide the feedback loop for IL-6 production on activated B cells. Using systems biology approach, we observed that IP-10 signature was quickly turned on after influenza vaccination in healthy individuals, and it was corresponding to the late neutralizing antibody and PC signature (90). Mice deficient for IP-10 showed reduced antibody titers against the model antigen hapten, further supporting the wide application of this macrophage-derived molecule in vaccine design (66).

Of note, targeting antigens to different subsets of APCs could lead to a differential class switching. Our preliminary data indicate that among the myeloid APCs generated from monocytes, DCs preferentially induce IgG-producing cells, whereas type 2 macrophages (M2) preferentially promote IgA-producing cells (Xu et al., unpublished). The mechanism of APC subset-mediated preferential class switching remains to be explored further. It will lead to a better understanding of vaccine design when a unique Ig subclass response is needed.

CONCLUDING REMARKS

The past decade has witnessed the important roles of DCs and macrophages in educating B cell activation, proliferation, and differentiation toward PCs. These APC subsets residing at distinct organs might be equipped different sentinels to initiate the prompt humoral response. For example, the subcapsular sinus macrophages, which form a thick lining beneath the capsular in the lymph node, represent the prime APCs to deliver combined signals to naïve B cells for priming. As compared to DC-targeted vaccines for T cell immunity that are applied for more than a decade (91), we are just beginning to design APC-targeted vaccines aiming at enhance antibody responses. As such, various studies have helped our understandings that the interplay of several distinct factors needs to be considered (1) selection of APC subsets as the target cells; (2) selection of appropriate surface receptors as the antibody target; (3) selection of adjuvant.

ACKNOWLEDGMENTS

The authors thank Katarina Kreymborg, Caroline Waltzinger, and Claire Dunn for critical reading of this manuscript. Wei Xu is a full-time employe of Hoffmann-La Roche Ltd. in the Switzerland.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 October 2013; accepted: 20 December 2013; published online: 06 January 2014.
*Citation: Xu W and Banchereau J (2014) The antigen presenting cells instruct plasma cell differentiation. Front. Immunol. **4**:504. doi: 10.3389/fimmu.2013.00504*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 27 December 2013
doi: 10.3389/fimmu.2013.00494

Long-lived plasma cells in autoimmunity: lessons from B-cell depleting therapy

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A large number of auto-immune diseases are treated with rituximab, an antibody against CD20 that depletes most of the B-cells in the organism. The response to this treatment depends largely on the disease and the type of lymphoid cells involved in the auto-immune process. We recently reported that B-cell depletion in immune thrombocytopenia induced the appearance of pathogenic long-lived plasma cells in the spleen, which were not present before treatment or in non-auto-immune conditions. The spleen of treated patients produced an excess of the cytokine B-cell activating factor, which in *in vitro*-cultured splenic cells, could increase the longevity of plasma cells. Our results suggested that, paradoxically, the B-cell depletion itself, by altering the splenic milieu, promoted the differentiation of short-lived auto-immune plasma cells into long-lived ones. We describe the cellular and cytokinic components of the splenic plasma cell niche, notably $CD4^+$ T cells and discuss possible survival factors that could be targeted simultaneously with rituximab-mediated B-cell depletion to interfere with plasma cell persistence.

Keywords: rituximab, plasma cell niche, BAFF/Blys, belimumab, autoreactive antibody

B-CELL DEPLETION, FROM MICE TO HUMANS

For the last decade, anti-CD20-induced B-cell depletion has been increasingly used to treat several auto-immune conditions such as rheumatoid arthritis, vasculitis, and immune thrombocytopenia (ITP), but the clinical results have been disappointing (1–4). Moreover, mouse models of B-cell depletion have added to the confusion by showing incomplete B-cell depletion in lymphoid organs, which suggests specific resistance of some tissue-resident B-cell subsets. The first model was a transgenic mouse expressing human CD20 and treated with an anti-hCD20 (2H7) monoclonal antibody (mAb). Despite complete depletion of circulating lymph nodes and peritoneal-cavity B-cells, a large fraction of B-cells remained in the spleen, mainly represented by marginal-zone B-cells whose resistance was not related to the dose of hCD20-depleting antibody (5, 6).

Two groups have developed anti-mouse CD20-depleting mAbs (6, 7). Using one of them (MB20-11 antibody, Ig2a), Hamaguchi et al. suggested that despite extensive depletion of all B-cell subsets in blood, spleen, or lymph nodes, the peritoneal-cavity provided a niche for B1 and conventional B lymphocytes, which were resistant to the anti-CD20 treatment. Of note, inflammation resulting in the migration of effector cells, mainly monocytes, facilitated the depletion of these peritoneal B-cells (8). Using another mouse antibody (18B12), one group reported the persistence of germinal-center B-cells in spleen on injection of the depleting antibody at the peak of the immune response (9). In both models (anti-human or mouse CD20 mAbs), lupus-prone mice showed incomplete B-cell depletion in secondary lymphoid tissues, which was explained by a defect in monocyte/neutrophil IgG-mediated phagocytosis (6, 7, 10, 11). These results suggested that specific resistance to B-cell depletion could occur under auto-immune conditions, the failure of such therapies being related to incomplete elimination of auto-reactive B-cell clones in secondary lymphoid organs.

In humans, as in the mouse, CD20 is expressed from pro-B to memory B-cells. Rituximab achieves almost complete peripheral B-cell depletion, with minimal numbers of $CD19^+$ cells being detectable in blood within 6 months after treatment. Residual circulating $CD19^+$ cells are mainly IgA plasmablasts, as documented by high-sensitivity flow cytometry (12). These cells were suggested to originate from mucosal tissues where they would have been spared from the B-cell depletion, but their presence was not associated with poorer response to treatment in the disease studied, rheumatoid arthritis.

We recently reported marked B-cell depletion in spleens up to 6 months after treatment with rituximab in ITP patients (see below), with only about 0.5% of residual $CD45^+CD19^+$ B-cells, mainly plasma cells (13) (**Figure 1A**). Thus, in humans, the spleen is not the site of germinal-center or marginal-zone rituximab-resistant B-cells, even in auto-immune conditions.

B-CELL DEPLETION IN ITP INDUCES DIFFERENTIATION INTO LONG-LIVED PLASMA CELLS

Immune thrombocytopenia is an acquired bleeding disorder mediated by pathogenic autoantibodies that enhance platelet destruction and limit their production. The major target of these autoantibodies is the platelet membrane glycoprotein IIb–IIIa (GpIIbIIIa, integrins alpha2b, and beta3), but other glycoproteins can be involved (e.g., GPIb-IX) (14). The spleen is the major

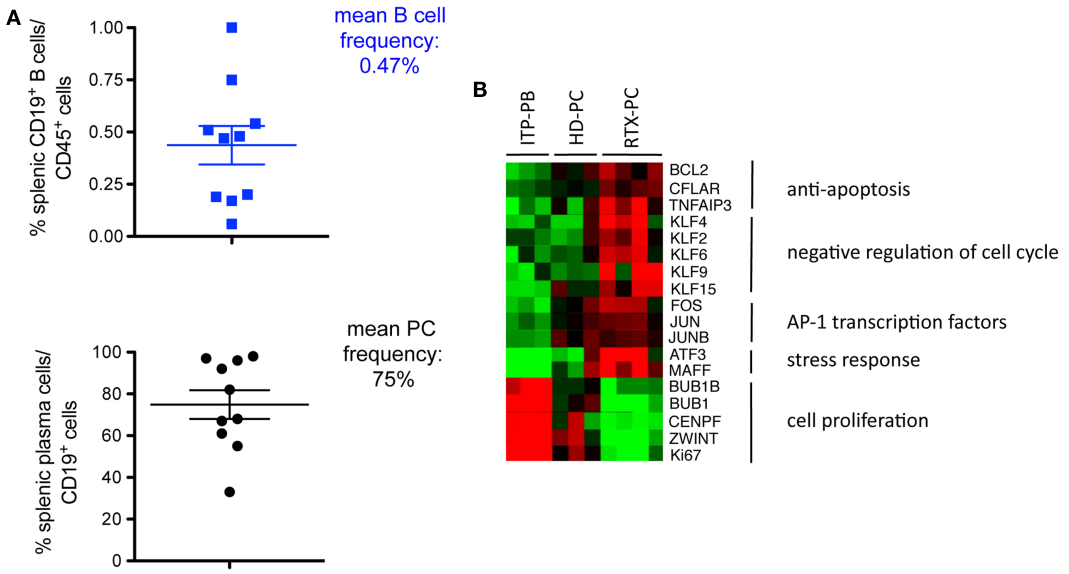

FIGURE 1 | Long-lived plasma cells in the spleens of ITP patients receiving rituximab. **(A)** Residual B-cells resisting rituximab treatment in spleens from ITP patients ($CD19^+$, % of $CD45^+$ spleen cells, upper part) are, for the most part, plasma cells, identified as $CD19^+CD20^-CD24^-CD27^+CD38^{high}$ (lower part) (10 patient's samples analyzed) (13). **(B)** Transcriptomic analysis of splenic plasma cells from four ITP patients receiving rituximab (RTX-PC) reveals a long-lived expression profile as compared with plasmablasts from three ITP patients not treated with rituximab (ITP-PB), and plasma cells from three healthy controls (HD-PC). Heatmap clustering of selected genes classified as anti-apoptosis factors, negative regulators of cell cycle, transcription factors of the AP1 family, stress response genes, and cell proliferation control and marker genes. Genes were selected from the supervised comparison of RTX-PC vs. ITP-PB (609 probes with a fold change >4 or <0.25, and $P < 0.05$). Columns represent individual samples, rows specific gene probes, with upregulated genes in red and downregulated genes in green (twofold scale) (13).

site of platelet destruction and is also considered the main site of auto-antibody production, thus seemingly containing all the players required to perpetuate the auto-immune reaction (15). Accordingly, for decades, splenectomy has been the "gold standard" of second-line therapy, resulting in a durable disease cure in two-thirds of patients.

We observed, as did others, that the spleen of ITP patients was the site of an intense B-cell response, with a considerable expansion of short-lived plasmablasts and active germinal-center reactions (13, 16). As described by many authors, plasmablasts generated during a T-dependent response in non-auto-immune conditions will migrate to the bone marrow and differentiate into short-lived plasma cells. Some of these short-lived plasma cells differentiate into long-lived plasma cells (LLPCs) and reside in this niche for a variable length of time. These cells, which represent only 0.5% of mononucleated cells in bone marrow, constitutively secrete antibodies and sometimes persist for decades in humans (17). Differentiation toward LLPCs was described in secondary lymphoid tissues at the site of an immune reaction. Also, from an auto-immune genetic mouse model (NZB/W, lupus-prone), the inflamed environment generated by an auto-immune disease was suggested to be a niche for LLPCs, the cells further perpetuating the local inflammation (17, 18). This observation raised the question as to whether the spleen could similarly represent a site for plasma cell persistence in ITP.

Splenectomy performed in ITP, as standard-of-care treatment or with primary failure of rituximab, provides a unique opportunity to study splenic plasma cells in different settings. We compared the transcriptomic profile of splenic plasma cells in healthy subjects and ITP patients receiving or not rituximab. Most splenic plasma cells in rituximab-receiving patients expressed a program similar to that of bone marrow LLPCs (13). They overexpressed anti-apoptotic factors (*BCL2*, *CFLAR*, *TNFAIP3*), negative regulators of the cell cycle, among which are multiple members of the Krüppel-like factor family (*KLF2*, *KLF6*, *KLF9*, *KLF15*). Transcription factors of the AP1 family (*FOS*, *JUN*, and *JUNB*) were also upregulated, as were genes involved in the unfolded protein response (*ATF3*, *MAFF*) (**Figure 1B**). By contrast, plasmablasts, found in ITP patients not receiving rituximab, showed a cell proliferation profile characterized by the expression of positive regulators or markers of the cell cycle (*BIRC5*, *CENPF*, *BUB1*, *BUB1B*, *ZWINT*, *CDC6*, *MKI67*). Surprisingly, analysis of normal plasma cells and plasma cells from ITP patients revealed an intermediate gene expression profile between short-lived plasmablasts and LLPCs. To determine whether this observation was due to a mixture of two populations, we analyzed plasma cells from healthy donors and ITP patients at the single-cell level. Unexpectedly, most cells expressed an intermediate profile between the two populations, with <15% of splenic plasma cells displaying a long-lived signature (13).

These results raised several questions. First, they suggested that an auto-immune inflammatory milieu *per se* does not systematically create a niche for LLPCs (17); second, that LLPCs may only be a minor component of the plasma cell pool in the normal spleen; third, that the B-cell depletion could induce a new microenvironment allowing for short-lived splenic plasma cells to differentiate

into long-lived ones. Remarkably, the presence of LLPC in the spleen has mainly been documented after B-cell depletion in mice (through irradiation and anti-CD20 treatment), a situation that, like with rituximab treatment, may have artificially induced their differentiation *in situ* (19, 20). Moreover, some of these post-rituximab splenic LLPCs secreted anti-platelet antibodies, thus explaining the treatment failure.

PLASMA CELL LIFESPAN: THE ESSENTIAL ROLE OF THE MICROENVIRONMENT

The persistence of LLPCs depends on signals from the microenvironment, including direct cell–cell contact and production of survival factors. Many different factors and cells have been described, both in mice and humans, as being essential for the survival of LLPCs in bone marrow; such factors include the cytokines a proliferation-inducing ligand (APRIL) and interleukin 6 (IL-6) and the chemokine CXCL12 secreted by stromal cells, which attracts CXCR4-positive plasma cells (21). In mice, megakaryocytes and eosinophils are involved in the survival of LLPCs in their bone marrow niche (22). LLPCs express very late antigen 4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1), as well as CD44 and P-selectin glycoprotein ligand 1 (PSGL-1), all involved in their survival. However, we still do not know what triggers the differentiation of a small number of short-lived plasma cells into LLPCs as they settle into the bone marrow.

APRIL and B-cell activating factor (BAFF) are two key cytokines that belong to the tumor necrosis factor family: they share receptors such as transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA); BAFF can also signal through BAFF receptor (BAFF-R), and APRIL can bind to heparan sulfate proteoglycans. BAFF-R is mainly expressed on immature and naive cells, whereas plasmablasts and plasma cells express TACI and BCMA, the latter markedly upregulated on bone marrow LLPCs (23). APRIL is probably the key survival factor for plasma cells, but various gene inactivation experiments have suggested, at least in the mouse, that BAFF and APRIL may substitute for each other in plasma cell maintenance (24). In addition to a survival function, these two molecules may play a role in differentiation from plasmablasts to plasma cells and possibly LLPCs.

With *in vitro* culture of splenic cells, we observed increased BAFF level in the medium from rituximab-treated spleen samples with B-cell depletion as compared to ITP spleens not exposed to rituximab, with no difference in APRIL secretion. Moreover, preliminary experiments showed that normal plasma cells survived better in *in vitro* cultures in the presence of BAFF (13). Indeed, increased BAFF concentration has been reported to likely be a direct consequence of B-cell depletion, its accumulation resulting from a lack of consumption by naive B-cells (25). Interestingly, CD138, a heparan sulfate, has been proposed to bind APRIL and concentrate it in the plasma cell niche (26). CD138 is a specific marker of LLPCs in bone marrow, but human splenic plasma cells are negative for surface expression of CD138 (27), while expressing it at the mRNA level (13). Therefore, BAFF may have a preferred

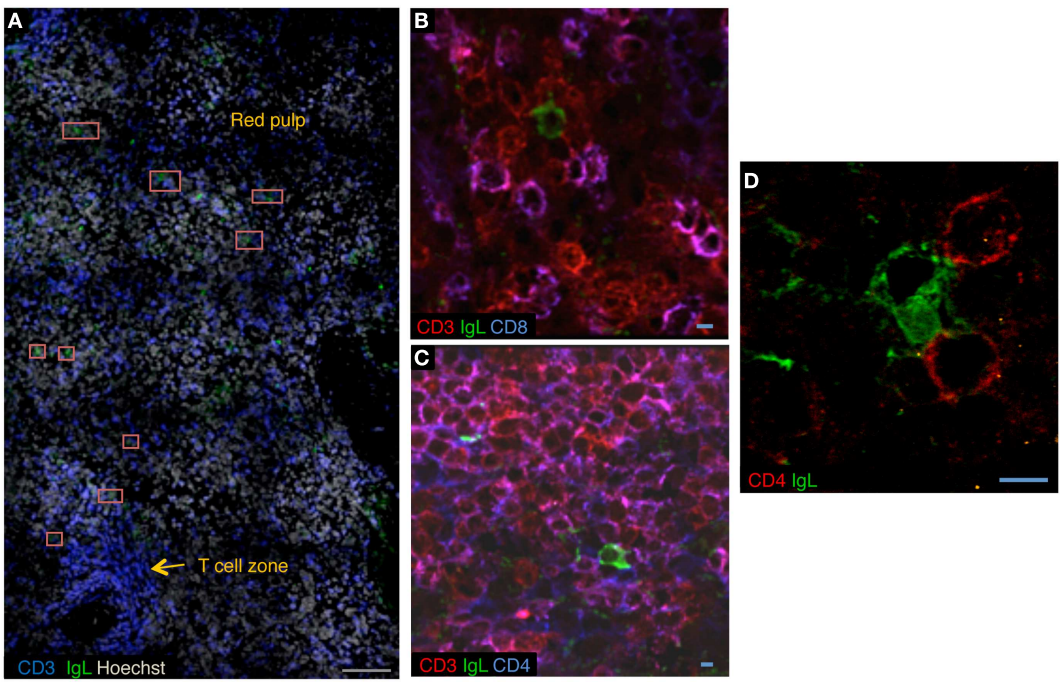

FIGURE 2 | A CD4⁺ T-cell niche for long-lived plasma cells in rituximab-treated spleens. **(A)** Confocal microscopy of sections of rituximab-treated spleen stained with anti-CD3 (blue), anti-kappa/lambda light chains (green), and Hoechst (cyan). Plasma cells are located adjacent to the T-cell zone and in red pulp. Red squares mark plasma cells. **(B,C)** Confocal microscopy of sections of rituximab-treated spleen stained with anti-kappa/lambda light chains (green), anti-CD3 (red), and anti-CD8 (blue) **(B)**, or anti-CD3 (red) and anti-CD4 (blue) **(C)**. **(D)** Sections of rituximab-treated spleen stained with anti-kappa/lambda light chains (green) and anti-CD4 (red). Data are representative of three spleen samples. Scale bars: gray 100 μm, blue 5 μm.

survival role in the context of the splenic plasma cell microenvironment and a specific role in plasma cell differentiation (26, 28).

The cellular components of the splenic plasma cell niche are not well established. In mice, basophils have been proposed to play a role in plasma cell survival by secreting BAFF and APRIL (29). Stromal cells in the human spleen secrete IL-6 (27). The B-cell depletion induced by rituximab provided us with a unique opportunity to investigate the splenic microenvironment of LLPCs by confocal microscopy. Plasma cells were unambiguously identified as cells strongly expressing kappa/lambda light chains and not CD20. We observed plasma cells in the periphery of the T-cell zone and in the red pulp (unpublished data, **Figure 2A**). Unexpectedly, in the three spleen samples studied, approximately 20% of plasma cells co-localized with $CD3^+$ T cells. In most cases, we observed interaction of one plasma cell with two or three T cells, either $CD4^+$ (**Figures 2B–D**) or possibly double-negative T cells (data not shown). In a co-culture system, $CD3^+CD4^+$ T cells isolated from rituximab-treated spleens did not increase the survival of autologous plasma cells [data not shown and Ref. (30)], which may suggest distinct roles for cells involved in direct contact, providing retention in a defined environment, and cells in close proximity, producing survival signals. A more thorough analysis of the splenic plasma cell niche after rituximab-induced B-cell depletion is in progress.

INTERFERING WITH AUTO-REACTIVE PLASMA CELL PERSISTENCE, A FUTURE GOAL IN AUTO-IMMUNE DISEASES

We demonstrated that B-cell depletion in ITP induced the differentiation of short-lived auto-immune plasma cells into long-lived ones in the spleen. This observation might be of general relevance. In fact, many immunosuppressive and/or biological agents largely used in auto-immune diseases (cyclophosphamide, mycophenolate mofetil, steroids) confer various degrees of B-cell depletion. Of note, rituximab failure in specific diseases such as lupus was often documented in conditions treated with various depleting treatments, which suggests that differentiation into LLPCs was already achieved. In contrast, interfering with the plasma cell survival niche at the time of B-cell depletion might greatly improve the success of these treatments. One first target could be BAFF, because increased level of BAFF accompanies B-cell depletion. Belimumab (monoclonal anti-BAFF antibody) has been approved for the treatment of lupus, with conflicting results (31). Thus, combined anti-CD20 and anti-BAFF therapy might be a first way to interfere with plasma cell persistence. Identification of key cytokines and accessory cells that promote plasma cell differentiation and/or constitute the plasma cell niche in B-cell depleted environments, both in mouse models and in human tissues, may allow for the development of new strategies in antibody-mediated auto-immune diseases.

ACKNOWLEDGMENTS

INSERM U783 is supported by the Ligue Nationale contre le Cancer ("Equipe labelisée"), the Foundation Princess Grace and by an ERC advanced investigator grant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 October 2013; paper pending published: 21 November 2013; accepted: 16 December 2013; published online: 27 December 2013.
*Citation: Mahévas M, Michel M, Weill J-C and Reynaud C-A (2013) Long-lived plasma cells in autoimmunity: lessons from B-cell depleting therapy. Front. Immunol. **4**:494. doi: 10.3389/fimmu.2013.00494*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 28 March 2014
doi: 10.3389/fimmu.2014.00129

Splenic B-1a cells expressing CD138 spontaneously secrete large amounts of immunoglobulin in naïve mice

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B-1a cells constitutively secrete natural antibody that provides immediate protection against microbial pathogens and functions homeostatically to speed removal of apoptotic cell debris. Although B-1a cells are especially prominent in the peritoneal and pleural cavities, some B-1a cells reside in the spleen. A small subset of splenic B-1a cells in naïve, unimmunized mice express CD138, a recognized plasma cell antigen, whereas the bulk of splenic B-1a cells are CD138 negative. Splenic B-1a cells *in toto* have been shown to generate much more antibody per cell than peritoneal B-1a cells; however, specific functional information regarding $CD138^+$ splenic B-1a cells has been lacking. Here, we find a higher proportion of $CD138^+$ splenic B-1a cells spontaneously secrete more IgM as compared to $CD138^-$ B-1a cells. Moreover, IgM secreted by $CD138^+$ splenic B-1a cells is skewed with respect to N-region addition, and some aspects of V_H and J_H utilization, as compared to $CD138^-$ splenic B-1a cells and peritoneal B-1a cells. The small population of $CD138^+$ splenic B-1a cells is likely responsible for a substantial portion of natural IgM and differs from IgM produced by other B-1a cell subsets.

Keywords: B lymphocytes, antibody, B-1 cells, IgM

INTRODUCTION

Murine B-1a cells represent a unique lymphocyte lineage distinguished by specific ontologic, phenotypic, and functional characteristics (1, 2). Although the human equivalent of mouse B-1a cells has been described recently (3, 4), most knowledge about B-1a cells has been generated from studies in mice. It has been shown that B-1a cells spontaneously and constitutively generate "natural" immunoglobulin (Ig), which constitutes the vast majority of resting serum IgM and about half of resting IgA (5). The natural Ig produced by B-1a cells differs from Ig produced by B-2 cells. B-1a cell Ig contains minimal N-region addition and little somatic hypermutation (6–9). As a result, B-1a cell Ig tends to be "germline-like"; that is, to accurately reflect germline sequences without the intervention of randomly added nucleotides or mutated residues. Because B-1a cell Ig reflects germline coding, the B-1a cell repertoire is to a large extent inherited. Therefore, those sequences that enhance an organism's survival to reproductive age are likely to be retained.

B-1a cell Ig is both polyreactive and autoreactive. Polyreactive natural Ig is always present and acts as an initial shield against many common infectious agents, particularly during the lag period that precedes the development of adaptive, high affinity antigen-specific antibody produced by germinal center B-2 cells (10–15). Autoreactive natural Ig has been implicated in the disposition of irreversibly damaged cells and noxious molecular debris and in so doing maintains homeostasis and prevents untoward inflammation (16).

B-1a cells are preferentially located at serosal surfaces, most prominently in the peritoneal cavity. However, B-1a cells are also located in the spleen, and splenic B-1a cells differ from peritoneal B-1a cells in a number of characteristics, including the intensity of Ig secretion (17–19). Through the unique property of self-renewal (20), mature B-1a cells can give rise to their own progeny in place, which suggests differentiation and migration in adult animals is minimal. Therefore, B-1a cells were considered for a time to represent static, Ig generating lymphocytes. However, recent reports suggest a richer life experience. Peritoneal B-1a cells may respond to specific and/or non-specific stimulation, migrate to the spleen, and may then return to the peritoneal cavity as memory B-1a cells (21–23). Further, a subset of splenic B-1a cells expresses CD138 (24) suggesting that appropriately stimulated B-1a cells may take a different path and differentiate in a plasma cell direction. $CD138^+$ B-1a cells differ from $CD138^+$ plasma cells in retaining B-1a-specific surface antigen expression. Identification of splenic B-1a cells that express CD138 raises the question of whether $CD138^+$ B-1a cells differ from $CD138^-$ B-1a cells, or whether these splenic B-1a cell populations behave similarly, especially with respect to the amount of Ig secretion.

MATERIALS AND METHODS

MICE

Male BALB/c-ByJ mice of 6–8 weeks age were obtained from the Jackson Laboratory. Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

CELL PURIFICATION AND FLOW CYTOMETRY

Splenocytes were obtained from 8 to 14-week-old BALB/c-ByJ male mice and stained with fluorescence-labeled antibodies to B220, CD5, CD23, and CD138. Splenic B cell populations

were sort-purified (BD Biosciences Influx) as follows: splenic B-2 cells, $B220^{hi}CD5^{-}CD23^{hi}CD138^{-}$; splenic $CD138^{+}$ B-1a cells, $B220^{lo}CD5^{lo}CD23^{-}CD138^{+}$; splenic $CD138^{-}$ B-1a cells, $B220^{lo}CD5^{lo}CD23^{-}CD138^{-}$. Post-sort analysis of the splenic B-1a and B-2 cell populations showed each to be ≥98% pure. The following rat anti-mouse antibodies were obtained from BD Biosciences: FITC-conjugated B220 (clone RA3–6B2), PE-Cy5-conjugated CD5 (clone 53–7.3), PE-conjugated CD138 (clone 281–2). The anti-mouse Pacific Blue-conjugated CD23 antibody (clone B3B4) was obtained from BioLegend.

ELISPOT ASSAY

ELISPOT assay was carried out as previously described (25). In brief, sort-purified, naive B cells were distributed onto MultiScreen*-IP Plates (Millipore) pre-coated with goat anti-mouse Ig (H + L) and then incubated in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin for 4 h at 37°C and 5% CO_2. Plates were treated with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotechnology Associates) and developed with 5-bromo-4-chloro-3-indolyl phosphate/p-NBT chloride substrate (KPL). IgM-secreting B cells were enumerated using Phoretix Expression software (Non-Linear Dynamics).

SINGLE CELL SEQUENCING AND ANALYSIS

Splenic $CD138^{+}$ B-1a and $CD138^{-}$ B-1a cells were sorted onto 48-well AmpliGrid slides (Advalytix). Reverse transcription and PCR (Qiagen OneStep RT-PCR) were carried out as described previously (8). The products were purified and then sequenced (Genewiz) using the MsVHE primer. Sequences were then analyzed using an online sequence analysis tool for VDJ sequences (IMGT, the international ImMunoGeneTics information system). Each of the sequences analyzed and reported in this manuscript, from each population, is characterized by a unique V, D, and J segment along with a unique CDR3. Sequences with identical V, D, and J segments as well as identical CDR3 regions were eliminated from consideration according to the criteria of Kantor et al. (26). CDR3 hydrophobicity was calculated using the online calculator GRAVY (http://www.gravy-calculator.de/index.php?page=file). Sequences are provided as Data Sheet in Supplementary Material.

RESULTS

We sort purified splenic B-1a cells to separate $CD138^{+}$ and $CD138^{-}$ populations using a variation on the gating strategy reported by Herzenberg and colleagues (24). The selected splenic populations were gated as $B220^{lo}CD5^{lo}CD23^{-}CD138^{+}$ ($CD138^{+}$ B-1a cells) and $B220^{lo}CD5^{lo}CD23^{-}CD138^{-}$ ($CD138^{-}$ B-1a cells), as depicted in **Figure 1**. Far fewer $CD138^{+}$ B-1a cells were recovered than $CD138^{-}$ B-1a cells with the former amounting to less than 1/100 the number of the latter. We questioned whether CD138 expression marks splenic B-1a cells that vigorously secrete Ig, as it does B-2 plasma cells. To address this, we tested IgM secretion of sorted splenic B-1a cells from naïve, unimmunized BALB/c-ByJ mice by ELISPOT assay. We found both $CD138^{+}$ and $CD138^{-}$ B-1a cells secreted IgM spontaneously, without stimulation, over a 4 h period. However, the frequency of IgM-secreting B-1a cells was significantly higher for $CD138^{+}$ B-1a cells in comparison to $CD138^{-}$ B-1a cells, as illustrated in **Figure 2A** and enumerated in **Figure 2B**. More than half of $CD138^{+}$ splenic B-1a cells (56%) spontaneously secreted IgM whereas the fraction of $CD138^{-}$ splenic B-1a cells that secreted IgM (5%) was much lower. Beyond frequency, the amount of IgM secreted by $CD138^{+}$ splenic

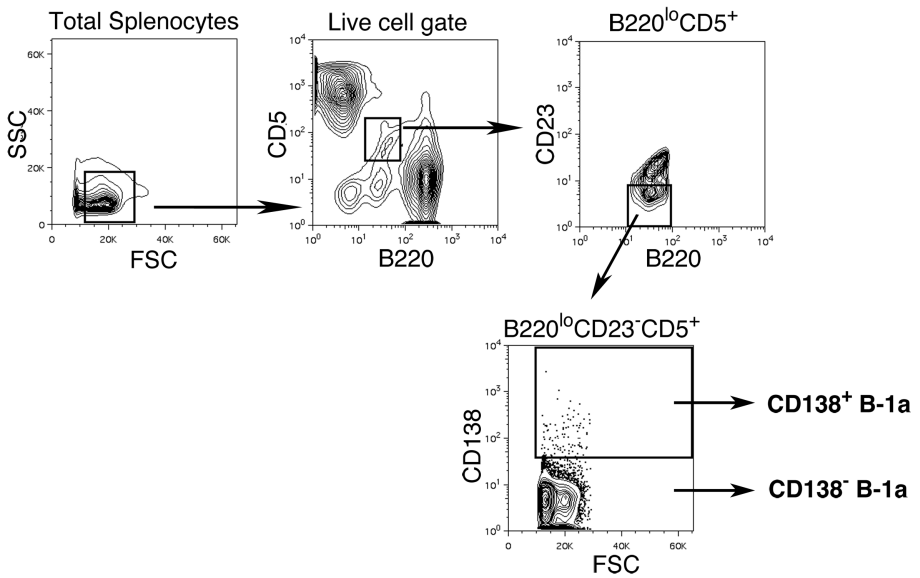

FIGURE 1 | Gating strategy for $CD138^{+}$ and $CD138^{-}$ splenic B-1a cells in naïve non-immune mice. Single cell suspensions of RBC lysed spleens from BALB/c-ByJ mice were prepared and stained with B220-FITC, CD5-PE-Cy5, CD23-Pacific Blue, and CD138-PE. Splenic $CD138^{+}$ and $CD138^{-}$ B-1a cells were gated as shown and sorted.

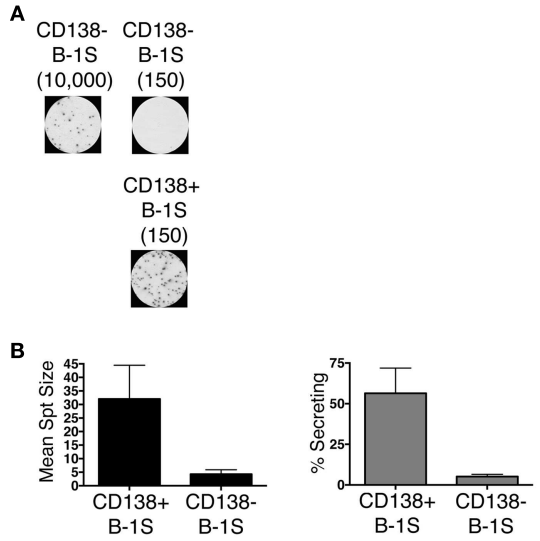

FIGURE 2 | CD138⁺ and CD138⁻ splenic B-1a cell IgM secretion in naïve non-immune mice. Sorted splenic CD138⁺ and CD138⁻ B-1a cells were evaluated for IgM secretion by ELISPOT. Cells were placed on the ELISPOT membrane for 4 h. **(A)** Representative experiment showing 10,000 or 150 CD138⁻ cells total placed per well as compared to 150 CD138⁺ cells total per well. **(B)** The spots were enumerated to determine mean spot area (black) and the percent of cells secreting IgM (gray). Results shown represent mean values of three independent experiments with lines indicating standard errors of the means.

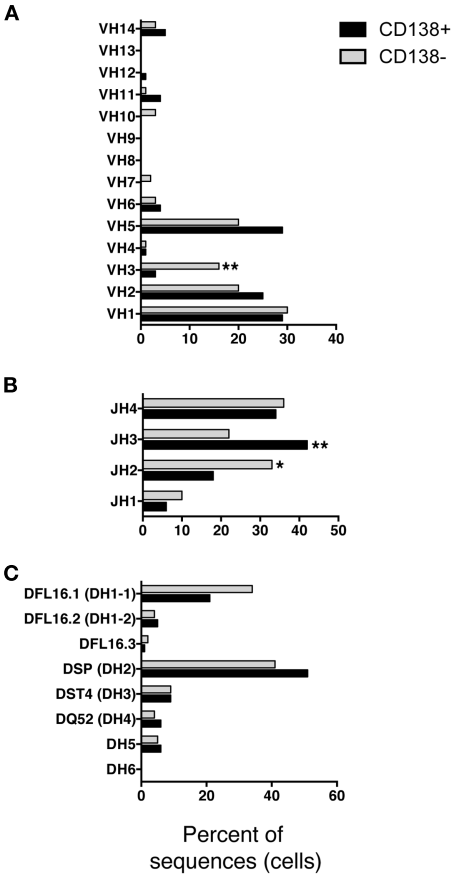

FIGURE 3 | VH, DH, and JH analysis of CD138⁺ and CD138⁻ splenic B-1a cells. Immunoglobulins were amplified by PCR from single sorted splenic CD138⁺ ($n = 77$) and CD138⁻ ($n = 92$) B-1a cells and evaluated for **(A)** V, **(B)** J, and **(C)** D segment heavy chain usage. The percent of cells (sequences) expressing each segment is displayed. Chi square and Fisher's exact tests were used to determine significance.

B-1a cells was significantly greater than that of CD138⁻ splenic B-1a cells, as judged by relative mean spot area (**Figure 2B**). CD138⁺ B-1a cells generated ELISPOTS that were more than six times as large as the ELISPOTS produced by CD138⁻ B-1a cells. Still, both CD138⁺ and CD138⁻ splenic B-1a cells secreted more IgM per cell than peritoneal B-1a cells (18).

We questioned whether IgM produced by CD138⁺ B-1a cells represents a selected repertoire in comparison to CD138⁻ B-1a cells. To address this, we sorted single cell CD138⁺ and CD138⁻ splenic B-1a cells and analyzed individual antibodies by PCR amplification and sequencing. We found overall similarity in V_H–D_H–J_H usage with several significant differences. Among V_H gene segments, V_H3 was expressed significantly less frequently by CD138⁺ splenic B-1a cells (3%; $n = 77$; **Figure 3A**) as compared to CD138⁻ splenic B-1a cells (16%; $n = 92$; **Figure 3A**) ($p = 0.003$). CD138⁺ splenic B-1a cells also expressed V_H3 less frequently than peritoneal B-1a cells [13%; $n = 56$; Ref. (8)] ($p = 0.02$). In contrast, V_H5 was expressed more frequently by CD138⁺ splenic B-1a cells (29%) as compared to CD138⁻ splenic B-1a cells (20%), although this difference did not reach the level of significance. Among D_H gene segments, DFL16.1 was expressed less frequently, and DSP was expressed more frequently, by CD138⁺ splenic B-1a cells as compared to CD138⁻ splenic B-1a cells although these differences were not significant (**Figure 3C**). Among J_H gene segments, J_H3 was expressed significantly more frequently (42%; **Figure 3B**) ($p = 0.005$) and J_H2 was expressed significantly less frequently (18%; **Figure 3B**) ($p = 0.03$), by CD138⁺ splenic B-1a cells as compared to CD138⁻ splenic B-1a cells (22 and 33%, respectively). The latter utilization of J_H3 and J_H2 by CD138⁻ splenic B-1a cells approximated that of peritoneal B-1a cells [18 and 27%, respectively, Ref. (8)]. Thus, distinctive V and J gene segment usage separated CD138⁺ splenic B-1a cells from CD138⁻ splenic B-1a cells and peritoneal B-1a cells.

Much attention has focused on N-region addition in B-1a cell Ig, which is typically severely limited in comparison to B-2 cell Ig. We questioned whether CD138⁺ B-1a cell Ig represents a selected subset of all B-1a cell Ig. To address this, we analyzed N-addition at the D–J and V–D junctions and determined CDR3 length. Analyzing the junctions separately, we found the average length of N-additions at the D–J junction of CD138⁺ splenic B-1a cell antibodies was larger than that of CD138⁻ splenic B-1a cells ($p = 0.03$ by Mann–Whitney U) (**Table 1**). This bespeaks increased diversity among antibodies expressed by CD138⁺ B-1a cells as compared to CD138⁻ B-1a cells. However, there were no significant differences between CD138⁺ and CD138⁻ splenic B-1 cell antibodies at V–D junction in terms of mean N-addition

length. Regardless, overall N-addition in CD138$^+$ splenic B-1a cell Ig was significantly different ($p = 0.003$ by Chi square analysis) from that of CD138$^-$ splenic B-1a cell Ig (**Figure 4B**), emphasizing that CD138 expression divides splenic B-1a cells into distinct populations.

In previous work, we analyzed N-addition frequency of Ig sequences amplified from peritoneal B-1a cells and from splenic B-2 cells (8). Antibodies expressed by both CD138$^+$ and CD138$^-$ splenic B-1a cells contained significantly more N-additions (**Figure 4A**) in comparison to antibodies expressed by B-1a cells in the peritoneal cavity, by Chi square analysis (**Figure 4B** and Ref. (8); $p < 0.0001$ and $p = 0.02$, respectively). Conversely, antibodies expressed by both CD138$^+$ and CD138$^-$ splenic B-1a cells contained significantly fewer N-additions in comparison to antibodies expressed by splenic B-2 cells, by Chi square analysis (**Figure 4B** and Ref. (8); $p = 0.0003$ and $p < 0.0001$, respectively). Thus, whereas Ig sequences from CD138$^+$ and CD138$^-$ splenic B-1a cells differ in N-addition, both populations of splenic B-1a cells differ from peritoneal B-1a cells and splenic B-2 cells, whose Ig contains less and more N-addition, respectively.

We further examined CDR3 length and found that despite differences in N-addition between CD138$^+$ and CD138$^-$ splenic B-1a cells, average Ig CDR3 lengths were not significantly different for the two populations (**Table 1**; **Figure 4C**). Moreover, average Ig CDR3 hydrophobicity indices were similar for CD138$^+$ and CD138$^-$ splenic B-1a cell antibodies (-0.63 ± 0.065 and -0.69 ± 0.064 SEM, respectively). Thus, the small CD138$^+$ splenic

Table 1 | Mean N-region addition and CDR3 lengths (±SD).

	V–D	D–J	CDR3 length
CD138$^+$ splenic B-1a	1.7 (±2.2)	1.8 (±1.9)	11.8 (±1.9)
CD138$^-$ splenic B-1a	2.3 (±2.8)	1.3 (±1.8)	11.6 (±2.6)

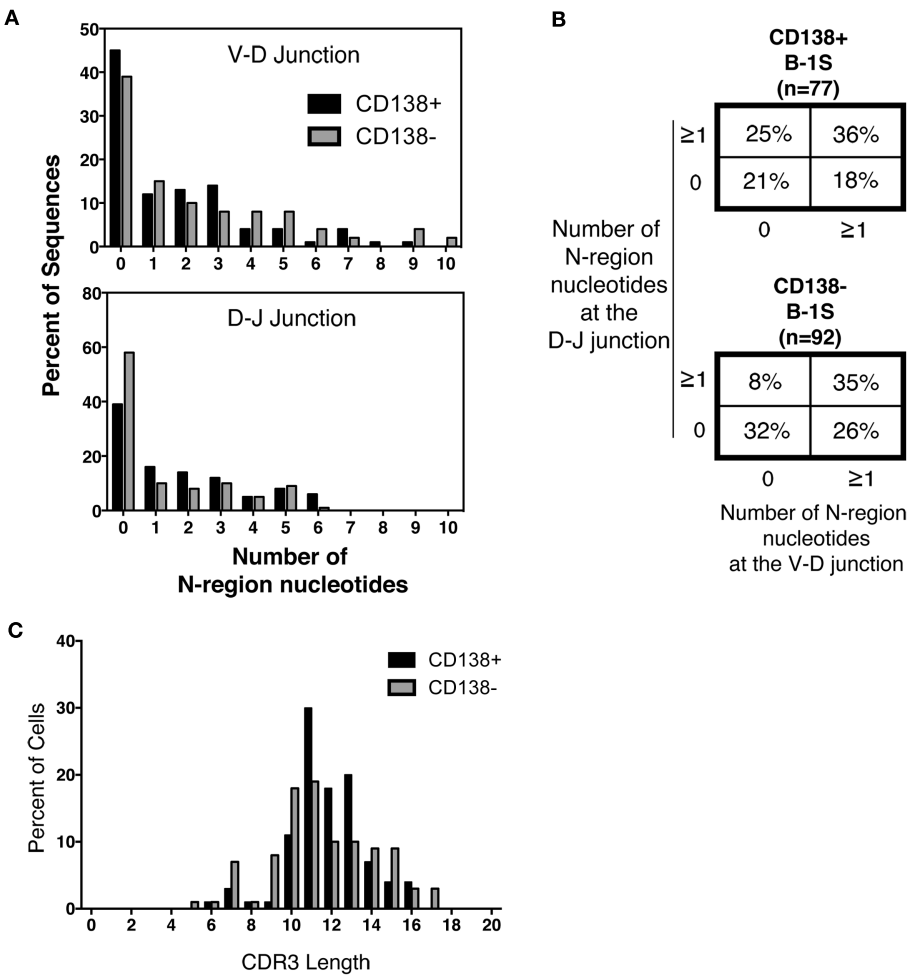

FIGURE 4 | N-region addition and CDR3 length analysis of CD138$^+$ and CD138$^-$ splenic B-1a cells. Immunoglobulins were amplified by PCR from single sorted splenic CD138$^+$ ($n = 77$) and CD138$^-$ ($n = 92$) B-1a cells and evaluated for N-region additions and CDR3 lengths. **(A)** The number of N-additions at each junction is shown. The graphs display the percent of sequences with 0–10 N-additions at each junction for CD138$^+$ (black bars) and CD138$^-$ (gray bars) splenic B-1a cells. **(B)** N-addition analysis of the V–D and D–J junctions together. **(C)** CDR3 length analysis of immunoglobulin sequences from CD138$^+$ (black bars) and CD138$^-$ (gray bars) splenic B-1a cells.

B-1a cell population expresses Ig that varies in Ig gene segment usage and is more diverse on account of increased N-addition, as compared to the dominant CD138$^-$ splenic B-1a cell population, but differs little in CDR3 length and CDR3 hydrophobicity.

DISCUSSION

Our results further characterize CD138-bearing B-1a cells, first identified by Yang et al. (24), which appear as a very small B cell population in the spleen. A large fraction of this small population secretes IgM, and an increased amount of IgM is secreted, in comparison with CD138$^-$ splenic B-1a cells and peritoneal B-1a cells. Thus, although their numbers may be small, it is likely that CD138$^+$ splenic B-1a cells make a substantial contribution to the circulating pool of natural antibodies. Therefore, the nature of the CD138$^+$ antibody repertoire is of interest in understanding the protective capacity of natural IgM.

The repertoire of these potently secreting CD138$^+$ splenic B-1a cells is somewhat skewed away from V_H3 and J_H2, and toward J_H3. This would seem to parallel the situation with the peritoneal B-1a cell repertoire, in which overall usage of V_H gene segments is similar to that of B-2 cells except for the key difference of increased V_H11 and V_H12 expression, the two V_H families responsible for PtC binding (27–29). In other words, repertoire skewing can show up in a limited way, which may be the case here with the preferential use of a very small number of V_H and J_H gene segments by CD138$^+$ splenic B-1a cells.

Prominent among variably expressed V_H gene segments is V_H3, which was found to be increased in NZM2410 anti-nuclear antibodies as compared to antibodies that did not bind nuclear components (30). This might suggest that CD138$^+$ splenic B-1a cells predominantly generate anti-microbial as opposed to self-reactive antibodies; however, in NZM2410 mice V_H5 is also increased in anti-nuclear antibodies (30) and among splenic B-1a cells V_H5 is utilized more frequently by CD138$^+$ B-1a cells as compared to CD138$^-$ B-1a cells, although this difference did not reach statistical significance. Thus, there is no clear evidence that CD138$^+$ B-1a cell Ig skews more toward or away from autoreactivity.

Both CD138$^+$ and CD138$^-$ splenic B-1a cell Ig sequences contained more N-addition than peritoneal B-1a cells' Ig sequences (and less N-addition than splenic B-2 cell Ig sequences), suggesting that the splenic B-1a pool differs from B-1a cells located elsewhere as well as from B-2 cells that share the splenic environment. Moreover, among splenic B-1a cell Ig, CD138$^+$ B-1a sequences contained more N-addition than CD138$^-$ sequences. It has been shown the natural IgM produced by B-1a cells is essential for early protection against bacterial and viral infections and that N-addition plays a substantial role in determining antibody diversity and effectiveness (10, 11, 13, 15). For example, the prototypical B-1a anti-phosphorylcholine (PC) antibody, T15, represents a germline sequence and has no N-addition (31). T15 has been shown to be protective against *Streptococcus pneumoniae* infection (31). The relationship between N-addition and antibody function is illustrated by the finding that after vaccination with heat killed pneumococci, mice that overexpress TdT generated an anti-PC response, but the anti-PC antibodies in this situation were not protective against *S. pneumoniae* infection (32). These findings highlight the importance of N-addition, which varies among antibodies spontaneously secreted by CD138$^+$ splenic B-1a cells, CD138$^-$ B-1a splenic B-1a cells, and peritoneal B-1a cells, in determining protection by natural antibody.

Circulating natural antibody is primarily generated by splenic B-1a cells, which differ in many characteristics from peritoneal B-1a cells (17–19). Among splenic B-1a cells, CD138$^+$ B-1a cells differ from CD138$^-$ B-1a cells in the frequency of secreting cells, the amount of antibody secreted, and the repertoire of antibody expressed. The combination of skewing with respect to V_H and J_H gene segments, and degree of N-region addition, suggests that the CD138$^+$ B-1a cell pool does not result from randomly triggered differentiation events applied to all splenic B-1a cells or all peritoneal B-1a cells, but rather results from a selective process whose origin remains unclear.

This raises the question of how the distinct splenic B-1a populations come about, and whether this represents selection from a pre-existing population or contribution from a new or different source. Previous work suggests several potential mechanisms. Peritoneal B-1a cells may migrate to the spleen following antigen-specific (or non-specific) activation (21–23, 33, 34). Herzenberg and colleagues have shown that these B-1a cells may become antibody secreting cells and/or return to the peritoneal cavity as memory B cells (21–23, 33, 34). In addition, we and others have suggested that the pool of B-1a cells changes with age, as fetal liver-derived B-1a cells are slowly replaced by bone marrow-derived B-1a cells in the adult expressing antibody with increased levels of N-addition (8, 35, 36), and the latter could preferentially give rise to splenic B-1a cells. A further possibility relates to the report of B-1 progenitor cells in the spleen that might give rise to mature B-1a cells *in situ* (37, 38). In fact, a combination of these mechanisms may be at play, whereby the fetal liver B-1a pool in the peritoneal cavity is replaced by bone marrow-derived B-1a emigrants over time, which then become activated and migrate to the spleen in a selective fashion. It will be of interest to determine whether the N-addition and other characteristics of CD138$^+$ B-1a cells change with advancing age. In sum, CD138$^+$ splenic B-1a cells constitute a distinct B-1a cell population that appears to play a substantial role in generation of natural antibody.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00129/abstract.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 17 January 2014; accepted: 14 March 2014; published online: 28 March 2014. Citation: Holodick NE, Vizconde T and Rothstein TL (2014) Splenic B-1a cells expressing CD138 spontaneously secrete large amounts of immunoglobulin in naïve mice. Front. Immunol. **5**:129. doi: 10.3389/fimmu.2014.00129*

This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 31 October 2014
doi: 10.3389/fimmu.2014.00535

B1b cells recognize protective antigens after natural infection and vaccination

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There are multiple, distinct B-cell populations in human beings and other animals such as mice. In the latter species, there is a well-characterized subset of B-cells known as B1 cells, which are enriched in peripheral sites such as the peritoneal cavity but are rare in the blood. B1 cells can be further subdivided into B1a and B1b subsets. There may be additional B1 subsets, though it is unclear if these are distinct populations or stages in the developmental process to become mature B1a and B1b cells. A limitation in understanding B1 subsets is the relative paucity of specific surface markers. In contrast to mice, the existence of B1 cells in human beings is controversial and more studies are needed to investigate the nature of these enigmatic cells. Examples of B1b antigens include pneumococcal polysaccharide and the Vi antigen from *Salmonella* Typhi, both used routinely as vaccines in human beings and experimental antigens such as haptenated-Ficoll. In addition to inducing classical T-dependent responses some proteins are B1b antigens and can induce T-independent (TI) immunity, examples include factor H binding protein from *Borrelia hermsii* and porins from *Salmonella*. Therefore, B1b antigens can be proteinaceous or non-proteinaceous, induce TI responses, memory, and immunity, they exist in a diverse range of pathogenic bacteria, and a single species can contain multiple B1b antigens. An unexpected benefit to studying B1b cells is that they appear to have a propensity to recognize protective antigens in bacteria. This suggests that studying B1b cells may be rewarding for vaccine design as immunoprophylactic and immunotherapeutic interventions become more important due to the decreasing efficacy of small molecule antimicrobials.

Keywords: B1b cells, antibody responses, bacterial infections, vaccines, B-cells

THE IMPORTANCE OF B-CELLS IN VACCINATION

B-cells carry a unique signature through the expression of a distinct surface immunoglobulin receptor. Under favorable circumstances, if the cognate antigen is engaged and appropriate secondary signals are received, the B-cell can differentiate into a plasma cell and secrete antibody through at least two pathways (1, 2). Depending upon the specificity, antibody can help prevent infections from establishing and protecting against a number of infectious diseases. B-cells can have additional activities during infection, which are not necessarily related to antibody production, where they provide signals through contact-dependent and -independent mechanisms as seen during experimental infections with the helminth *Nippostrongylus brasiliensis* (3). Such antibody-independent activities of B-cells are clearly important in infectious and non-infectious diseases (4–6). Thus, B-cells are important modulators of the host response and the growing and extending interest in the effector activities of B-cells is a welcome expansion of our understanding of the activities of this cell type.

Nevertheless, it is the antibody-mediated effector functions of B-cells that are estimated to save >2 million lives yearly (7). Antibody is behind the elimination of smallpox and the drastic reductions in the prevalence of measles, polio, diphtheria, tetanus, and a plethora of other infections for which vaccines exist, bringing tremendous economic and social benefits (8). Moreover, once infections have been encountered and natural immunity acquired, then the levels of antibody often correlate to the levels of protection against reinfection (9). Vaccines and antibody typically protect at the first encounter with a pathogen, usually before clinical signs are apparent and when bacterial numbers are at their lowest. In contrast, antibiotics are used when bacterial burdens are toward their peak and when clinical signs are more prominent. This game of numbers is probably a key reason why antimicrobial resistance is more common than resistance to a vaccine. As we head toward an era where increased resistance means existing antimicrobials will be less efficacious, there will be an increasing reliance on antibody-mediated mechanisms to protect us. To achieve this requires an efficient way to identify protective antigens. This is an important concept as separating out which antigens are protective from those antigens which are not is a timely, complex, and costly process (10). Therefore, understanding how to efficiently identify protective antigenic targets on pathogens will be a valuable tool for the future control of infection. We propose that understanding

the nature and targets of B1 cells, particularly B1b cells, is one such route for this. In this review, we discuss elements associated with B1 cells and infection, with a major emphasis on the relationship between bacterial antigens and B1b cells. This is in part to maintain a focus in the review, but also because other elements of B1 cell biology, particularly B1a cell biology, such as their development, role in housekeeping functions, and in diseases, such as autoimmunity, have been elegantly reviewed elsewhere in detail (11–36).

THE ROLE OF ANTIBODY IN INFECTIONS AND RESPONSES TO VACCINATION

Virtually, all vaccines work through the induction of antibody. The key point here is that, in general, antibody needs to be pre-existing at the time of pathogen encounter indicating the importance of inducing a persisting plasma-cell response to maintain this protective blanket of antibody. It is clearly desirable to induce B-cell memory to complement these activities and to augment antibody levels after antigen re-encounter, but responses to vaccination with T-independent (TI) antigens such as purified capsular polysaccharides show that robust memory is not essential for vaccines to work (37). Antibody induced to T-dependent (TD) antigens, such as proteins, is induced in two waves. Initially, after antigen encounter extrafollicular (EF) IgM is induced, which is typically of modest affinity as at the earliest time after antigen encounter, it is not derived from germinal centers (GCs; see below). Slightly later, the first IgG is detected, which increases in affinity with time as the GC makes a greater contribution (1, 2). Nevertheless, IgM is normally present with IgG to make a significant contribution to protection (38–42). In mice, the isotype of IgG induced can reflect the nature of the immune response. IgG3 is the dominant switched isotype after TI antigens, whereas IgG1 and IgG2a reflect T helper (Th) 2 and Th1 responses, respectively (38). *In vivo*, the predominant IgG isotype to a single antigen can vary depending upon the antigenic context in which it is encountered by the immune system (43). A relationship between the direction of Th responses and the direction of IgG switching is less clear in human beings, although some responses are more associated with certain IgG isotypes, for instance IgG2 and IgG4 antibodies are commonly found to LPS O chain and helminths, respectively (44, 45).

Multiple experimental models of infection show that IgM is critical for much of the short- and long-term protection afforded after natural infection and that the functional roles of IgM and IgG are likely to be synergistic (40, 46). The high avidity of pentameric IgM means that it is efficient at activating complement, whereas not all IgG isotypes are equally efficient at doing this (47). In response to most infections or proteinaceous vaccines, IgG titers will rise over many weeks, whereas IgM titers typically remain steady or fall. High affinity IgG is not induced by purified capsular polysaccharides (48).

For instance, the value of IgG has been demonstrated in studies using antibody generated during a natural non-typhoidal *Salmonella* infection or by an experimental protein vaccine against this infection that can induce TI and TD responses (46, 49). In these studies, the consistent observation was that IgG could account for up to 95% of the protection observed in wild-type (WT) mice, although surprisingly the additional benefit of IgG was not necessarily related to it being of high affinity. However, the role of IgG in the absence of IgM was not assessed in these studies. An additional consideration is that cell-free, antibody-dependent, complement-mediated bactericidal killing in mice may not be equally active against all infections. This is notable in *Salmonella* infections, where mouse serum is not effective at killing the organisms in bactericidal assays *in vitro* as human serum is, and so the true value of IgM and antibody *per se* may be underrepresented in murine systems (50). A further consideration is that the amount of antibody induced to a single antigen can affect outcome. Individuals with HIV/AIDS have a known increased susceptibility to invasive non-typhoidal *Salmonella* infections (51). The reason for this was recently attributed to elevated titers of inhibitory IgG to LPS and removal of this antibody enabled bacterial killing by the remaining non-inhibitory antibodies (52). A similar observation has been made in patients with bronchiectasis and a *Pseudomonas aeruginosa* infection (53). A proportion of these patients have markedly elevated titers of IgG2 to LPS O chain that inhibits cell-free or cell-dependent killing of bacteria. Therefore, efficient protection after infection or vaccination requires antibody to selective targets, present in the right amount and of the right isotype.

THE DEVELOPMENT OF ANTIBODY RESPONSES TO T-DEPENDENT AND INDEPENDENT ANTIGENS

There are classically three types of antigens (54–56). Some, such as LPS, can act as TI type I antigens, which induce specific and non-specific antibody responses through direct stimulation of the B-cell. A second class of antigens is TI type II antigens. Antigens that fall into this group include purified capsular polysaccharide vaccines such as those generated from pneumococcus and the experimental antigen, haptenated Ficoll. Typical haptens include 4-hydroxy-3-nitrophenyl acetyl (NP) and variations of this such as DNP and TNP. A feature of TI–II antigens is that within a single molecule, there are multiple repeats of the same epitope. This means that there is spatial co-localization of the same epitope and when a B-cell encounters the antigen, then multiple surface B-cell receptors are engaged in parallel (57). This drives a strong signaling response within the cell and abrogates the necessity for T-cell support to generate an antibody response. The third class of antigens is TD antigens, which are generally proteins. This is then processed and presented to T-cells via MHCII to enable their recruitment into the response. Conjugation of a TI–II antigen to a protein carrier can convert it into a TD antigen, a process that typically requires physical linkage (58, 59). The consequences of T-cell involvement in the generation of antibody responses are dramatic, since it ultimately results in the induction of productive GCs and the generation of greater amounts of switched antibody of a higher affinity and the generation of robust long lived and memory B-cell responses. Furthermore, conjugating a TI antigen to a protein carrier enables responses to capsular polysaccharide vaccines to be induced in certain groups, such as infants, in whom responses would otherwise be refractory (60, 61). The concept of classes of antibody responses has been the focus of recent discussion (62). This has led to the development of the idea of a TI–III response, which displays unique features, as well as sharing some with other types of B-cell responses. The distinct

features of the TI–III response focus on the support provided by additional bone marrow-derived cells including neutrophils, monocytes, mast cells, and basophils and are induced after infection with bloodborne or gut bacteria. They result in the production of EF antibody and show a marked dependence on TLR signaling for their development.

In mice, responses to model TI-2 and TD alum-precipitated antigens such as NP-chicken gamma globulin or ovalbumin, the kinetics of the immune response has been well characterized for both primary and recall responses and below is a short synopsis based on this non-exhaustive list of references (1, 2, 63–69) (**Figure 1**). It is likely most of the core features of this response reflect what happens in human beings. After antigen encounter, dendritic cells within the T-zone present and prime T-cells within the first 24 h to enable their rapid differentiation to T follicular helper (Tfh) cells. The dendritic cell subset involved in Tfh priming, Ig switching, and other T effector functions may not always be the same since different subsets can provide these functions in different sites concurrently. For instance, we have found that after flagellin immunization $CD103^+$ dendritic cells prime for IgA and IgG in the mesenteric lymph node but not the spleen, and that after *Salmonella* infection monocyte-derived dendritic cells help prime for IFNγ-expression in T-cells but not IgG2a responses (70, 71). In parallel, B-cells that have engaged antigen interact with primed Tfh cells also within the T-zone. Depending upon the signals received, B-cells can essentially have one of the three fates. They can die, or while still in the T-zone start to differentiate and proliferate. A proportion of these cells will migrate to the follicles and with the adequate recruitment of Tfh cells start to form GCs. Other B-cells will migrate to EF sites such as the red pulp of the spleen or the medulla of lymph nodes to generate plasmablasts and ultimately plasma cells. The kinetics are important for this process and so by around 5 days after immunization, an initial wave of short-lived IgM and IgG (predominantly IgG1 in mice) plasma cells is readily detectable in EF sites. These peak by around day 7 before gradually falling over the coming days (72). With slightly delayed kinetics, GCs form within the follicles and contribute the majority of plasma cells by a week or so after immunization. The key points that are applicable to the current discussion are that after proteinaceous molecules there is a parallel development of EF and GC responses, and that there is a robust EF switched IgG response.

What is less appreciated is that the process for the generation of responses to TI–II antigens is similar, but with accelerated kinetics. Thus, after antigen encounter, B-cells still migrate through the T-zone and start to proliferate in this site. Furthermore, some B-cells migrate to the follicles to form GCs that abort around the fourth day after immunization as they lack T-cell help to maintain them. Robust EF plasma-cell responses develop with a rapid expansion of the plasmablast population and the immunoglobulin isotypes most commonly detected are IgM and (in mice) IgG3. There are two major consequences of failing to induce Tfh and GC, mainly the longevity of the antibody response is shorter than that commonly seen to protein antigens, and upon reencountering the same antigen, an accelerated and augmented secondary response is absent. This is a significant clinical problem since hyporesponsiveness to a second immunization with a TI–II vaccine is often seen. Thus, the mechanics of TD and TI antibody responses show many key similarities.

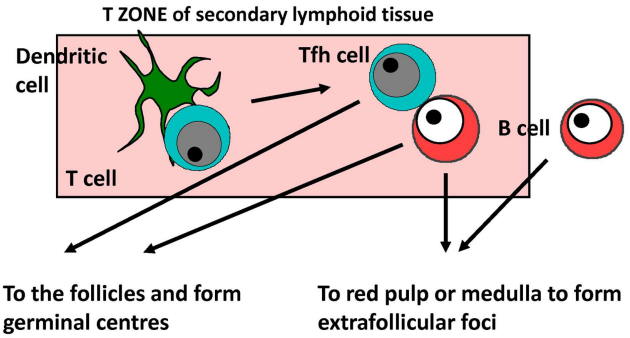

FIGURE 1 | The development of antibody responses. Specific antibody responses develop in secondary lymphoid tissues. B-cells responding to T-dependent and T-independent antigens migrate through the T-zone before producing antibody through the germinal center (GC) or extrafollicular (EF) pathway. Tfh cell – T follicular helper.

THE DISTINCT FLAVORS OF B-CELL SUBSETS

In human beings and mice, there are multiple B-cell subsets, including follicular B-cells and marginal zone (MZ) B-cells, as well as transitional B-cells (17, 31, 73–77). Follicular and MZ B-cells are known as B2 cells. MZ B-cells contain naïve and antigen-experienced B-cells. Follicular B-cells in both species express IgM, IgD and are $CD21^{int/lo}$ and $CD23^{hi}$. The level of diversity in B-cell receptor usage is greatest in follicular B-cells and accounts for most of the enormous antibody diversity seen in B-cells. Their productive engagement in immune responses shows a clear dependence on T-cells. MZ B-cells also share some similarities between mice and human beings in terms of phenotype with both subsets being IgM^+, $IgD^{lo/-}$, $B220^{hi}$, $CD21^{hi}$, and $CD23^{lo}$. Neither B-cell subset has the capacity to self-renew. There are differences between follicular and MZ B-cells. These differences include the sites where they reside, the more limited antigen repertoire recognized by MZ B-cells, the immaturity of the MZ B-cell compartment after birth, and finally, the capacity of MZ cells to respond to TI–II antigens. These latter two points make the responsiveness of MZ B-cells of significant clinical interest as TI vaccines have poor or negligible efficacy in infants, which correlates with limited MZ B-cell responses.

In mice, there are a number of additional B-cell subsets that are recognized and these come under the umbrella term of B1 cells (17, 22, 23, 26, 28, 31, 33). They are most associated with some serosal sites such as the peritoneal cavity, although they can also be detected in secondary lymphoid tissues and so are probably more widespread than currently appreciated (78, 79). A key feature that distinguishes B1 subsets from B2 subsets is their capacity to self-renew. Thus, transfer of B1 populations into lymphopenic mice will result in their expansion over a period of weeks. This means that it is possible to make B1 chimeric mice by transferring peritoneal cells into a Rag-deficient mouse [e.g., Ref. (80)]. In the steady state in the peritoneal cavity of a WT mouse, about 50% of B-cells are B1 cells, and we find there is an approximate ratio of B1a

cells to B1b cells of 2:1 (46, 81). A factor that complicates the study of B1 cells is their more elusive phenotype. They express CD19, low levels of IgD but relatively high levels of IgM. They can express CD43 and CD9, are negative or low expressers for other markers such as CD21 and CD23, and are mostly intermediate for B220 (27, 31, 82–84). In the spleen, many of their characteristics are similar to transitional B-cells when at a transitional T1 stage. Identification of B1a cells is substantially aided by their modest expression of CD5, although in mice expressing CD5 is not necessarily exclusive to B1a cells (85). Another marker closely associated with B1 cells is CD11b, but the frequency of B-cells expressing CD11b can be variable; for instance, during some infections, it alters from that seen during steady-state conditions (46). It is unclear whether the differences in CD11b expression mark distinct B1 subsets or reflect differing developmental stages (86–88). Nonetheless, these factors make examining B1 cells more complicated, particularly in secondary lymphoid tissues, and the often necessary reliance on CD11b as a positive marker, though understandable, is likely to mean their true numbers are underestimated.

EVIDENCE FOR B1 CELLS IN HUMAN BEINGS

In human beings, the identification of B1 cells has proven to be a contentious issue (89–94). Part of the reason for this relates to the difficulty in accessing sites such as the peritoneal cavity, where B1 cells are enriched in mice. Nonetheless, currently, there is limited evidence for an exact replication of the murine phenotype for B1 cells in human beings, particularly for B1a cells (89, 91, 95, 96). One report has identified a rare population of B-cells in patients with chronic variable immunodeficiency disease (97) and cells in other non-human primates (NHP) share common B1b markers (98). B-cells in human fetal cord blood and adult blood with a $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ phenotype, but with a variable expression of CD5, CD86, IgM, and IgD have been identified as human B1 cells (89). The definition of these cells is not solely based on surface markers as these cells also had other properties associated with B1 responses. These include (i) the ability to spontaneously secrete IgM antibody to antigens associated with B1 responses such as phosphorylcholine, a known target of B1a-derived antibody in mice and (ii) the ability to readily interact and prime T-cells. Interestingly, the probability of a human B1 cell having either or both properties correlates to its expression of CD11b. Spontaneous IgM secretion is more associated with $CD11b^{-}$ cells and T-cell modulating activity associated with $CD11b^{+}$ human B1 cells. Nevertheless, others have suggested that $CD27^{+}CD43^{+}CD70^{-}$ B-cells may reflect a pre-plasmablast differentiation state of cells present in the blood (93). It is tempting to speculate how cumulative exposure to antigen may influence the phenotype and frequency of B1 cells in different species since B1 cells are more readily detectable in animals kept in controlled environmental conditions. Therefore, currently, there is not a universally accepted definition for B1 cells in human beings as there is for mice.

MICE CONTAIN MULTIPLE B1 SUBSETS

B1 cells can have phagocytic and antigen-presenting functions, secrete cytokines to modulate the host and produce antibody, with these properties not necessarily mutually exclusive within individual cells (32, 99–101). Most work on B1 cells in mice has focused on B1a cells, and their activities have been well reviewed by a number of authors (see above). B1a cells express CD5 and, at least in the steady state, are the dominant B1 subset in the peritoneal cavity. They play a major role in maintaining host immunological tone and homeostasis, but also make striking contributions to the control of infections, such as influenza (15, 31, 102, 103). What is striking about the role of B1a response to influenza is that they contribute antibody through two pathways. The first is through the production of natural antibody, which is generated independent of the presence of cognate antigen. The second is the generation of antibody during infection. Influenza infection results in the accumulation of B1a cells to sites of infection and their production of antibody specific to the pathogen. B1a cells are able to generate IgM and IgA antibodies of modest affinity that can be poly-reactive. Features of the B1a response are atypical since it does not necessarily require the differentiation of B1a cells into CD138-expressing plasma cells or the loss of B1a cell markers, and the majority of the B1a antibody induced is not specific to influenza (104). This suggests a robust early and innate, if "blind" antibody response, which functions to limit infection. While this may simply be a "panic" response to a pathogen, it is also possible that these cells also modulate immunity through phagocytic or contact-dependent mechanisms.

The reported ability of B1a cells to secrete antibody after antigen exposure, but without differentiation into plasma cells, raises questions regarding the nature of antibody-secreting cells. This has only been addressed in any detail for CD5+ B1a cells, and is also an area where reports do not necessarily converge. Based on *in vitro*, *ex vivo*, and adoptive transfer studies, Rothstein and colleagues have shown that antibody-secreting B1a cells in the peritoneal cavity show limited expression of genes associated with plasma cells, such as blimp-1 and CD138 (105). Furthermore, IRF4-deficient peritoneal B1 cells can still secrete IgM, whereas splenic B1 cells do not (106). Nevertheless, experiments using Blimp-1 reporter mice failed to support these findings, although Blimp-1-deficient mice retain the capacity to generate some immunoglobulin (107, 108). The differences in these studies may reflect the different approaches taken or other factors. What is lacking is a detailed comparison between plasma cells generated from B-cells of different origins that compare more than the antibody isotype they produce or antigens they initially responded to, although this is clearly a technical challenge.

B1b CELLS MAKE IMPORTANT CONTRIBUTIONS TO PROTECTIVE IMMUNITY IN MICE

Much of the work examining the activities of B1a cells have focused on their role in autoimmunity, while their roles in controlling infection are less studied. B1a cells do not necessarily function in isolation and, for instance, they can collaborate with B1b cells to combat pneumococcal infections (80). Despite sharing many phenotypic features with B1a cells, such as the generation of natural antibody to self-antigens (109), there are key differences between B1a and B1b cells. There is evidence that each of these cell types has a different developmental pathway and infection may increase a progenitor population that has the potential to generate progeny with a B1a-like phenotype (26–28, 30, 31, 33, 110–116). Another

difference between B1a and B1b cells is that the latter tend to exhibit a greater level of junctional diversity compared to B1a cells suggesting a broader repertoire of antigens is recognized by these cells, although this may be a more complex picture than previously envisaged (117–120). This then leads to a key question. What antigens are recognized by B1b cells and are they clinically relevant?

The first studies showing a potential clinical benefit of B1b responses came from experiments using a murine model of relapsing fever caused by the spirochete *Borrelia hermsii* (121, 122). This bacterial infection in mice results in a pronounced bacteremia, with bacterial numbers reaching $>10^{6-7}$ bacteria/ml blood, but there is much less colonization of tissues by bacteria (123). A key reason for this is the high degree of antigenic variation in certain bacterial surface proteins (124). The infection relapses because antibody develops to the dominant clone and as this is controlled it enables the outgrowth of an antigenically distinct minor clone, which goes on to cause another episode of fever. This can occur multiple times, but typically each further round of bacteremia is less severe than the one that preceded it. This suggests there are protective antigens present that are unrelated to the variable proteins and that the antibody response to these protective antigens develops at a slower rate, due to potential reasons such as antigen density or epitope availability. IgG can contribute to protection in this model but an absolute requirement for IgM has been demonstrated (121–123). A role for B1b cells in protection against *B. hermsii* was identified after infection of a range of genetically altered mice (IL7−/− mice that are deficient in follicular B-cells), splenectomized mice, and B-cell chimeric mice (121). Protection was TI and reinfection of T-cell-deficient mice revealed that a TI memory response was induced. Significantly, B1b chimeras generated by transfer of B1b cells from convalescing mice into Rag1-deficient hosts were more efficient at controlling infection than equivalent chimeras generated using naïve B1b cells. This finding of TI memory was reproduced in a separate model involving immunization with a polysaccharide antigen from *Enterobacter cloacae* (125).

The unequivocal demonstration of a role for B1b cells in controlling this infection raised questions regarding which antigen was the target for this protective antibody. The obvious candidates were the variable surface proteins from this pathogen, which contribute to immune evasion, but this turned out not to be the case. The target of the protective B1b antibody was a conserved protein, the Factor H binding protein (fHbp), and the response to this protein could develop in the absence of T-cells (126). Antibody to fHbp accumulates slowly with time and protection is only achieved when levels of antibody to this antigen reaches a certain level. This is sufficient to explain why each round of bacteremia tends to be less severe than the one before. This is an important finding and the concept it identifies has far reaching implications for understanding protective immunity during natural infection and for vaccinology. It means that targeting cell wall localized immunodominant antigens is not necessarily required to generate immunity to different strains of the same organism. There are manifold forms of fHbp produced by different bacterial genera and these show significant variability at the protein level. This protein is of interest to vaccinologists as it is one of the protective antigens included in the Bexsero vaccine used against group B meningococcus (127). Alugupalli and colleagues extended their findings on fHbp from *B. hermsii* by generating mice with a humanized immune system, so that all B-cells are derived from human progenitors (128). They showed these chimeras generated IgM antibody to *B. hermsii* and to fHbp and that protection was dependent upon B-cells. Although not formally shown, the antibody response in these chimeras is likely to have been induced in a TI manner. This is a landmark study since it demonstrates that human B-cells are capable of generating anti-protein antibody responses in a manner that resembles the process in mice.

Parallels can be observed between B1b cell responses in mice and the B-cell response in other species to TI–II antigens, such as capsular polysaccharides (78, 80). Classically, splenic MZ B-cells are associated with the generation of antibody to such antigens, in part because of the poor responses to capsular polysaccharide vaccines observed in asplenic adults and infants, who lack a mature MZ B-cell compartment (74, 129). Nevertheless, mice deficient in MZ B-cells can still make robust responses to TI–II antigens (78, 130) so this does not negate the potential for B1b cells to be involved in such responses. Also, B1b cells are detected in the spleen after immunization with TI–II antigens and they can contribute directly or through interactions with MZ B-cells (131, 132). Although B1b cells can recognize haptenated Ficoll, the antigen itself is not a natural antigen and it is typically absent from the environment meaning that in the absence of immunization, animals are naïve to it. This has been exploited to identify the presence of cells that phenotypically and functionally resemble B1 (both B1a and B1b cells) in NHP (African green monkeys and cynomolgus monkeys) (98). NHP B1 cells have a similar phenotype to cells in mice and are $CD11b^{hi}$ forward scatter hi, $CD21^{lo/-}$ and CD19hi and can upregulate CD27 upon antigen encounter. Like B1b cells, in mice, they respond to TNP-Ficoll and produce IgM and IgG but little IgA.

In an earlier pivotal study, Haas and colleagues demonstrated that capsular polysaccharide from *Streptococcus pneumoniae* is a B1b antigen and that B1b-derived antibody was sufficient to protect against infection (80). Purified pneumococcal polysaccharide is currently used as a vaccine in human beings against these infections and such responses can be long lived (133, 134). The response in B-cells also differs depending whether the vaccine is conjugated or not (135). Additional studies using α-1,3 dextran from the Gram-negative bacterium *E. cloacae* have identified B1b cells as the principal reservoir of memory to bacterial polysaccharide antigens (125). Indeed, B1b cells maintain the potential to respond to pneumococcal polysaccharide throughout life (136), with the mechanisms underlying the responsiveness of B1b cells under active investigation (137). Therefore, identifying targets of B1b cells may help in developing strategies to improve vaccination against pneumococcus in the elderly, who are disproportionately susceptible to these infections and in whom vaccination with pneumovax has not had a dramatic effect on the incidence of infection (138). It is likely that the number of infections where B1b responses are important will expand. For instance, putative evidence suggests that the characteristic response of the organism *Ehrlichia muris* at least in part involves B1b cells (40, 139). Collectively, studies such as those highlighted above show that B1b

antigens are present in a plethora of bacterial genera of major clinical importance and that these antigens can be key targets of protective immunity.

SALMONELLA INFECTION AS A MODEL TO STUDY B1b CELLS

In murine systems, B1b cells can make a significant contribution to protection. Our own work has examined the B-cell response in the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (49). Clearance of primary *S.* Typhimurium infections in mice requires an intact and persisting CD4 T-cell response regulated by the transcription factor T-bet (140–144). Antibody can protect against secondary infection or after immunization (46, 81, 145, 146). We came to study *S.* Typhimurium indirectly as a development of our use of model antigens such as ovalbumin and the bacterial TLR5 ligand flagellin (43, 147). Using these antigens, we could show how altering the context in which an antigen was encountered could significantly alter the T- and B-cell response to it. Through immunohistological examination of the splenic plasma-cell response after systemic *S.* Typhimurium infection, we noted that the response was highly atypical (**Figure 2**). In particular, we noted that the bacterium induced a rapid EF plasmablast response (49). On day 3 after *S.* Typhimurium, the majority of IgM plasmablasts in the red pulp were in cell cycle, and their numbers had already increased >10-fold. In parallel, on day 3, there was also a 3–4-fold increase in IgG2a-switched plasmablast numbers, rising to around 20-fold higher by day 4, when most IgG2a cells are in cell cycle. In contrast to EF responses after immunization with alum-precipitated proteins (148), the induction of the IgM response was CD40L-independent, whereas switching was CD40L dependent [(49); data not shown]. Atypically, the EF IgM and IgG response in WT mice occurred in the absence of a detectable GC response, which was not observed until the infection was largely cleared (around day 35 in the described model). Therefore, for at least 3 weeks, this model allows the assessment of an EF response in the absence of a confounding influence from GC.

The speed and extent of the EF response and the T-cell independence of its induction had two major implications. First is that there is a significant availability of antigen for B-cells to access, despite the organism having an intracellular life-style and only inducing a low grade bacteremia. Second, it suggests there is likely to be a significant precursor B-cell pool that is enriched for antigens present within this bacterium. Identifying which antigens were targets of the atypical B-cell response was greatly aided by the characterization of the prolonged antibody response to the major porin proteins from *S.* Typhi OmpF and OmpC. Work by Lopez-Macias, Isibasi and colleagues showed that immunization of human beings and mice with purified, soluble porins induced long-lived bactericidal antibody against typhoid (149, 150). These proteins were good candidates as targets of the EF antibody response in that they naturally share features of TI–II antigens such as existing as oligomers and thereby containing repeating epitopes (151). Immunization with purified porin proteins from *S.* Typhimurium showed that these proteins induced a TI response and induced protective antibody primarily through the presence of an additional porin, OmpD, which is absent in *S.* Typhi (46). Characterizing the peritoneal B-cell response to porin proteins and

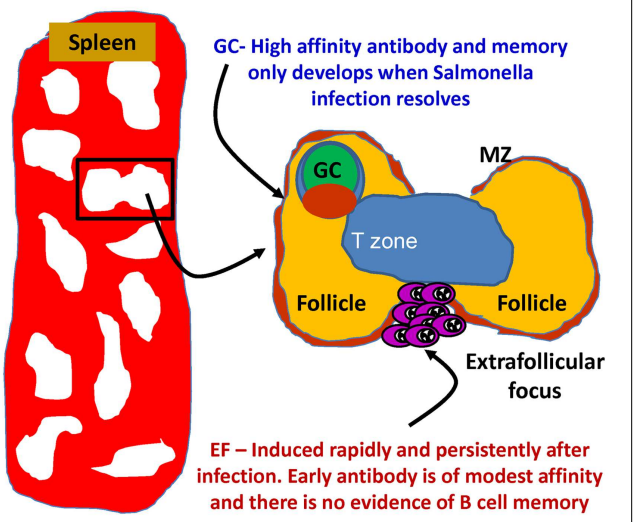

FIGURE 2 | The development of antibody responses in mice after *Salmonella* infection. After infection, there is a rapid induction of extrafollicular responses, so that IgM and IgG plasma cells are readily detectable by day 3. These events occur in the absence of germinal center (GC) responses that are absent until infection has all but cleared. MZ – marginal zone.

live *S.* Typhimurium showed that both antigens induced a B1b cell response. In contrast, heat-killed bacteria or purified, monomeric flagellin did not. Immunization with TLR grade LPS also induces features of a B1b response, although it is unclear how much of this is antigen-specific or induced through its mitogenic effects. In responses to other Gram-negative bacteria, LPS may induce a B1a response (152). A marker commonly used to help identify B1b cells is CD11b. After infection of WT, but not T-cell-deficient mice, the majority of B1b cells was not CD11b+. Thus, it is possible that examination of the response in WT mice using CD11b may under-estimate the numbers of B1b cells, although other reasons may help explain this, such as the maturity of the B1b cells after infection or that additional B1 lineages are recruited to the response.

This model allowed the investigation of other key questions regarding B1b cells, one of which is whether multiple B1b antigens exist within the same species. This was investigated using the Vi antigen from *S.* Typhi. This antigen, in purified form, is used as a vaccine against typhoid in human beings (146). Since *S.* Typhi is a human pathogen with limited infectivity in non-primates, it was necessary to examine the response in mice to *S.* Typhimurium engineered to express Vi antigen and to purified Vi itself (81, 153). This allowed a combined approach to be used where the response to Vi in the context of the bacterium or after immunization with the purified antigen could be examined. These experiments confirmed the TI nature of the response to Vi and showed that it induced a B1b cell response. Furthermore, the antibody generated by B1b chimeras was sufficient to provide protection against challenge with *S.* Typhimurium expressing Vi. Thus, the same species

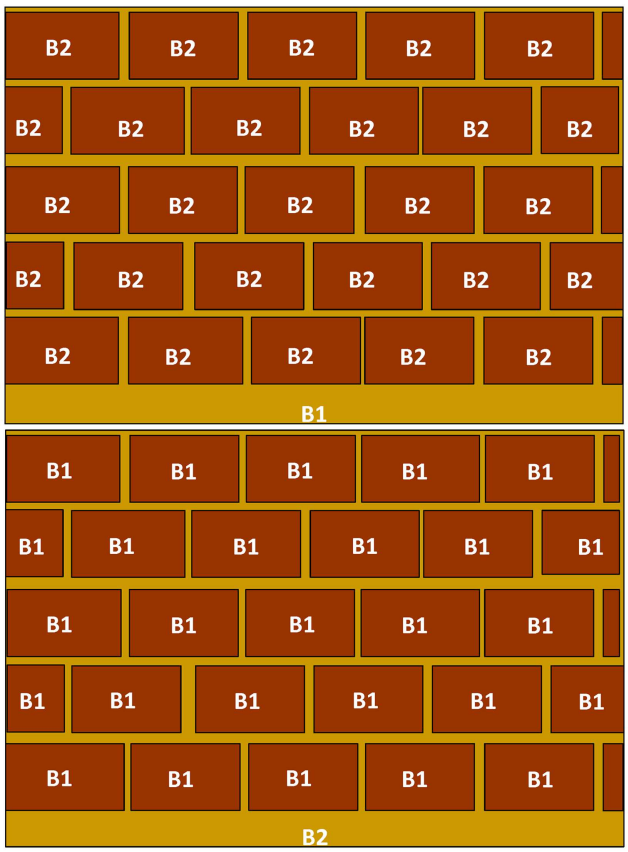

FIGURE 3 | The relative contribution of antibody from B1 and B2 cells. Antibody provides a wall of protection against infection. Antibody to many B1(b) antigens is also protective and suggests that a wide repertoire of responses are not always necessary to protect against infection. Since antibody to many antigens is not protective, it may be that antibody derived from B1 cells provides greater protection in relative terms than that from B2 cells, particularly follicular B-cells. In the context of this figure, the mortar fills the gaps between the bricks, which provide the majority of protective coverage.

of bacterium contains multiple B1b antigens, suggesting they may be more widely distributed than was perhaps originally perceived when the pioneering studies were being undertaken.

CONCLUSION

The evidence clearly shows that B1 cells play an important role in providing antibody against infection. Between the B1 subsets, B1b cells are most associated with providing responsive antibody during natural infection or after vaccination. The targets of B1b cell-derived antibody appear to have a disproportionate likelihood of also being protective antigens and many of these antigens are known to induce protective responses in human beings. This strongly supports the concept that the B-cell receptor usage by B1b cells is not random. Furthermore, the antigens recognized by B1b cells are not limited to any one genus or Gram classification. The detection of multiple protective B1b antigens in a single species suggests that B1b antigens are widespread. Therefore, even allowing for the incomplete understanding of B1 responses in human beings examining B1b antibody responses in mice is likely to be a rewarding avenue for identifying putative vaccine candidates.

From a personal perspective, there remain a number of unresolved questions regarding the contribution of antibody from and the biology of B1 cells. How complete is our understanding of the nature of selection of B1 cells? How can they be selectively enriched to recognize protective antigens and epitopes? How widespread are B1b cells in human beings? How great is the contribution of antibody from B1b cells to protection after vaccination or natural infection compared to B2 cells and do B1b cells primarily target bacteria and is this restricted to cell wall antigens? Thus, if we think of the protection provided by antibody as a wall then most of a wall is occupied by the bricks, with the gaps between filled by mortar. If antibody to B1b antigens is sufficient to protect against most infections, then the coverage offered by this would be sufficient to protect against the majority of bacterial threats (**Figure 3**). The remaining spaces would be filled by antibodies derived from other B-cell subsets and contribute to protection against antigens such as soluble toxins where there is no evidence of any contribution of B1b cells. How does conjugating a B1b antigen, such as pneumococcal polysaccharide to a typical protein carrier like CRM197, alter the responsiveness of B1b cells to this antigen? Furthermore, how well can B1b cells switch? Experiments using *Salmonella* show how closely the TI induction of the response and the TD switching in the same response are coupled and so we suspect they are two features of the same B-cell population. Is this really the case or are there two parallel responses induced concurrently? Finally, can B1b cells take part in the GC response? The expansion in the numbers and types of antigens recognized by these cells, the models available, and the intensive investigations underway in human beings make the coming years an exciting time for this field and have implications for both basic and applied immunology, microbiology, and vaccinology.

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Conflict of Interest Statement: Adam F. Cunningham has a patent on OmpD – Pub. No.: WO/2010/029293 International Application No.: PCT/GB2009/002159: Non-typhoidal *Salmonella* vaccines (http://patentscope.wipo.int/search/en/WO2010029293). Granted and currently under license with NVGH and held in South Africa, China, Brazil, Canada, US, and UK. The other co-authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 05 June 2014; accepted: 10 October 2014; published online: 31 October 2014. Citation: Cunningham AF, Flores-Langarica A, Bobat S, Dominguez Medina CC, Cook CNL, Ross EA, Lopez-Macias C and Henderson IR (2014) B1b cells recognize protective antigens after natural infection and vaccination. Front. Immunol. **5**:535. doi: 10.3389/fimmu.2014.00535*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 18 December 2013
doi: 10.3389/fimmu.2013.00460

A replicative self-renewal model for long-lived plasma cells: questioning irreversible cell cycle exit

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Plasma cells are heterogenous in terms of their origins, secretory products, and lifespan. A current paradigm is that cell cycle exit in plasma cell differentiation is irreversible, following a pattern familiar in short-lived effector populations in other hemopoietic lineages. This paradigm no doubt holds true for many plasma cells whose lifespan can be measured in days following the completion of differentiation. Whether this holds true for long-lived bone marrow plasma cells that are potentially maintained for the lifespan of the organism is less apparent. Added to this the mechanisms that establish and maintain cell cycle quiescence in plasma cells are incompletely defined. Gene expression profiling indicates that in the transition of human plasmablasts to long-lived plasma cells a range of cell cycle regulators are induced in a pattern that suggests a quiescence program with potential for cell cycle re-entry. Here a model of relative quiescence with the potential for replicative self-renewal amongst long-lived plasma cells is explored. The implications of such a mechanism would be diverse, and the argument is made here that current evidence is not sufficiently strong that the possibility should be disregarded.

Keywords: plasma cell, cell cycle, self-renewal, quiescence, myeloma, monoclonal gammopathy, gene expression, lifespan

INTRODUCTION

Entry into cell cycle quiescence accompanies the final phenotypic maturation of plasma cells (1). In short-lived plasma cells the maintenance of quiescence is rendered irrelevant by cell death. In contrast long-lived plasma cells may potentially survive for the lifetime of the organism (2, 3), and the mechanisms maintaining quiescence in such populations are of much greater significance. The current paradigm holds that cell cycle exit is permanent and irreversible for all plasma cells (2, 4, 5). However it may be timely to re-evaluate this paradigm and consider an alternate possibility: that long-lived "memory" plasma cells adopt a quiescent state, controlled by their niche environment, but maintain the potential for replicative self-renewal through cell division. The implications of such a mechanism are diverse, and the evidence against is potentially not sufficiently strong that the possibility should be disregarded. Recent progress in generating and maintaining long-lived human plasma cells *in vitro* may help to address some aspects of this issue.

DISCUSSION

LIFESPAN OF PLASMA CELLS

The nature of plasma cell lifespan and the concept of irreversible cell cycle exit accompanying terminal differentiation are intertwined. Early observations of potential plasma cell longevity were largely set aside in favor of the view of continuous generation of short-lived plasma cells (6, 7); and in the context of cell cycle exit coupled to functional differentiation, and imminent cell death the concept of irreversible cell cycle exit is natural and follows the prevailing pattern in other short-lived hemopoietic effectors.

The critical transition in our understanding of plasma cell longevity came with the studies of Manz et al. (8) and Slifka et al. (7), whose works combined to provide proof of the existence of long-lived plasma cells, which preferentially resided in the bone marrow and made a central contribution to long-term humoral immunity. Subsequent work from other labs in mouse models has also pointed to extended lifespans, although the half-life predictions vary somewhat with the type of assay and vaccination strategy employed, and in recent data include dynamic changes in long-lived plasma cells in response to systemic inflammation (5, 9, 10). In earlier continuous tritiated-thymidine incorporation studies in rat, antibody-secreting cells in the bone marrow showed more general labeling reaching near 40% by 10 days (11), but it has been argued that these experiments may have overlooked long-lived quiescent plasma cells since antigen-specific populations were not assessed (12).

Serological studies in human combined with the persistence of plasma cells after therapeutic B-cell depletion point to significant lifespans for human bone marrow plasma cells (13). While direct evidence of plasma cell longevity in man is limited, *in vitro* generated human plasma cells can certainly persist as non-dividing cells for months (14). A view of the bone marrow plasma cell compartment, encompassing the decay of antibody titers after therapeutic B-cell depletion, would include a heterogenous mix of plasma cell populations, many with relatively short half-lives in the region of $<$100 days, as well as populations of longer-lived cells persisting well beyond this time-frame. In human bone marrow such heterogeneity is potentially reflected in phenotypic differences in bone marrow plasma cells (G. Arumugakani and A. Rawstron, personal communication).

DIFFERENTIATION AND THE PERMANENCE OF CELL CYCLE EXIT

While the shift toward a general acceptance of long-lived bone marrow plasma cell has occurred, the paradigm that all plasma cells have irrevocably exited cell cycle has remained (2, 4, 5). Terminal differentiation as a concept encompasses the acquisition of high functional specialization and the loss of potential for alternate cell fates. This is frequently linked to irrevocable cell cycle exit. This clearly pertains in the context of short-lived effector cells that die soon after completing differentiation and exiting cell cycle. In contrast in long-lived cells functional specialization is not necessarily linked to irrevocable cell cycle exit (15–19). Schwann cells provide a well-studied example of cells with high functional specialization that enter a quiescent rather than post-mitotic state, and can re-enter cell cycle in response to injury or growth factor stimulation (18). However the ability of differentiated cell populations to re-enter cell cycle also extends to other systems traditionally viewed as terminally differentiated such as cardiac myocytes (15, 16, 19). Recently such concepts have also been extended to tissue resident macrophage populations (20–22). Given that such examples exist in other tissues with complex organization, and in immunological populations conventionally viewed as deriving from hemopoietic repopulation, it is reasonable to re-evaluate the paradigm that all differentiated plasma cells have necessarily exited cell cycle in an irrevocable fashion.

While there is little evidence directly in support of long-lived plasma cells undergoing self-renewal by cell division there is also relatively scant data in direct opposition. Several early studies demonstrated that plasma cells but not their proliferating precursors were resistant to hydroxyurea in short-term cultures (23–25). This is consistent with cell cycle exit, but does not distinguish the nature of this quiescence. Phenotypically mature human plasma cells generally lack Ki67 expression by flow cytometry (26), but low levels of proliferating plasma cells are detectable (27), equally flow cytometry only identifies a small fraction of hemopoietic stem cells (HSCs) as detectably in cell cycle (28). The need for long-lived plasma cell renewal may be different in organisms with extended lifespans and longer intervals to sexual maturity, but in the murine studies a slight accumulation of BrdU-labeled antigen-specific plasma cells over time appears to be present in the continuous feeding arm of the original Manz et al. study (8). Additionally an interesting recent study examined pulsed BrdU incorporation into bone marrow plasma cells under a range of immunization scenarios (5). At 60 and 100 days after primary immunization a very low, but above background, level of BrdU incorporation into antigen-specific plasma cells appears to be present. While such data can be interpreted in the context of the prevailing paradigm as newly formed plasma cells arising from B-cell differentiation, it can also be argued that the cell of origin from which a BrdU-labeled plasma cell derives is not known in such studies, particularly after an extended period post immunization. Some or all of the BrdU labeling could derive from incorporation into pre-existing plasma cells that arose following the initial immunization.

CELLULAR RELATIONSHIPS, THE NICHE, AND CELL CYCLE

Under a range of experimental conditions the process of cell cycle exit during plasma cell differentiation occurs in an orderly fashion (14, 29–31), suggesting that the core mechanism is a common feature shared by all plasma cells regardless of cell of origin or activating stimulus. Whether such consistency pertains to the maintenance of cell cycle exit in differentiated plasma cells is less certain. The ability of cellular environment to influence plasma cell biology is a central theme of long-term plasma cell survival and the bone marrow plasma cell niche is critical to the persistence of these cells (32, 33). This niche is in part defined by stromal cells that also support multipotent hemopoietic precursors (34). In addition to stromal cells many more fluctuant hemopoietic populations have been implicated as contributing factors to plasma cell survival (34–38). This complexity has been summarized in the concept of the multicomponent plasma cell niche (39).

For the principle other long-lived quiescent bone marrow population, the HSC, a remarkably close relationship exists between the niche environment and quiescent state, with a range of niche factors implicated in maintaining quiescence (40–44). Using HSCs as an example, if replicative self-renewal can occur in plasma cells then it is likely that triggers will include changes in niche factors that link quiescence to survival, and physiologic signals that promote transient proliferation. If both quiescence and survival are directly linked to niche occupancy, then the partial displacement of a long-lived plasma cell from its niche, or the depletion of limiting niche factors by competing populations could represent a trigger for replicative self-renewal.

CELL CYCLE IN PLASMA CELL DIFFERENTIATION

In general clonal expansion precedes plasma cell differentiation representing an intrinsic feature of an effective immune response. In model systems plasma cell differentiation is a process directly linked, at a mechanistic level, to cell division (23, 45–47). Indeed whether plasma cell differentiation can occur in the absence of cell division is uncertain. Elegant modeling approaches indicate that the fate adopted by differentiating B-cells is determined in a stochastic fashion linked to cell division (48).

While cell division is thus an essential component of the plasma cell differentiation program, this program does not complete if exit from cell cycle is prevented (1, 30). This is not a unique feature of plasma cell differentiation, but a general one of differentiation programs. Indeed much more is known of how cell cycle control intersects with transcriptional programs of cellular differentiation in other lineages (49). General features are that control of G1 progression and regulators of the RB/E2F pathway are central to these processes, but show lineage specific relationships. Furthermore cell cycle regulators can directly impact on transcriptional regulation and the execution of differentiation programs (49).

Direct experimental evidence indicating that cell cycle exit is necessary for plasma cell differentiation comes from analysis of p18INK4C, whose presence in differentiating B-cells is essential for plasma cell differentiation (30). p18INK4C (CDKN2C) is a repressor of the CyclinD-CDK4/6 complex and thus controls the initial phosphorylation of RB which is required for subsequent hyperphosphorylation by Cyclin-E-CDK complex and G1/S progression (1). CDKN2C is induced in parallel with BLIMP1 (PRDM1), the principle transcription factor defining the committed transition to plasma cell differentiation (50). CDKN2C deficient B-cells induced to undergo plasma cell differentiation express BLIMP1 and initiate the gene expression program of plasma cell

differentiation, but fail to complete the process of differentiation appropriately, and never achieve the high secretory activity of terminally differentiated plasma cells (30). In a more recent analysis the same laboratory has extended these investigations to examine the transition of highly proliferative "intermediate plasma cells" to mature plasma cells, the former cell state being similar to plasmablasts. CDKN2C was progressively induced from activated B-cell to intermediate and then mature plasma cell state, while CDKN1B (p27KIP1) was selectively induced in mature plasma cell in which both CYCLIN-D2 and -E were also maintained (50). Failure to induce CDKN2C led to cell death amongst "intermediate plasma cells." These data suggest that cell cycle inhibitors may act sequentially in the differentiation process, and indicate that apoptotic pathways provide a fail-safe controlling plasmablast that do not appropriately exit cell cycle.

A direct link between the differentiation program and cell cycle exit also lies in the ability of BLIMP1 to repress *MYC* (51, 52). BLIMP1 expression accumulates with time during plasma cell differentiation (53), and is well established as a critical regulator of this process necessary to mediate the genetic reprograming from B-cell to plasma cell (54, 55). BLIMP1 is implicated in the repression of multiple targets associated with cell proliferation (54), and can bind at the promoters of genes associated with kinetochore function (*SPC25* and *CENPH*) (56), which are also repressed at the plasmablast to plasma cell transition (14), providing an additional mechanism by which it may impact on cell division in plasma cells. Expression of BLIMP1 accumulates during differentiation, precedes the entry into cell cycle quiescence, and is abundantly expressed in mouse and human proliferating plasmablast populations (14, 31, 50, 53). BLIMP1 can recruit several different chromatin modifiers (57–59), which together provide the potential for either labile or stable epigenetic regulation. In a cumulative expression model the gradual increase of BLIMP1 may be linked to sequential and progressively more stable extinction of different gene expression programs. Under such a model the proliferative program would represent a late target repressed once BLIMP1 levels pass a particular threshold at the plasmablast stage. The inability of BLIMP1 to compensate for p18INK4C (50), suggests that the role of BLIMP1 in cell cycle control could lie on the one hand in attenuating the impetus for cell cycle progression and on the other in reinforcing the decision through epigenetic repression of proliferation genes. Nonetheless at present little is known of how the transcriptional program of plasma cell differentiation intersects with the machinery of cell cycle control and exit. While BLIMP1 acts as a regulator of terminal differentiation in both B- and T-cell lineages (55, 60, 61), this transcription factor can also play roles in cellular populations with stem cell properties (62–65). Thus BLIMP1 expression *per se* is not necessarily strictly linked to irreversible cell cycle exit.

BLIMP1, BCL6, AND MYELOMA PLASTICITY

During B-cell differentiation BLIMP1 is involved in counter-regulatory relationships with PAX5 (66–68), SPIB (54, 69), and BCL6 (54, 70, 71). These regulatory interactions may be further modified by BACH2 dependent BLIMP1 repression (72–75). Together these interactions play critical roles in limiting and modulating PC differentiation. A notable feature of the "intermediate plasma cells" generated during murine splenic immune responses, was the apparent co-expression of BLIMP1 and BCL6 in a significant fraction of cells (9%), when analyzed at single cell level (50). However this co-expression was likely to represent a transient state as most cells expressed either factor in a mutually exclusive fashion. Interestingly, in a myeloma cell line model co-expression of BCL6 and MTA3 can drive a phenotypic reversion with B-cell antigen expression (76). Although evidence for BCL6 expression in primary myeloma is limited, some evidence of expression has been observed (77). While BCL6 expression and phenotypic reversion may provide an explanation for observations in advanced myeloma, and myeloma cell lines, it is important to distinguish between such transformed states and those operating in normal plasma cells, and early plasma cell neoplasms. In cell lines and advanced myeloma several co-operating oncogenic events contribute to extensive cell cycle deregulation (78, 79). In addition loss of epigenetic control, with a global increase in DNA hypomethylation, is a feature of the monoclonal gammopathy of undetermined significance (MGUS) to myeloma transition (80). Cell proliferation itself imposes a burden on maintaining epigenetic regulation, requiring the re-establishment of appropriate control with each division (81). Thus rapid proliferation is likely to contribute to plasticity of gene expression in advanced myeloma, and may be intrinsically linked to reversion to an "intermediate plasma cell" or plasmablast-like state. Indeed in some instances patients present *de novo* with disease that blurs the boundaries between "plasmablastic myeloma" and "plasmablastic lymphoma," leading to significant diagnostic and therapeutic questions (82). However, in normal plasma cells, and early neoplasia the balance is strongly in favor of maintaining appropriate epigenetic control and if cell cycle re-entry does occur, then limiting the frequency of this event. Thus, while consideration of phenotypic plasticity and its transcriptional control is relevant in advanced plasma cell malignancies, this must be distinguished from the control processes potentially operating in normal plasma cells, and early plasma cell malignancy under consideration here.

NUCLEAR ORGANIZATION AS A LINK TO STABLE CELL CYCLE EXIT

A classical feature of mature plasma cells is the organization of the nucleus such that dense chromatin condensation is observed in a ring like pattern at the nuclear periphery (83). This generates the "clock-face" nucleus of the mature plasma cell. The significance of this nuclear architecture is unknown, but compaction of heterochromatin can provide a mechanism contributing to stable gene silencing.

A precedent for this in relation to cell cycle exit can be found in the process of senescence. Cellular senescence is characterized by the expression of a range of cell cycle control proteins, some but not all of which are shared with plasma cells, and the reorganization of the cell nucleus with compaction of silenced chromosome domains into senescence associated heterochromatic foci (SAHF) (84). These domains do not occur in reversibly arrested cells, and are enriched for E2F target genes (84). SAHFs derive from the organization of facultative heterochromatin, associated with tri-methylated H3-K9 and tri-methylated H3-K27, into ordered structures rather than from epigenetic spreading (85–87). Thus if the organization of the plasma cell nuclear heterochromatin

can be shown to resemble that of SAHFs and contain E2F target genes, it would support a process of irreversible cell cycle exit amongst such cells. If this were the case, failure to establish such highly ordered nuclear structure would be predicted amongst long-lived plasma cell that retain the potential for cell cycle entry, providing a potential mechanistic distinction between such populations.

GENE EXPRESSION CHANGES ASSOCIATED WITH PLASMABLAST TO PLASMA CELL TRANSITION

One limiting factor in analyzing plasma cell differentiation has been the inability to generate and maintain quiescent mature plasma cells *in vitro*. The resolution of this problem allowed analysis of the gene expression changes associated both with the differentiation and survival of human plasma cells over time (14). Cell cycle control was amongst the largest and most coherently regulated genetic programs. As expected, genes linked to cell cycle progression, mitosis, and cytokinesis were induced in activated B-cells maintained in plasmablasts and silenced in quiescent plasma cells. Although mRNA expression is not necessarily an accurate readout of protein expression and cell cycle control, nonetheless the gene expression changes linked to the transition from proliferating plasmablast to quiescent and long-lived plasma cells provide an indication of the cell cycle state and the potential processes operating to maintain quiescence (**Figure 1**).

MYC and CDKN2C

Consistent with the proposed pattern of regulation from prior studies (52), *MYC* expression was repressed at the plasmablast to plasma cell transition. *CDKN2C* (*p18INK4C*) followed a pattern of expression consistent with its role in murine differentiation being induced initially at the activated B-cell to plasmablast transition and then further induced at the plasmablast to plasma cell transition. Interestingly over the following weeks *CDKN2C* expression appeared to decay slightly amongst *in vitro* plasma cells that maintained cell cycle quiescence, and was expressed at only very low level in bone marrow derived plasma cell populations (data not shown) (14). A similar pattern is observed in data from another analysis of plasma cell associated gene expression (http://amazonia.transcriptome.eu/) (31). The data in human plasma cells would suggest therefore that *CDKN2C* expression is not maintained.

CDKN1A and CDKN1B

Amongst other negative regulators of the G1/S cell cycle checkpoint *CDKN1A* (*p21CIP1/WAF1*) was initially modestly expressed

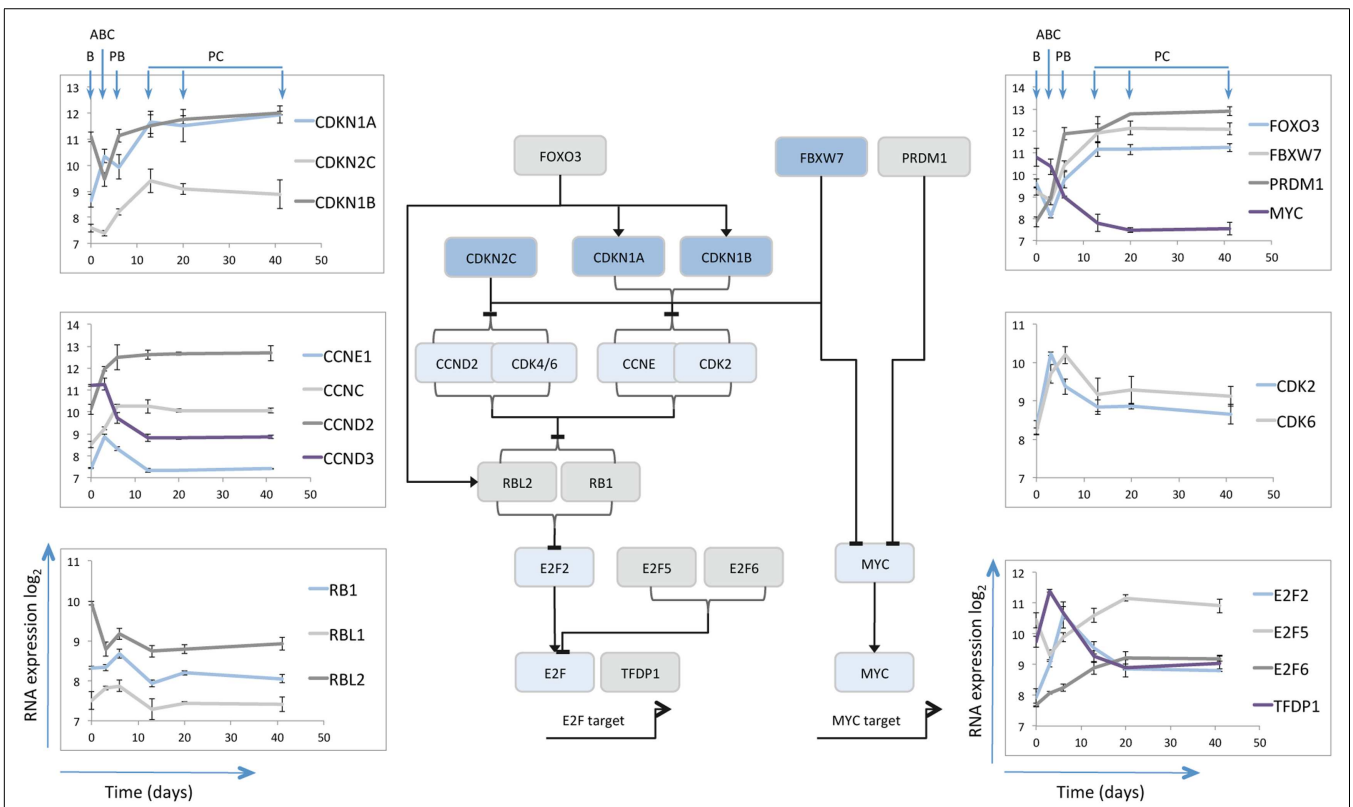

FIGURE 1 | Schematic illustration of selected cell cycle regulators. The central line diagram provides a broad summary of relationships between the selected cell cycle regulators as identified in referenced literature. General positive (arrow heads) and negative (horizontal lines) regulatory interactions are indicated, without specific reference to the nature of the regulatory mechanism. The pattern of mRNA expression of the illustrated cell cycle regulators is shown in the scatter plots as indicated, with data derived from Cocco et al. (14). The *y*-axes show $\log_2$ mRNA expression values, the *x*-axes time in days, the B-cell (B), Activated B-cell (ABC), Plasmablast (PB), and Plasma cell (PC) time points are illustrated for the left and right hand top panels.

in resting B-cells induced in activated B-cells maintained at slightly lower level in plasmablasts and then sustained at high level in plasma cells. *CDKN1B* (*p27KIP1*) was expressed in B-cells, repressed in activated B-cells and induced and then maintained in plasmablasts and plasma cells. These data would therefore support the contention that CDKN1B (p27KIP1) co-operates with CDKN2C (p18INK4C) in contributing to G1/S checkpoint regulation (50), but suggest that in human plasma cells CDKN1A also contributes. Given the gradual loss of *CDKN2C* following differentiation the expression patterns support the argument that CDKN1A and CDKN1B are preferentially involved in restraining cell cycle progression once quiescence is established. Additionally the interaction of these proteins with the differentiation program may extend beyond the control of cell cycle. Thus in oligodendrocyte differentiation CDKN1B promotes cell cycle exit, while CDKN1A is linked to expression of the differentiated cell program in cells which have already exited cell cycle (88).

E2F5 and 6

The expression patterns of *CDKN2C* and *CDKN1A/B* at the plasmablast/plasma cell transition point reflect the central theme of G1/S control in cellular differentiation and senescence programs (49, 89), with common convergence on the control of RB phosphorylation and repression of E2F transcription factors. The E2F family includes both activators and repressors of transcription, which dimerize with transcription factor DP1 (TFDP1) to bind at common consensus sequence (90). *TFDP1* is dynamically regulated showing maximal expression in activated B-cells, but sustained expression at lower levels in plasma cells. The mRNA for activatory family member *E2F2* shows a similar pattern but with maximal expression in plasmablasts, rather than activated B-cells, and is progressively repressed with sustained plasma cell longevity. In contrast the repressive E2F family members *E2F5* and *E2F6* are distinguished by maximal expression in plasma cells. Co-expression of E2F5 and E2F6 in the absence of E2F7 is observed in quiescent rather than senescent cells (91). While E2F7 can provide a link between RB and cellular senescence programs (91), *E2F7* mRNA was not induced upon plasma cell differentiation.

E2F family members, including E2F6, show extensive overlap in binding of DNA elements across the genome (92). However the specific expression of *E2F6* mRNA in plasma cells is of interest since E2F6 has been shown to repress E2F and MYC target genes in G0 in complex with TFDP1 and polycomb group proteins (93). Indeed E2F6 can be found in combination with several distinct functional partners implicated in the regulation of chromatin state such as EZH2, BMI1, and L3MBTL2 (94–96). Each of these genes is expressed at some level in plasma cells, but *BMI1* is notable for showing a progressive increase over time. Thus the induced expression of *E2F6* upon plasmablast to plasma cell differentiation may support the maintenance of the quiescent cell state and has the potential to contribute to epigenetic repression of cell cycle genes (93, 96).

Cyclins

While *Cyclin-E* (*CCNE*) mRNA was induced acutely in activated B-cells and then repressed, *Cyclin-D3* (*CCND3*) decreased rapidly during the differentiation. *Cyclin-D2* (*CCND2*) mRNA by contrast increased rapidly and was maintained at stable levels in quiescent plasma cells that no longer expressed genes associated with active cell proliferation. This is similar to the expression pattern reported in murine plasma cells (50). *CDK6* showed a spike of expression in activated B-cells and plasmablasts and was maintained at low level in mature plasma cells.

The re-entry from quiescent G0 into the G1 phase of the cell cycle can be regulated by a CCNC/CDK3 complex (97). While *CDK3* expression was not detected, CDK2 also acts as a potential alternate CCNC partner (98), and its mRNA was expressed at similar levels in plasma cells as in resting B-cells. CCNC can also act in complex with CDK8 to regulate RNA-polymerase II phosphorylation (99), but *CDK8* mRNA was minimally expressed throughout the differentiation series. In human HSCs CCNC acts in control of quiescence, with overexpression of CCNC linked to reduction and loss of CCNC to enhancement of HSC quiescence (100). It is therefore notable that *CCNC* expression increased on transition from plasmablast to plasma cell state, possibly providing a mechanism along with sustained *CCND2* mRNA expression, to poise plasma cells for cell cycle re-entry.

TP53, RB1, and RBL1/2

TP53, RB1 and their homologs are central to pathways of cell cycle control, and both TP53 and RB dependent pathways co-operate in the robust cell cycle exit of senescence (89). CDKN1A is a core TP53 target and its transcriptional up-regulation could be indicative of a TP53 dependent pathway in plasma cells. At mRNA level *TP53* showed modest induction in activated B-cells, at the peak of proliferative response but was subsequently present at very low level.

RB1 was expressed at modest levels up to the plasmablast stage, but decreased at mRNA level on plasma cell maturation. *RBL1* (p107) was minimally expressed throughout differentiation, while *RBL2* (p130) displayed the most sustained expression levels. Interestingly a functional co-operation between CDKN1B and RBL2 has been identified in hemopoietic cells, and in B-cells combined deficiency of these genes leads to hyper-responsiveness to mitogenic stimuli (101).

FBXW7 and FOXO3

Beyond genes encoding proteins that are direct controllers of cell cycle progression and exit, other genes notably induced at the plasmablast to plasma cell transition include *FBXW7* and *FOXO3* both of which play important roles in the control of HSCs (102–104). FBXW7 is a component of the SCF-ubiquitin ligase complex and is responsible for targeting several cell cycle promoting factors including MYC and CCNE for degradation (105). Thus expression of FBXW7 can provide a mechanism to actively control pro-proliferative stimuli.

The FOXO family of transcription factors, including FOXO3, is linked to control of cell cycle progression, apoptosis, and stress resistance (106). FOXO3 can drive cell cycle exit through transcriptional regulation of CDKN1B (p27) and RBL2 (p130) (107), both of which as noted above are expressed in plasma cells. Additionally FOXO3 can induce CITED2 (108), a transcriptional co-regulator, which in HSCs is implicated in cellular survival pathways (109), and is also induced at the plasmablast to plasma cell transition.

FOXO3 is itself a target of the PI3K pathway via AKT or SGK1 (110, 111). PI3K signaling is necessary for earlier stages of plasma cell differentiation (112), but has not been examined in detail in differentiated plasma cells. If FOXO3 expression is linked to control of CDKN1B and RBL2 in plasma cells then activation of the PI3K/AKT pathway may provide one means of promoting cycle re-entry.

Combined expression pattern

The gene expression changes during human plasmablast to plasma cell transition and subsequent maintenance are consistent with the model that cell cycle exit is established through the combined action of CDKN2C (p18), CDKN1A (p21), and CDKN1B (p27) (50), with the latter two showing more consistent expression once cell cycle exit is established. The sustained expression of *RBL2* offers a potential direct co-operation with *CDKN1B* (101). In parallel expression of *E2F5* and *E2F6* provides additional regulators that can maintain G0 quiescence (90). While *FOXO3A* identifies a potential upstream control pathway for negative cell cycle regulators (106), which is known to be responsive to PI3K/AKT signaling (110), *FBXW7* expression provides the potential for degrading mediators of cell cycle progression (105). In parallel the up-regulation of *CCNC* and sustained expression of a potential CDK partner (*CDK2*) as well as *CCND2* and *CDK6* mRNAs, could comprise a latent trigger for G0/G1 re-entry (97, 98). At a population mRNA level the induction of plasma cell quiescence is therefore associated with expression of several tiers of cell cycle repressive genes, accompanied by retained expression of cell cycle activators. This suggests the potential for regulated cell cycle re-entry.

MONOCLONAL GAMMOPATHY: AN EXPECTED MANIFESTATION OF SELF-RENEWING PLASMA CELL POPULATIONS?

Prior to the development of aggressive myeloma, the majority of patients pass through a clinically detected or retrospectively identifiable period of indolent plasma cell neoplasia (113, 114). At the earliest stages this is characterized by the presence of a paraprotein and very low percentages of phenotypically aberrant plasma cells in the bone marrow, and is known as MGUS (115). The evolution from indolent to aggressive myeloma is thought to occur with both linear and branching patterns (79, 116–118).

In the context of the prevailing paradigm of plasma cell differentiation, the irreversible nature and the requirement for cell cycle exit in order to complete differentiation should oppose malignant transformation of mature plasma cells. This presents a potential conundrum when considering the pathogenesis of plasma cell malignancies, in particular the more indolent precursor states. There are two principle models of plasma cell malignancy: the first is that neoplastic plasma cells acquire sufficient oncogenic deregulation to escape the normal process of cell cycle exit and thus have the potential to clonally expand and progress over time (78, 79); the second is that the phenotypic plasma cell population of the malignancy is supported by a population of clonally related B-cells that act as tumor progenitors to maintain the myeloma plasma cell population (119–123). Given the complexity of human disease, each of these models may pertain in at least some instances. However while B-cell populations related to the malignant plasma cell clone are widely described (124, 125), they are not universally detected (126). Clonotypic B-cells in the peripheral blood have been found to possess early but not late oncogenic events of the myeloma clone (127). Furthermore myeloma cells expressing CD138 maintain the tumor upon transplantation into immunodeficient mice (128); and these results have been extended in recent studies showing that the capacity to establish and sustain the tumor in immunodeficient mice resides in malignant plasma cells, some of which lack CD138 expression, but not in clonotypic B-cells (129–131). Overall therefore there is strong evidence that in many instances of myeloma the disease is maintained in the neoplastic plasma cell compartment.

Given that in established myeloma the disease is maintained amongst plasma cells, earlier indolent plasma cell neoplasms including MGUS are also likely to be maintained by the neoplastic plasma cells themselves. However in this context the fraction of cells in cycle is very low, as are the number of transforming oncogenic events, while the accumulation of oncogenic events associated with cell cycle deregulation occurs later in plasma cell transformation (78, 79, 115, 118).

In MGUS and early myeloma, phenotypic differentiation is not prevented and the majority of clonal plasma cells are quiescent. In the context of a model of irreversible cell cycle exit for normal plasma cells, the initiating oncogenic event in MGUS must therefore be sufficient to establish an abnormal quiescent, but not post-mitotic state, which is not observed in normal plasma cells. A unifying model in plasma cell neoplasia has proposed that a point of convergence for initiating events is aberrant expression of D-type cyclins, and hence the deregulation of the cell cycle (132). The mechanism of deregulation and the cyclin affected differs according to the nature of the underlying molecular abnormality, but the common event is aberrant D-type cyclin expression. Effects on p18INK4C and other negative regulators of D-type cyclins and G1/S phase progression are implicated in mediating the downstream effects of such deregulation in neoplastic plasma cells, but the cells in the early phases of disease are minimally proliferative (78). How D-type cyclin deregulation suffices to establish an aberrant state of quiescent but not irreversible cell cycle exit, while the capacity to fully differentiate is maintained, remains unclear. Direct transcriptional effects of CCND1 on cell cycle gene expression have been recently reported in other lineages and may contribute (133, 134), but have yet to been investigated in plasma cells.

In contrast if transit into the long-lived bone marrow plasma cell population is linked to entry into a relative quiescent state, accompanied by an intrinsic capacity for replicative self-renewal, then the initiating oncogenic event in plasma cell malignancies driving D-type cyclin expression would not be required to deregulate an entire process of irreversible cell cycle exit. Instead it would act on a differentiated and quiescent, but not post-mitotic population to reduce the threshold for cell cycle re-entry. Over time such populations would be expected to expand relative to other long-lived plasma cells leading to gradual dominance of the clone, but given a low overall rate of cell cycle re-entry would exhibit slow clonal progression. Thus a relative quiescence model would fit with the prevailing concept of plasma cell neoplasia, and would provide a simple explanation for why small neoplastic plasma cell

populations arise quite frequently and only progress to myeloma at a low rate.

SUMMARY AND PERSPECTIVES

A REPLICATIVE SELF-RENEWAL MODEL OF LONG-LIVED PC MAINTENANCE

In a replicative self-renewal model long-lived plasma cells reside in the bone marrow niche in a quiescent state, expressing a range of cell cycle regulators, but can be triggered by cellular and immunological cues into undergoing transient episodes of replicative self-renewal at a low frequency (prevalence <1% of plasma cells) (**Figure 2A**). A combination of sibling-rivalry and external competition for limited niche factors would then act to constrain population expansion and link further long-term survival to the re-establishment of quiescence. For any individual antigen-specificity the frequency of plasma cell replicative self-renewal would be low, and occurring randomly would not be predicted to result in global impact on antibody titers at any single time-point. In the context of a plasma cell precursor with an expressed oncogene the threshold for cell cycle re-entry would effectively be reduced. However the plasma cell would remain constrained both by a requirement for physiological cues to trigger cell cycle re-entry, and by a dependence on niche signals for survival (**Figure 2B**). This would be expected to result in only gradual clonal expansion, which over time would manifest as a paraprotein and preferential clonal representation. Additional contributory mechanism, that have not been addressed here, could be envisaged from expressed oncogenes conferring apoptotic resistance leading to preferential survival of both daughter cells despite a normal self-renewal threshold. In either case the impact of the expressed

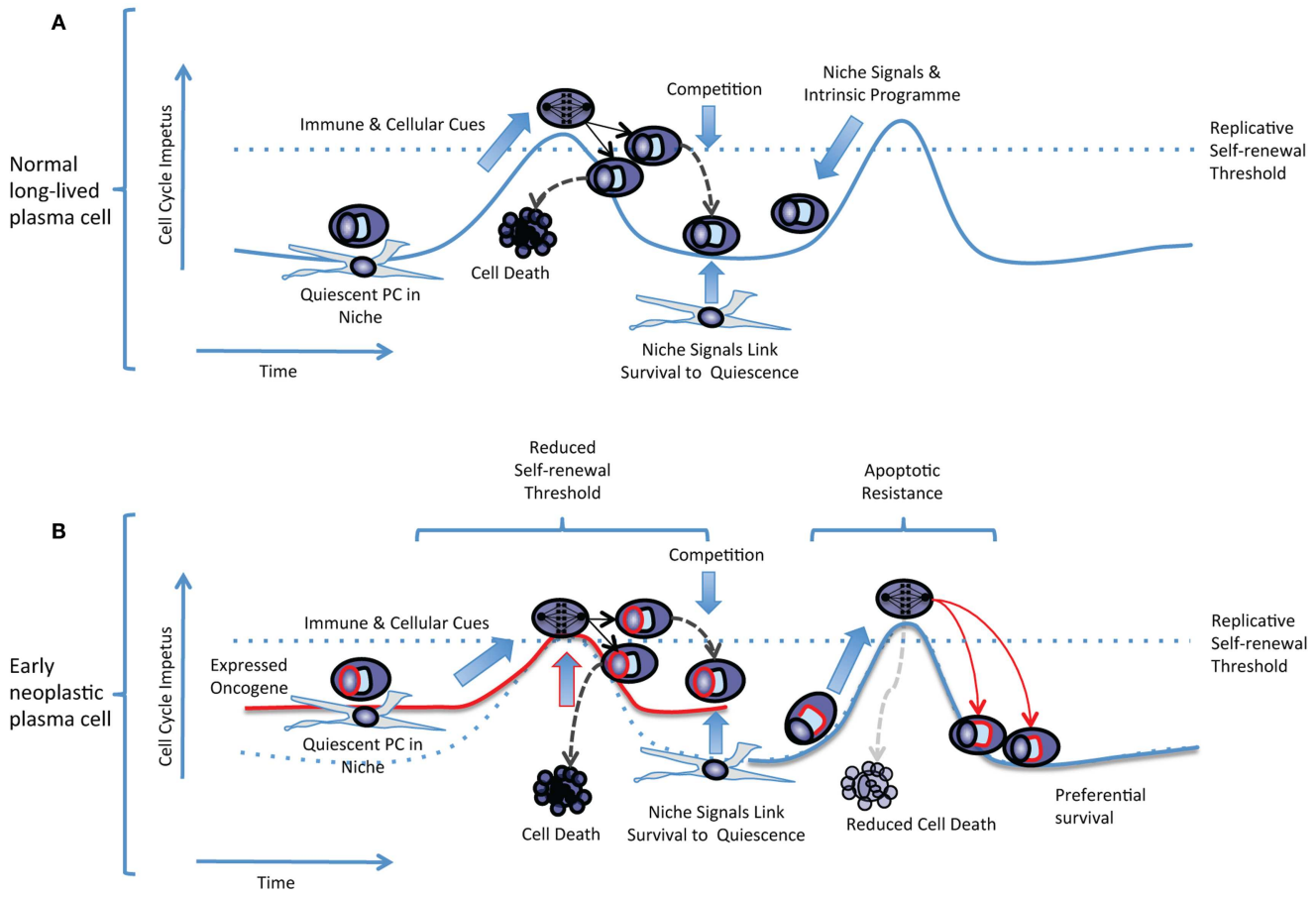

FIGURE 2 | The replicative self-renewal model of long-lived PC maintenance. **(A)** A normal quiescent plasma cell is illustrated residing in a niche environment. The replicative self-renewal threshold is illustrated as a dotted blue line running across a hypothetical curve representing the cell cycle impetus in the quiescent plasma cell. The vertical axis represents the cell cycle impetus and the horizontal axis represents time. Niche signals and the plasma cell intrinsic program combine to establish the quiescence threshold and oppose cell cycle re-entry. Immune and cellular cues intermittently overcome the self-renewal threshold, leading to entry into cell cycle, mitosis, and generation of daughter cells that compete for survival. Successful competition for niche survival signals is linked to the re-establishment of quiescence. Failure to compete for niche occupancy leads to cell death limiting population expansion. **(B)** In an early neoplastic plasma cell expression of an oncogene such as CCND1 (illustrated as a red line surrounding the "nucleus") reduces the effective replicative self-renewal threshold by raising the steady-state level of cell cycle impetus. Dependence on immune and cellular cues still limits cell cycle re-entry, and niche dependence contributes to the re-establishment of quiescence. In an alternate non-mutually exclusive scenario expression of an anti-apoptotic oncogene (illustrated as a red line surrounding the "secretory apparatus") leads to preferential survival of daughter cells but the normal replicative self-renewal threshold for cell cycle entry is retained.

oncogene would reflect a partial deregulation of a normal feature of human plasma cell biology rather than a gross perturbation of the normal differentiation process. Over time such populations would be expected to expand relative to other long-lived plasma cells. However, given a retained dependence on niche signals and capacity for establishing the normal quiescent state, would only gradually manifest as dominant clones and exhibit slow clonal progression, thus helping to explain the population of patients with MGUS who are at low overall risk of disease progression. The transition to progressive disease would be expected to arise from acquisition of additional oncogenic events eliminating the dependence on physiologic cues for replicative self-renewal, delaying re-entry into quiescence, and establishing niche independence.

PREDICTIONS OF THE MODEL

Predictions of the replicative self-renewal model would include that: (i) a population of phenotypically mature plasma cells with markers of cell cycle should be evident at low frequency in normal bone marrow, these should include cells secreting antibodies specific for prior/historic vaccine immune responses, (ii) conditions should be identifiable that promote an exit from quiescent state and re-entry of plasma cells into short bursts of re-proliferation, (iii) niche factors promoting plasma cell survival should support quiescence, and alterations in these factors should impact on cell cycle regulatory machinery as well as survival, (iv) loss of quiescence should be accompanied by cell death if quiescence is not re-established, and (v) expression of myeloma associated oncogenes should allow plasma cell differentiation and cell cycle exit but lower the threshold for cell cycle re-entry allowing enhanced clonal re-proliferation.

PERSPECTIVE

The model of relative quiescence and replicative self-renewal in long-lived plasma cells is presented here as an alternative to the currently prevailing paradigm of irreversible cell cycle exit. The latter has deep roots in plasma cell biology, and it can be argued that no direct evidence against the prevailing paradigm currently exists, but equally in the context of long-lived plasma cells how strong is the evidence in its favor? Self-renewal is an established concept amongst other memory lymphocyte populations (135, 136) and has recently been extended to tissue resident macrophages (20–22). While terminal differentiation is generally seen as a process opposing such a self-renewal capacity, quiescence with the capacity for replicative self-renewal has parallels in other cellular systems with equivalently high functional specialization (15–19). Testing these opposing models will provide a deeper understanding of the nature of cell cycle exit in plasma cells, the intersection of this process with the core plasma cell differentiation program, and the mechanism of early plasma cell neoplasia.

ACKNOWLEDGMENTS

I thank Dr. Gina Doody for discussion, advice, and critical review of the manuscript and Dr. Martin Turner, Dr. Andy Rawstron, and Dr. Gururaj Arumugakani for additional discussion and advice. This work was supported by a CRUK Senior Clinical Fellowship (C7845/A10066).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 October 2013; paper pending published: 26 October 2013; accepted: 02 December 2013; published online: 18 December 2013.
*Citation: Tooze RM (2013) A replicative self-renewal model for long-lived plasma cells: questioning irreversible cell cycle exit. Front. Immunol. **4**:460. doi: 10.3389/fimmu.2013.00460*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 12 March 2014
doi: 10.3389/fimmu.2014.00103

Autophagy in plasma cell pathophysiology

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Plasma cells (PCs) are the effectors responsible for antibody (Ab)-mediated immunity. They differentiate from B lymphocytes through a complete remodeling of their original structure and function. Stress is a constitutive element of PC differentiation. Macroautophagy, conventionally referred to as *autophagy*, is a conserved lysosomal recycling strategy that integrates cellular metabolism and enables adaptation to stress. In metazoa, autophagy plays diverse roles in cell differentiation. Recently, a number of autophagic functions have been recognized in innate and adaptive immunity, including clearance of intracellular pathogens, inflammasome regulation, lymphocyte ontogenesis, and antigen presentation. We identified a previously unrecognized role played by autophagy in PC differentiation and activity. Following B cell activation, autophagy moderates the expression of the transcriptional repressor Blimp-1 and immunoglobulins through a selective negative control exerted on the size of the endoplasmic reticulum and its stress signaling response, including the essential PC transcription factor, XBP-1. This containment of PC differentiation and function, i.e., Ab production, is essential to optimize energy metabolism and viability. As a result, autophagy sustains Ab responses *in vivo*. Moreover, autophagy is an essential intrinsic determinant of long-lived PCs in their as yet poorly understood bone marrow niche. In this essay, we discuss these findings in the context of the established biological functions of autophagy, and their manifold implications for adaptive immunity and PC diseases, *in primis* multiple myeloma.

Keywords: antibody, autophagy, endoplasmic reticulum, multiple myeloma, plasma cell, proteostasis, unfolded protein response, XBP-1

INTRODUCTION

The biology of plasma cell (PC) differentiation is a unique model for scientists to investigate the complex connections between metabolism, stress, proteome plasticity, and cellular renovation. In particular, the regulation of antibody (Ab) production is a valuable paradigm for the molecular wirings controlling protein folding and assembly in professional secretory cells. Moreover, the bone marrow PC niche, which provides lifelong Ab memory, depends on as yet incompletely understood intrinsic and environmental components, whose derangement is instrumental to the development of multiple myeloma.

We recently discovered an unanticipated essential function played by autophagy during PC differentiation, disclosing new links with endoplasmic reticulum (ER) homeostasis and Ab production. Moreover, autophagy emerges as an intrinsic requirement of long-lived PCs and long-term immunity (1). Scope of the present review is to discuss the newly identified role of autophagy in PC pathophysiology, in perspective of recently established autophagic functions across stress biology, cell differentiation, immunity, and cancer.

Abbreviations: Ab, antibody; Ag, antigen; EM, electron microscopy; ER, endoplasmic reticulum; ERAD, ER associated degradation; Ig, immunoglobulin; PC, plasma cell; SILAC, stable isotope labeling in cell culture; SLR, SQSTM1-like receptor; TLR, Toll-like receptor; UPR, unfolded protein response

THE STRESS OF PLASMA CELL DIFFERENTIATION

Plasma cells are the terminal effectors of adaptive immunity endowed with the unique ability to secrete Abs capable of neutralizing pathogens and toxins. Upon encounter with antigens (Ags), B cells differentiate into short-lived PCs in secondary lymphoid organs (e.g., spleen and lymph nodes). Most of these effector cells die within few days. In addition, T cell-dependent responses induce the germinal center reaction, which generates a second wave of plasmablasts, secreting high-affinity, class-switched Abs, and capable of acquiring lifelong survival in dedicated bone marrow niches. Long-lived PCs maintain immunological memory of Ab-inducing Ags, yielding prompt protection against pathogens and their toxic products (2).

From a biological standpoint, PCs are professional secretory cells dedicated for massive synthesis, assembly, and secretion of Abs. To accomplish this mission, upon activation, B cells must reshape their proteome. To this aim, a powerful genetic program silences B cell identity, through the repression of genes encoding the transcription factors Pax5 and Bcl-6, and establishes PC function, inducing the transcriptional regulators IRF4 and PRDM1/Blimp-1 (3). Early during differentiation, XBP-1, a key ER stress transducer and transcription factor of the unfolded protein response (UPR), drives ER expansion to augment the folding capacity of this organelle and accommodate intensive immunoglobulin (Ig) synthesis in the secretory pathway (4, 5).

In addition to ER stress, other stresses are constitutive of full gear Ab production in PCs. For example, oxidative protein folding causes redox stress, counterbalanced by antioxidant responses (6, 7). After the blastic phase, in post-mitotic PCs, additional stress may ensue from the impossibility of diluting damaged organelles through cell division, as demonstrated in other non-dividing terminally differentiated cells (8). We found that PCs also experience profound *proteasome stress* (9). Indeed, although Ig-synthetic activity requires intense proteasome-dependent degradation of Ab byproducts, during their differentiation short-lived PCs display a progressive, remarkable reduction of proteasome expression, which leads to accumulation of poly-ubiquitinated proteins, at the expense of free ubiquitin – an additional stress referred to as *ubiquitin stress* (10) – and stabilization of proapoptotic factors (11, 12). This apparently paradoxical lack of adaptation may serve as a built-in mechanism to reduce the apoptotic threshold and limit PC lifespan and the duration of Ab responses (13). We also noted that PC differentiation confers exquisite sensitivity to proteasome inhibition, rendering PCs as sensitive to proteasome inhibitors as multiple myeloma cells, disclosing a general characteristic of PCs, rather than a feature of malignancy (9). Attenuating general protein synthesis by the otherwise toxic agent cycloheximide reduces proteasome sensitivity in differentiating plasmablasts, indicating protein synthesis as a key determinant of the proteolytic burden on proteasomes in PCs (14). Such a challenged protein homeostasis (*proteostasis*) may explain why the first-in-class proteasome inhibitor bortezomib reduced Ab responses (12) and attenuated autoAb-mediated pathology in a mouse model of lupus (15). Clearly, basic stress biology in PCs is instructive on putative targets against PC dyscrasias (see below).

AUTOPHAGY: FROM BULK DEGRADATION TO SELECTIVE RECYCLING

Autophagy is a highly conserved self-digestive strategy that envelops cytoplasmic contents in a double-membrane vesicle, the *autophagosome*, delivered to the lysosome (in animal cells) or to the vacuole (in plant and yeast cells) for subsequent degradation and recycling. The prime function of autophagy in unicellular organisms is to sustain cellular metabolism in conditions of nutritional starvation (16, 17). This metabolic role is conserved in metazoa, where autophagy is an essential source of energetic equivalents (18). An exemplar case, autophagy-incompetent newborn mice fail to resist the physiologic early neonatal starvation (19). Autophagy also provides building blocks for cellular renovation, and is crucially involved in differentiation and development (18, 20). In mammals, autophagy is essential for embryogenesis (21) and lineage differentiation, as demonstrated, for example, in adipocytes, erythrocytes, and lymphocytes (20).

By contrast with the ubiquitin–proteasome system, autophagy has long been viewed as a bulk non-selective process, with the only exception of chaperone-mediated autophagy. Its recently recognized capacity to ensure cellular quality control by clearing toxic and damaged macromolecules and organelles disclosed an unanticipated level of selectivity (22, 23). Selective autophagic degradation has been reported for a number of endogenous supramolecular structures: peroxisomes (*pexophagy*) (24), protein aggregates (*aggrephagy*) (25–27), ribosomes (*ribophagy*) (28), mitochondria (*mitophagy*) (29–31), lipid droplets (*lipophagy*) (32), secretory granules (*zymophagy*) (33), and midbody remnants after cytodieresis (34).

To target selected cargoes, autophagy makes use of adapter proteins acting as receptors. To mediate selective autophagy, these proteins must: (i) recognize substrates via a ubiquitin-binding activity; (ii) cross-link the cargo with the autophagic machinery via an LC3-interacting region; and (iii) polymerize (23, 35). Ubiquitination is thus used not only to convey individual proteins to the proteasome, but also for selective recognition by autophagic receptors, e.g., p62 and NBR1 during mitophagy and aggrephagy. While the prime tag for proteasomal degradation is a chain of ubiquitins covalently linked through their K48 lysine residues, K63-linked poly-ubiquitin tags may be preferentially associated with autophagic degradation, although additional post-translational modifications may contribute to direct cargoes to autophagy (23). In most cases, the ubiquitin ligases involved in autophagy remain to be identified. Hitherto established mammalian autophagic receptors include SQSTM1/p62, NBR1, optineurin, NDP52, and Nix. Adaptor proteins are also being characterized, which interact with autophagy receptors to recruit and assemble more Atg proteins, so as to shape the growing autophagosome (*phagophore*) around the cargo (36).

Virtually, all cellular membranes have been proposed to contribute to autophagosome biogenesis. Among them, the ER is an established membrane source for the phagophore (37). The ER may also undergo autophagic degradation: autophagic trimming of excess ER (*reticulophagy*, *ER-phagy*) counterbalances pharmacological stress-induced ER expansion in yeast (38). More recently, a mechanism mediating both mitophagy and ER-phagy has been described in HeLa cells (39). However, defining the physiological significance of reticulophagy in mammals is biologically relevant. As described below, our recent work on autophagy in PCs furthers this view by defining ER-phagy as an essential determinant of PC biology and Ab immunity (1).

ROLES OF AUTOPHAGY IN INNATE AND ADAPTIVE IMMUNITY

In the immune system, autophagy serves diverse innate and adaptive functions, including microbe clearance, Ag presentation, and the regulation of inflammation and lymphocyte development (40–42). The co-optation of autophagy to destroy intracellular microbes, i.e., *xenophagy*, already present in unicellular organisms (43), likely represents the most ancient form of immune defense. Xenophagy has been shown to restrict the growth of bacteria (*L. monocytogenes*, *S. flexnerii*, *S. typhimurium*) (42). The infectious phagosome is intracellularly recognized through internal toll-like receptor (TLR) signaling (44). Then, infected cells can promote phagosome–lysosome fusion or target cytosol-invading bacteria for autophagic degradation (36). A number of autophagic receptors, including SQSTM1/p62, NDP52, and optineurin, have been shown to specifically recognize ubiquitinated bacteria within the cytosol (23, 36), hence the idea that SQSTM1-like receptors (SLRs) constitute a new family of innate pattern recognizing receptors (45). Autophagy may play additional antimicrobial activities through SLRs, e.g., by generating microbicidal peptides via incomplete digestion of ribosomal protein precursors

during *M. tuberculosis* infection (46). Autophagy also mediates viral recognition and destruction. For example, capsid proteins of the neurotropic Sindbis virus are degraded via p62-dependent autophagy (47).

Autophagy is also involved in the modulation of the inflammatory response. In particular, autophagy may both stimulate and inhibit the activity and output of the inflammasome. While basal autophagy prevents inflammation, e.g., by limiting mitochondrial generation of reactive oxygen species and the resulting inflammasome activation (48, 49), induction of autophagy can promote inflammation, mediating the inflammasome-dependent unconventional release of the *endogenous pyrogen*, IL-1β, which in turn can intensify autophagy (50). Autophagy may also yield negative feedback loops to prevent destructive inflammation, e.g., moderating IL-1β release by targeting inflammasomes and pro-IL-1β for degradation (51, 52).

Autophagy also serves adaptive immune functions, including the regulation of lymphocyte ontogenesis and homeostasis. Atg proteins have been shown to maintain normal numbers of $CD4^+$ and $CD8^+$ T cells, and fetal hematopoietic stem cells (41, 53, 54). First, $Atg5^{-/-}$ bone marrow chimeric mice revealed defects in T cell development and peripheral homeostasis, and impaired activation-induced proliferation (55). Although activated T cells require autophagy, negative controls may come into play to temper autophagy, preventing detrimental effects. Indeed, components of the extrinsic apoptotic cascade, namely FADD and caspase 8, were found to limit autophagy by interacting with the Atg5–Atg12 complex, thereby sustaining viability of activated T cells (56). In following studies, the development of mature T cells was found to require an active negative control on the intracellular production of reactive oxygen species, which in turn relies on efficient mitophagy (55, 57). Such mitochondrial quality control maintains mature naïve T cell homeostasis through Beclin-1 stabilization by the class III phosphoinositide-3 kinase Vps34 (58, 59). Moreover, in activated T cells, autophagy is induced to maintain ATP levels, proliferation, and the release of cytokines (60).

Autophagy is also important for B cell development: irradiated $Rag1^{-/-}$ recipients repopulated with fetal liver progenitors lacking the essential autophagic factor Atg5 have low counts of peritoneal B-1 B cells, due to defective transition of pro- to pre-B cells (61). Moreover, mice with conditional deletion of *Atg5* in mature B cells ($Atg5^{f/f}$CD19-Cre) show normal numbers of mature B lymphocytes and a normal ratio of marginal zone to follicular B cells, but reduced maintenance of B-1a cells in the periphery (1, 61).

A number of studies have implicated autophagy in different Ag presentation pathways. The delivery of exogenous Ags for MHC class II presentation to $CD4^+$ T cells has been shown to depend on autophagy (62). Indeed, MHC class II-loading compartments receive continuous input from autophagosomes, and autophagy has been shown to positively control $CD4^+$ T cell priming (63–65). Moreover, thymic epithelial cells deliver self Ags to MHC class II-loading compartments through the autophagic machinery. This task is essential to build self-tolerance, as its disruption leads to defective elimination of autoreactive T cells and autoimmunity (66). Furthermore, autophagy has been shown to mediate $CD8^+$ T cell priming *in vivo* through cross-presentation of phagocytosed Ags, normally routed through the MHC class II pathway, on MHC class I (67, 68). However, autophagy is not a universal Ag-presenting pathway, as we proved it dispensable for presentation by B cells to cognate T cells in the germinal center (see below) (1).

We hypothesized that autophagy may play an additional adaptive immune function in terminal PC differentiation, based on the specific biology of Ab-secreting cells (9). First, PC differentiation is expected to require a high degree of proteome plasticity. In support of this notion, we had generated quantitative evidence that both protein translation and degradation increase remarkably in primary activated B cells (14). Second, we had observed that such an increased demand for protein degradation is not met by a corresponding increase in proteasome capacity, which instead decreases dramatically (11, 12, 14), and reasoned that this would call for complementary protein degradation routes. Having the capacity to compensate for proteasome insufficiency (69), autophagy was an obvious candidate. Third, most, if not all, stresses experienced by PCs are known to be relieved by autophagy (9, 22). The following paragraphs illustrate our findings, unveiling the crucial role served by autophagy in the differentiation, function, and viability of PCs, required for humoral immunity, and the underlying mechanism, linking ER homeostasis with Ig synthesis and energy metabolism.

AUTOPHAGY SUSTAINS Ab RESPONSES AND IS ESSENTIAL TO LONG-LIVED PCs

When we assessed overall autophagic activity in differentiating PCs, we found strong induction of autophagy following B cell activation, both *ex vivo* and *in vivo*. In keeping with a developmental program, similar to UPR transcripts, Atg mRNAs increased concertedly during PC differentiation. The use of GFP-LC3 transgenic mice revealed intense autophagy also in long-lived bone marrow PCs (1). Encouraged by these observations, to assess the functional relevance of autophagy in PC ontogenesis, we first investigated Ab responses in $Atg5^{f/f}$CD19-Cre mice. These mice showed reduced IgM and IgG responses in both T-independent and T-dependent immunization experiments, demonstrating a positive role of autophagy in Ab responses mediated by short-lived PCs. A parallel independent study confirmed these findings, by showing significantly diminished Ab titers in the same mouse model during Ag-specific immunization, parasitic infection, and mucosal inflammation (70).

Inspired by the observation of high autophagic activity also in long-lived PCs, we then asked whether Atg5 is required for long-term humoral immunity, by assessing if $Atg5^{f/f}$CD19-Cre mice show defects in bone marrow PC populations. These mice had normal bone marrow PC counts, apparently arguing against a role for autophagy in long-lived PCs. However, the genomic quantification of Cre-mediated deletion of *Atg5* disclosed that while in splenic B cells most *Atg5* alleles had undergone Cre-dependent recombination, bone marrow PCs displayed normal amounts of the non-deleted allele. Hence, an efficient Darwinian selection for autophagy-competent PCs had occurred, demonstrating that autophagy is absolutely required to establish or maintain long-lived PCs. Moreover, despite a normal size of the bone marrow PC pool, $Atg5^{f/f}$CD19-Cre mice revealed a defect in long-term Ab immunity, as they had virtually absent Ag-specific long-lived PCs

in the bone marrow 11 months after T-dependent immunization. Altogether, the data establish autophagy as a novel determinant of the PC memory compartment (1).

Being essential to generate class-switched and high-affinity memory B cells and long-lived PCs, we also checked the germinal center reaction in NP-CGG-immunized *Atg5*$^{f/f}$CD19-Cre mice, but found it normal (1). This evidence mapped the requirement of autophagy specifically to PCs. Moreover, since the germinal center response requires Ag presentation by B cells to cognate T cells, this data demonstrated that autophagy is dispensable for soluble Ag presentation in B cells, in spite of its Ag-presenting role in other contexts (discussed above).

The above findings further our understanding of the intrinsic molecular competence required for PCs to achieve extended survival in the bone marrow (2). Hitherto recognized components of such competence comprise the chromatin modifier Aiolos (71), the transcriptional regulators Blimp-1 (72) and XBP-1 (73), and the anti-apoptotic molecule Mcl-1 (74). The identification of autophagy as a novel molecular requirement of bone marrow PCs and of long-lived humoral immunity is in keeping with its established capacity to grant extended survival to quiescent progenitors and highly specialized terminally differentiated cell types, such as neurons (75).

An important matter of future investigation is the precise level at which autophagy is required in memory PC ontogenesis, i.e., the survival of non-resident long-lived plasmablasts, their migration to the bone marrow, or the maintenance of resident long-lived PCs in the medullary niche. Moreover, it would be interesting to determine if autophagy also plays a role in maintaining the other memory compartment of B cell immunity, i.e., non-Ig-secreting memory B cells.

AUTOPHAGY CONTAINS PC DIFFERENTIATION AND Ab PRODUCTION THROUGH SELECTIVE ER-PHAGY

The easiest conceivable explanation for autophagy sustaining Ab immunity was to hypothesize it to be required for PC differentiation. Confuting this hypothesis, Atg5-deficient B cells apparently underwent normal PC differentiation (1). The exact molecular role of autophagy in developing PCs was gaged by an unbiased comparison of the proteome of autophagy-competent vs. incompetent activated B cells by stable isotope labeling in cell culture (SILAC). Importantly, the proteome of differentiating PCs was completely labeled in as little as 3 days upon activation, not only enabling this approach, but also convincingly demonstrating the highest proteome plasticity inherent to this differentiation program. SILAC proteomics of Atg5$^{-/-}$ PCs revealed a selective and rather exclusive expansion of the ER proteome, including Igs. An autophagic regulation of the size of the ER was demonstrated by two independent electron microscopy (EM) approaches: classical EM and an EM cytochemistry technique designed to stain and unbiasedly quantify the ER (76). Short treatment with distal autophagy inhibitors was sufficient to increase ER proteins in wild type differentiating PCs, unveiling the first case of physiologic reticulophagy in mammals (1).

Attesting to the functional relevance of the identified autophagic regulation of the ER in PC differentiation, Atg5$^{-/-}$ PCs had higher UPR signaling than wild type PCs, associated with higher expression of Blimp-1 and Ig transcripts, indicating that autophagy restricts the expression of two key determinants of PC differentiation, XBP-1 and Blimp-1, and of Igs. Providing mechanistic insight, pharmacological ER stress in wild type differentiating PCs was sufficient to increase Blimp-1 and Ig expression beyond the putatively maximal levels associated to PC differentiation. As a result, Atg5$^{-/-}$ PCs translated, assembled, and secreted more Abs over time, disclosing an unsuspected regulatory circuit of PC function negatively controlled by autophagy (1, 75) (see **Figure 1**). It will be important to dissect the mechanisms underlying ER-phagy during PC differentiation.

In cellular models of protein folding diseases, autophagy has been shown to dispose of polymeric misfolded protein aggregates in the ER, constituting an alternative form of ER associated degradation (ERAD) (77, 78). Noticeably, instead, in differentiating PCs, autophagy does not serve a similar quality control function in the secretory pathway, nor does it remove dysfunctional ER, as

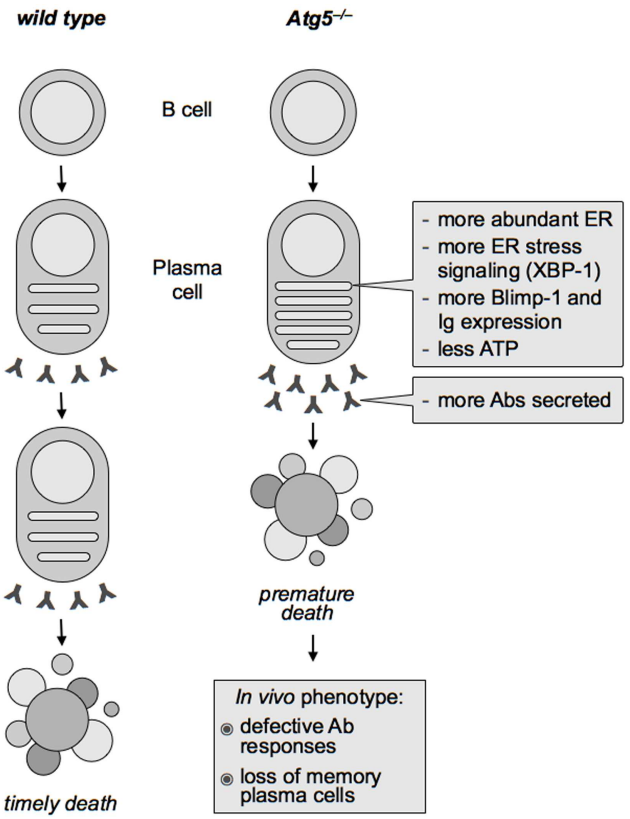

FIGURE 1 | Effect of Atg5 deficiency on plasma cell differentiation and Ab immunity. In differentiating plasma cells, autophagy limits the expansion of the endoplasmic reticulum (ER), providing the first physiological case of mammalian ER-phagy. As a result, Atg5$^{-/-}$ plasma cells show more abundant ER than wild type counterparts and more intense ER stress signaling (including higher expression of the essential plasma cell transcription factor, XBP-1), which in turn enhances the expression of the key plasma cell transcriptional regulator PRDM1/Blimp-1 and of immunoglobulins (Ig). Hence, despite increased Ig production, autophagy-deficient plasma cells have less ATP and live a shorter life, yielding defective Ab responses *in vivo*.

Atg5$^{-/-}$ PCs show normal Ig assembly, and do not accumulate Ig aggregates, but rather display higher capacity in their expanded secretory apparatus (1).

These findings imply that PCs are programed to become more productive Ab factories than actually observed, with autophagy acting as a physiologic brake on their differentiation and function. How is this reconciled with the defective Ab responses mounted by *Atg5*$^{f/f}$CD19-Cre mice? This paradox is solved by the observation that Atg5$^{-/-}$ PCs have less ATP and live a shorter life than wild type counterparts. Hence, autophagy accomplishes a sensible trade-off between viability and function, setting Ab production to sustainable levels (1, 75).

Can the higher Ig-secreting potential of PCs be demonstrated *in vivo*? A proof-of-principle experiment was the immunization with the T-independent hapten NP-ficoll, which, in our hands, yielded higher anti-NP Ig titers in *Atg5*$^{f/f}$CD19-Cre mice, despite normal PC counts, in line with the higher secretory activity of Atg5$^{-/-}$ PCs observed *ex vivo*. Unlike other Ags, NP-ficoll has been shown to persist and cause continual B cell activation (79). Hence, repeated rounds of PC differentiation may have surpassed the otherwise dominant impact of reduced PC viability on Ab titers, witnessing the hypersecretory effect of Atg5 deficiency at the single PC level (1, 75).

The discovery of a novel autophagy-centered regulatory network balancing PC activity and survival *in vivo* implies an unsuspected plasticity of Ab responses, potentially exploitable to tune their duration and intensity. A number of immune signaling molecules can regulate autophagy (41, 45), supporting this possibility, and offering opportunities to search for molecular targets to modulate Ab responses, of therapeutic use against autoimmune diseases.

AUTOPHAGY IN MULTIPLE MYELOMA

A matter of intense scientific debate, the role of autophagy in cancer is complex. Genetic defects of autophagy have been linked with tumorigenesis, establishing the notion that autophagy is a tumor suppressive pathway (80). Oncosuppressive mechanisms of autophagy include protection against the accumulation of oncogenic mutations (81–83) and reactive oxygen species, mainly through mitochondrial homeostasis, and reduction of necrosis and local inflammation (84). While in healthy cells, autophagy may suppress tumor initiation, established cancers may subvert autophagy to cope with intrinsic (e.g., metabolic), environmental (e.g., hypoxic), or pharmacological stress (e.g., induced by cytotoxic agents). This may explain why pharmacological inhibition of autophagy may be beneficial against cancer, being toxic to tumor cells and sensitizing them to chemotherapy (80, 85).

Multiple myeloma is a valuable model to investigate the role of autophagy in cancer, especially in perspective of integrated cancer proteostasis. Indeed, myeloma represents the paradigmatic neoplasm responsive to proteasome inhibitors, prototypical negative proteostasis regulators, although a substantial proportion of patients fail to respond, and resistance inevitably ensues (9, 86–88). We demonstrated that the exquisite proteasome sensitivity of normal and malignant PCs stems from an unfavorable ratio between proteasome workload and overall capacity (9, 11, 12), and termed this feature *proteostenosis* (13). Moreover, we found that myelomas with the highest sensitivity to proteasome inhibition are those expressing fewer active proteasomes in spite of the highest degradative workload, both features being causal to such inherent vulnerability (89). Noticeably, the degradative burden is a relatively neglected source of cellular stress, particularly in cancer (90). In myeloma, we found that recent protein synthesis saturates the limited capacity of the ubiquitin–proteasome system, causing the buildup of ubiquitin conjugates, and is a crucial determinant of proteasome stress (14). It is noteworthy that *lymphoplasmocytic lymphoma* (*Waldenstrom's macroglobulinemia*), another B cell cancer known to produce high levels of IgM, also proved vulnerable to proteasome inhibition (91–93), and the clinical use of bortezomib yielded encouraging results (94, 95).

Autophagy and the ubiquitin–proteasome system are integrated strategies that cooperate to maintain cellular proteostasis (69). This notion is sufficient to predict that multiple myeloma and *Waldenstrom's macroglobulinemia* may be as dependent on autophagy as they are on the ubiquitin–proteasome system. This prompted different laboratories to target autophagy in order to overcome resistance to proteasome inhibitors and achieve myeloma cell death, with controversial results. While the blockade of autophagy appears toxic against human myeloma lines, the combined inhibition of the proteasome and autophagy may be synergic or antagonistic, depending on the molecular level of autophagic inhibition (88, 96–98). Experimental discrepancies may be partly explained by the recent discovery of a vital circuit blocking autophagy, disclosing that deregulated autophagy can turn maladaptive in multiple myeloma cells. In brief, human myeloma lines depend for their survival on IRF4, which, through a caspase 10-dependent mechanism, prevents excessive autophagy from executing non-apoptotic myeloma cell death (99). This data suggests that autophagy may play both adaptive and maladaptive roles in myeloma, depending on its intensity, or its targets. A better understanding of the basal, adaptive function of autophagy in myeloma cells is needed to harness this pathway against cancer. Our identification of a new homeostatic function of autophagy, essential for the maintenance of long-lived PCs in the bone marrow, the normal counterpart of multiple myeloma (1, 75), prompts to test if PC tumors are at least as dependent on autophagy for their survival, and provides a framework for dissecting the precise function of autophagy in normal and malignant bone marrow PCs. The diverse homeostatic activities of autophagy in different lineages and diseases hitherto defined suggests that PC-specific, and possibly myeloma-specific, autophagic circuits may be identified, linking organelle homeostasis, stress responses, and energy metabolism, to disclose new molecular therapeutic targets.

ACKNOWLEDGMENTS

We thank Federica Loro and all our lab members for creative discussions. We are particularly grateful to Holger Auner, Paolo Cascio, Stefano Casola, Marco Colonna, Paolo Dellabona, Luigi Maiuri, Giampaolo Merlini, Anna Mondino, Andrea Orsi, Giovanni Palladini, Niccolò Pengo, Fulvio Reggiori, Eelco van Anken, Skip Virgin, and Kathy Weilbaecher. Work in the Cenci Lab was supported by the Multiple Myeloma Research Foundation (Senior Research Award 2010), the Italian Ministry of Health (Giovani Ricercatori 1143560), and the Italian Association for Cancer

Research (AIRC, Investigator Grant 14691 and Special Program Molecular Clinical Oncology 5 per mille n. 9965).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 January 2014; accepted: 26 February 2014; published online: 12 March 2014.
*Citation: Oliva L and Cenci S (2014) Autophagy in plasma cell pathophysiology. Front. Immunol. **5**:103. doi: 10.3389/fimmu.2014.00103*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 18 February 2014
doi: 10.3389/fimmu.2014.00065

Cytokine-mediated regulation of plasma cell generation: IL-21 takes center stage

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During our life, we are surrounded by continuous threats from a diverse range of invading pathogens. Our immune system has evolved multiple mechanisms to efficiently deal with these threats so as to prevent them from causing disease. Terminal differentiation of mature B cells into plasma cells (PC) – the antibody (Ab) secreting cells of the immune system – is critical for the generation of protective and long-lived humoral immune responses. Indeed, efficient production of antigen (Ag)-specific Ab by activated B cells underlies the success of most currently available vaccines. The mature B-cell pool is composed of several subsets, distinguished from one according to size, surface marker expression, location, and Ag exposure, and they all have the capacity to differentiate into PCs. For a B-cell to acquire the capacity to produce Abs, it must undergo an extensive differentiation process driven by changes in gene expression. Two broad categories of Ags exist that cause B-cell activation and differentiation: T cell dependent (TD) or T cell independent (TI). In addition to the B-cell subset and nature of the Ag, it is important to consider the cytokine environment that can also influence how B-cell differentiation is achieved. Thus, while many cytokines can induce Ab-secretion by B cells after activation with mimics of TD and TI stimuli *in vitro*, they can have different efficacies and specificities, and can often preferentially induce production of one particular Ig isotype over another. Here, we will provide an overview of *in vitro* studies (mouse and human origin) that evaluated the role of different cytokines in inducing the differentiation of distinct B-cell subsets to the PC lineage. We will place particular emphasis on IL-21, which has emerged as the most potent inducer of terminal B-cell differentiation in humans. We will also focus on the role of IL-21 and defects in B-cell function and how these contribute to human immunopathologies such as primary immunodeficiencies and B-cell mediated autoimmune conditions.

Keywords: human B cells, differentiation, plasma cells, cytokines, IL-21, immunodeficiency, autoimmune diseases

INTRODUCTION

The humoral arm of the immune system is critical for providing protective antibodies (Abs) against infection pathogens. The Ab pool is maintained by long-lived plasma cells (PCs), which continuously secrete Abs following their formation in response to exposure to specific antigen (Ag). In 1948, Fagraeus was the first to report that PCs are the outcome of terminal B-cell differentiation and demonstrated their importance to Ab production *in vitro* (1). We now know that B cells are capable of secreting multiple Ig isotypes (IgM, IgG, IgA, IgE) and subclasses of these isotypes (IgG_{1-4}, IgA_{1-2}) following the receipt of appropriate stimulate. However, today – 65 years later – our understanding of the complexities of PC development remains incomplete.

PLASMA CELL FORMATION: THE IMPORTANCE OF T CELLS, CYTOKINES, AND TRANSCRIPTION FACTORS

Plasma cells are generated as a result of cognate interactions between Ag-specific B cells, $CD4^+$ T helper cells, and dendritic cells in response to foreign Ags (**Figure 1**). These interactions can drive B cells to become low-affinity short-lived, predominantly IgM-secreting, plasmablasts that provide an initial wave of protection against invading pathogens. More importantly though, they also lead to the formation of germinal centers (GCs), which are specialized structures in the follicles of secondary lymphoid tissues where somatic hypermutation (SHM) of immunoglobulin (Ig) variable region genes and selection of high-affinity B cells occurs. These selected high-affinity variants can then differentiate into long-lived memory B cells or PCs (2, 3) (**Figure 1**). This differentiation event is in part mediated by T follicular helper (Tfh) cells, a distinct subset of $CD4^+$ T cells characterized by expression of the transcriptional repressor B-cell lymphoma-6 (Bcl-6), the surface markers CXCR5, PD-1, ICOS, and CD40 ligand (CD40L), and production of various cytokines including interleukin-4 (IL-4), IL-10, and IL-21. Tfh cells localize to follicles and GCs – where they are termed "GC Tfh cells" – where they can interact with B cells and instruct their maturation into memory cells or PCs (4–6).

The differentiation of activated B cells into PC is regulated by transcriptional programs and networks that are influenced by numerous inputs and microenvironmental factors. These include the nature of the Ag and of the responding B-cell subset, the location in which Ag encounter occurs, and the accessory cells involved (7, 8). The key transcription factors involved in regulating

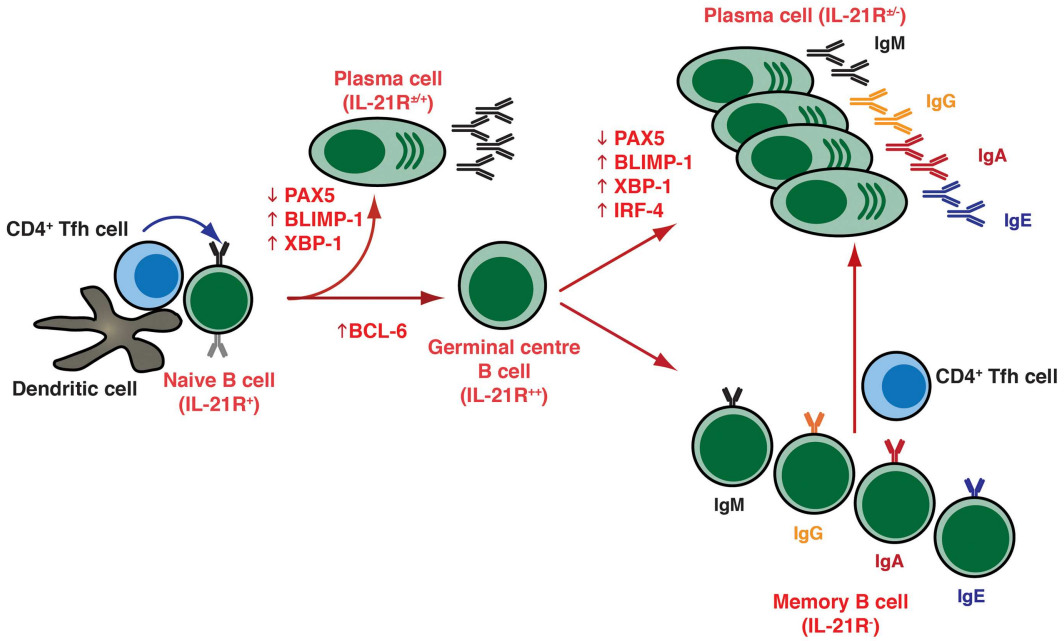

FIGURE 1 | T cell dependent B-cell differentiation. Following the receipt of signals provided by the microenvironment [e.g., Ag, $CD4^+$ T (Tfh) cells, DC], naïve B cells undergo activation and can initially differentiate into either extrafollicular short-lived Ab-secreting plasma cells (secreting predominantly IgM), or can seed a germinal center (GC). Within GCs, B cells undergo somatic hypermutation of their Ig V region genes and only those B cells with the highest affinity are selected to then differentiate into long-lived memory B cells or plasma cells that are capable of secreting a variety of Ig isotypes, including the switched isotypes IgG, IgA, and IgE. The outcome of the GC reaction is heavily influenced by Tfh cells, especially those within the GC itself. These cells are not depicted on the figure but they contribute greatly at this stage of B-cell differentiation. Following re-encounter with the initiating Ag, memory B cells rapidly differentiate into plasma cells. The differentiation of naïve B cells to these distinct effector fates is controlled by the balanced expression and regulated function of various transcription factors, including (but not exclusively) PAX5, BCL-6, BLIMP-1, XBP-1, and IRF4.

PC formation include the transcriptional repressors Bcl-6 and B-lymphocyte induced maturation protein (BLIMP)-1, encoded by the PRDM1 gene, as well as transcription factors PAX5, X-box-binding protein-1 (XBP-1), and IFN-induced regulatory factor 4 (IRF4) (**Figure 1**) (7, 8). Thus, while Bcl-6 is expressed in GC B cells and is required for the GC formation (9–11), it blocks PC differentiation and maintains a GC B-cell fate by suppressing expression of BLIMP-1, which is considered the master regulator of PC differentiation, being required for – or at least correlated with – PC commitment in mice and humans (**Figure 1**) (12–15). BLIMP-1 expression controls PC differentiation by restraining the mature B-cell gene expression program by down-regulating a set of genes including MHC, CIITA, PAX5, and CMYC, which result in a decrease of MHC class II expression, loss of B-cell identity, and cessation of proliferation, respectively (8, 14). BLIMP-1 may also co-ordinate expression of XBP 1, which allows expansion of the secretory apparatus necessary for high-level protein synthesis in PC differentiation (**Figure 1**) (16).

Cytokines represent a diverse group of small soluble proteins that can function as growth and differentiation factors in autocrine or paracrine ways. Cytokines exhibit considerable redundancy, in that many cytokines share similar functions. Through binding to specific cell surface receptors, they initiate signal transduction pathways that are critical for a diverse spectrum of functions, including induction of immune responses, cell proliferation, differentiation, and apoptosis. The key contribution of cytokines to B-cell differentiation lies in their ability to modulate expression of these transcription factors such that they regulate Ig secretion by B cells activated with mimics of T cell dependent (TD) (e.g., CD40L) or T cell independent (TI) [e.g., engaging the B-cell receptor (BCR), Toll-like receptors (TLRs)] stimuli *in vitro* and, by extension, *in vivo*. The effects of cytokines on B-cell differentiation is evidenced not only by the magnitude of the Ab response but also the quality, in terms of the particular Ig isotype(s) induced. Although many cytokines are capable of promoting B-cell differentiation, the relative roles of specific factors, and the hierarchy of the interactions between several cytokines, has only emerged in the last 10 years.

DISCOVERY OF T CELL-DERIVED FACTORS AS CRITICAL MEDIATORS OF B-CELL DIFFERENTIATION AND PC GENERATION

The concept that cross-linking of the BCR initiates B-cell activation and facilitates these cells to respond to T-cell-derived soluble factors and undergo proliferation and differentiation to become Ab-secreting cells was first appreciated in the 1970s (17–20). The different factors were classically grouped as T cell-replacing factors, some of which influence the replication of B cells (B-cell growth factor), while others directly cause B-cell differentiation to Ab-secretion cells (B-cell differentiation factor) (21). While it gradually emerged that these T cell-derived factors are Ag non-specific, genetically non-restricted, and are indeed involved in the

differentiation of B cells into Ab-secreting cells, at this time no single factor had been isolated or molecularly cloned, and it remained unknown how many factors were actually involved in, or required for, B-cell terminal differentiation (22, 23).

The molecular revolution of the 1980s saw the cloning and characterization of several cytokines – IL-2, IL-4, IL-5, IL-6, IFNs – which had B-cell growth and differentiation capacity (**Table 1**). This continued into the 1990s with the discovery of IL-10, IL-12, IL-13, IL-15, TNFα, BAFF, and APRIL, which could promote various aspects of B-cell function (**Figure 2**; **Table 1**). Thus, these cytokines enhanced proliferation and induced isotype switching, PC formation, and Ig secretion by activated B cells (22–47) (**Table 1**; **Figure 2**). Importantly, this era also saw the identification of CD40L – transiently expressed on the surface of activated $CD4^+$ T cells – which, together with these cytokines, was revealed to be a critical regulator of many facets of B-cell biology (48). Specifically, while CD40L (or anti-CD40 mAb) itself had minimal effect on Ab-secretion by murine and human B cells, Ab-secretion could be induced in an isotype specific manner in the presence of exogenous cytokines (**Figure 2**; **Table 1**). Thus, IL-4 and IL-13 directs naïve human B cells to switch to IgG_4 and IgE expression and production, while IL-4 exerts a similar effect for inducing IgG_1 and IgE by murine B cells (**Table 1**), with IL-5 acting synergistically with IL-4 in these murine B-cell responses (25, 28, 29, 41, 49–51). The significance of these *in vitro* findings was underscored by the generation of IL-4 deficient mice, which had significantly reduced production of IgE following nematode infection (52). Interestingly, IL-4-induced IgE production by human B cells could be enhanced by IL-6 or TNFα (33, 45), or inhibited by IL-8 (53), IL-12 (54), or IFN-α or IFN-γ (33, 40). While murine B cells were initially reported to be unresponsive to IL-13 (55), subsequent studies noted that IL-13 could enhance Ab production by murine B cells *in vivo* and that it acts directly on B cells *in vitro* to increase survival, thereby increasing Ab production (56). Additional support for a role for IL-13 in modulating murine B cells came from the analysis of IL-13 transgenic mice, which exhibited substantially increased levels of serum IgE, even in the absence of IL-4 (57). Similarly, while deficiency of either IL-4 or IL-13 reduced the levels of Ag-specific IgE, combined deficiency of both IL-4 and IL-13 resulted in undetectable levels of IgE (58). Thus, it is likely that IL-4 and IL-13 co-operate in both mice and humans to regulate Ig class switching, especially to IgE. IL-10 also strongly modulated the behavior of human B cells, significantly increasing the levels of IgM, IgG_1, and IgA secreted by human B cells stimulated through CD40 or the BCR (42). IL-10 was also found to induce class switching in human naïve B cells to IgG_1 and IgG_3 (59), and together with TGF-β promoted switching to IgA (31). IL-10 also mediated the differentiation of GC and memory B cells to PCs (**Table 1**) (26). The ability of IL-4, IL-10, and IL-13 to induce isotype switching reflected their abilities to upregulate expression of activation induced cytidine deaminase (AICDA), an enzyme critical for class switch recombination, while IL-10 mediated PC generation by inducing BLIMP-1 (7, 8, 60). The effects of IL-10, however, appear to be species specific because serum Ig levels were unaffected in mice that were either deficient for IL-10 or that expressed IL-10 from a transgene (61, 62). Similar to CD40L, the membrane bound form of TNF-α was also found to be transiently expressed on human activated $CD4^+$ T cells, and could co-stimulate polyclonal Ig secretion induced in human B cells co-cultured with mitogen-stimulated $CD4^+$ T cells, or their membranes, together with IL-4 (27, 63) (**Table 1**).

IL-2 has had a long history of being documented of enhancing Ig secretion by activated human B cells (**Table 1**) (38, 39). Consistent with the structural and functional similarities between IL-2 and IL-15, it was not surprising that IL-15 could also stimulate proliferation and induce secretion of IgM, IgG_1, and IgA, but not IgG_4 or IgE, by CD40L-primed B cells. This activity of IL-15 was comparable to that of IL-2 (24).

More recently, the TNF-related molecule BAFF, and its homolog APRIL, has emerged as a global regulator of B-cell development and function (64–66). While a primary role for BAFF lies in the ability to promote the survival of B cells at the transitional stage of development (65), both BAFF and APRIL can also induce the molecular events associated with isotype switching to IgG and IgA, and to IgE in the presence of IL-4. Furthermore, the secretion of these Ig isotypes occurred when the B cells also received signals through the BCR (64, 66). BAFF and APRIL can also sustain the survival of PCs *in vivo* and *in vitro* (66, 67). BAFF functions by binding to the surface receptors BAFF-R, TACI, or BCMA; APRIL can also activate B cells by binding to TACI and BCMA (65, 66) (**Table 1**). Interestingly, these effects of BAFF and APRIL appear to be mediated through different receptors. Thus, the pro-survival effects of BAFF on transitional and naïve B cells are delivered through BAFF-R, while this effect on PCs occurs predominantly through BCMA. On the other hand, BAFF-R and TACI mediates isotype switching to IgG, IgA, and IgE induced by BAFF and APRIL, respectively (64–66). Lastly, heparan sulfate proteoglycans can also act as a receptor for APRIL, and this appears to be important for mediating the pro-survival effects of APRIL on BM PCs (67–69).

Collectively, it is clear that myriad cytokines and combinations thereof, are capable of eliciting activation and terminal differentiation of human B cells to differing extents. However, with the discover of IL-21 in 2001, and the subsequent characterization of its function on human and murine B cells during the following decade, the physiological significance of many of these factors in initiating humoral immune responses needs to be re-addressed as IL-21 has emerged as the most potent inducer of B cell differentiation.

PLEIOTROPIC EFFECTS OF IL-21 ON HUMAN AND MURINE B-CELL DIFFERENTIATION

IL-21 belongs to the type I family of cytokines that also includes IL-2, IL-4, IL-7, IL-9, and IL-15, all of which bind to and form a complex with the common γ-chain (γc) and their private receptors (70–73). The IL-21 receptor (IL-21R) is expressed by fibroblasts, keratinocytes, and intestinal epithelial cells, but more importantly is also expressed on lymphocytes (T, B, NK cells), macrophages, and dendritic cells, and the levels of expression can be increased following cellular activation (70, 71, 74–77). IL-21 is predominantly produced by activated $CD4^+$ T cells and NKT cells (78–80), with the greatest production being by Tfh and GC Tfh cells (4–6). Akin to most cytokines, IL-21 exerts its effect by activating Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways, specifically Jak1 and Jak3, and

Table 1 | Contribution of different cytokines to the *in vitro* behavior of human B cells.

Cytokine	Effect on B cells	Reference
CD40L	Induces activation, blastogenesis, proliferation	(25)
IL-2	Enhances proliferation of CD40L-stimulated B cells Co-operates with other cytokines/stimulatory factors to enhance differentiation of activated B cells	(25, 26, 30, 36, 38, 39, 44, 104, 144, 145)
IL-4	Enhances proliferation induced by CD40L, BCR engagement Induces expression of AICDA Induces CSR, preferentially to IgG1, IgG4, and IgE	(25, 33, 37, 40, 45)
IL-6	Promotes survival and function of *in vitro*-derived as well as primary and malignant plasma cells	(32, 44–47)
IL-10	Enhances proliferation induced by CD40L, BCR engagement Induces expression of AICDA, BLIMP-1 Induces CSR, preferentially to IgG1, IgG3 Co-operates with TGF-β to induce CSR to IgA Promotes differentiation of B cells to become plasma cells secreting IgM, IgG, IgA	(25, 26, 31, 34, 42, 59, 60, 144)
IL-12	Induces B cells to differentiate into IgM-secreting cells Co-operates with IL-6 to augment IgM secretion Suppresses IL-4-induced IgE production	(32, 54)
IL-13	Enhances proliferation induced by CD40L, BCR engagement Induces expression of AICDA Induces CSR, preferentially to IgG1, IgG4, and IgE Effects essentially overlap with those of IL-4	(28, 29, 41, 50)
IL-15	Enhances proliferation of B cells stimulated with CD40L or BCR engagement Induces secretion of IgM, IgG1, and IgA by CD40L-stimulated B cells Magnitude of the effect was comparable to IL-2	(24)
IL-21	Currently, the most potent cytokine identified capable of regulating human B-cell function Enhances proliferation induced by CD40L, BCR engagement Induces expression of AICDA, BCL-6, BLIMP-1, XBP-1 Induces CSR, preferentially to IgG1, IgG3, and IgA1 Promotes differentiation of B cells to become plasma cells secreting IgM, IgG, IgA, and IgE Synergizes with IL-4 for CSR to IgG and secretion of IgE Sustain survival of primary plasma cells present in secondary lymphoid organs Growth and survival factor for malignant plasma cells (i.e., myeloma) Requires functional STAT3 to induce plasma cells differentiation	(60, 71, 76, 83, 84, 96–101, 103, 106, 107, 121)
IFNα, IFNγ	Inhibits CD40L-induced B-cell proliferation Inhibits IL-4 induced IgE secretion IFNα primes activated B cells to differentiate into precursors of plasmablasts, that become plasmablasts in response to IL-6	(25, 33, 36, 40)
TNFα	Membrane TNFα expressed by CD4+ T cells acts as a co-stimulus to promote B-cell differentiation induced by CD40L and IL-4	(27, 33, 63)
BAFF/APRIL	BAFF promotes survival of transitional B cells, as well as of early plasma cells and some malignant plasma cells BAFF and TACI can induce CSR to various isotypes, and can induce secretion of these Ig's when combined with BCR signaling and cytokines (e.g., IL-4, IL-10, IL-15)	(64–66)
TGFβ	Inhibits IL-4 induced IgE secretion Can induce CSR to IgA, in combination with IL-10	(31, 33)

CSR, class switch recombination.

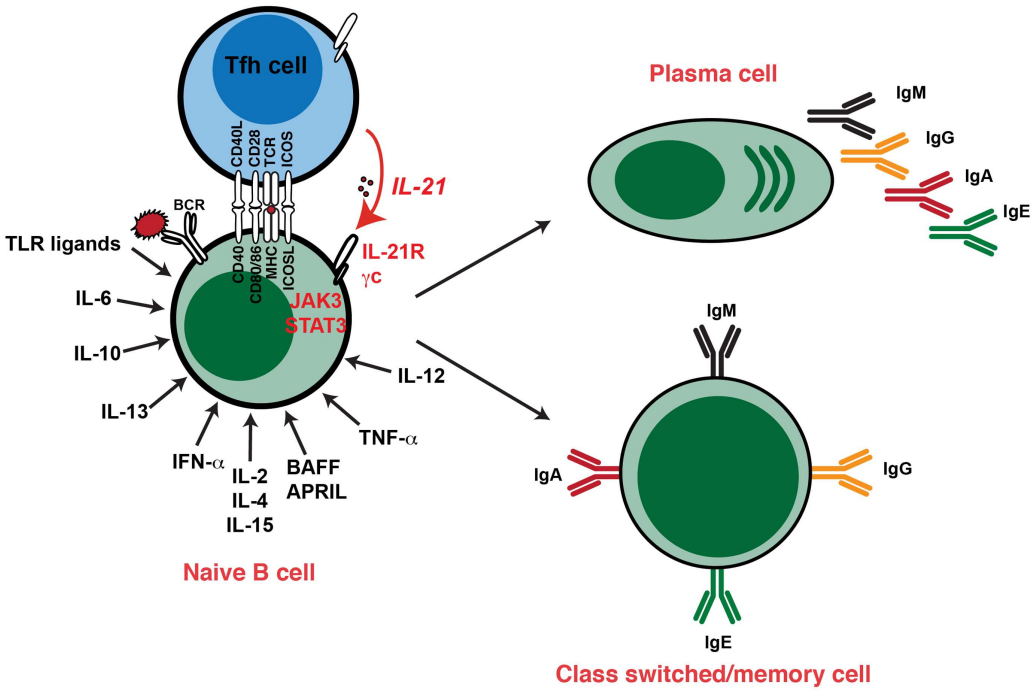

FIGURE 2 | Cytokine-induced differentiation of human B cells *in vitro*: requirement for IL-21 signaling *in vivo*. *In vitro* studies demonstrated that human B cells could undergo events such as Ig class switching and differentiation to become Ig-secreting cells following stimulation with a diverse range of cytokines. However, analysis of individuals with hypomorphic mutations in genes encoding STAT3, γc (*IL2RG*), JAK3, or IL-21R have revealed that this pathway – activated by IL-21 – is critical for the generation of memory B cells and the establishment of Ag-specific Abs *in vivo*. Thus, although cytokines such as IL-4, IL-13, IL-10, and BAFF/APRIL are strong B-cell growth and differentiation factors, their function is insufficient to compensate for impaired IL-21/IL-21R signaling *in vivo* in the setting of generating robust, long-lived Ag-specific Ab, and memory responses. Consequently, IL-21-mediated B-cell activation is a necessary and sufficient step in the generation of protective long-lived humoral immune responses in humans.

STAT1, STAT3, and to a lesser extent STAT5 (72, 81–84). The initial description of IL-21 hinted at its B-cell tropism, inasmuch that Parrish-Novak et al. showed that IL-21 significantly co-stimulated proliferation of human blood B cells induced by anti-CD40 mAbs (71). Since then, several studies have confirmed that IL-21 is an important regulator of B-cell activation, proliferation, PC differentiation, and Ab-secretion in both mice and humans.

THE ROLE OF IL-21 IN MURINE ACTIVATED B-CELL PROLIFERATION, APOPTOSIS, PC DIFFERENTIATION, Ab-SECRETION, AND MEMORY B-CELL FORMATION

In a seminal study, Ozaki et al. demonstrated that the IL-21 signaling pathway is involved in regulating Ab production and isotype switching (85). They showed that IL-21R$^{-/-}$ mice, despite having normal lymphoid development, have significantly diminished total serum and Ag-specific IgG_1 titers but elevated IgE levels in response to TD Ag immunization compared to wild-type animals. Ag-specific IgG_{2b} and IgG_3 serum levels were also decreased whereas IgG_{2a} and IgM titers were largely unaffected in the absence of the IL-21R. The decreased IgG_1 response appeared to result from a reduction in the generation of Ag-specific IgG_1 producing PCs. These *in vivo* data established that IL-21 has a critical role in inducing IgG_1 production, while concomitantly suppressing IgE responses. Strikingly, IL-4$^{-/-}$IL-21R$^{-/-}$ double-knockout mice displayed a more severe phenotype, characterized by a more dramatically reduced IgG response. Furthermore, the strong up-regulation of IgE secretion in IL-21R$^{-/-}$ mice was abrogated in IL-4$^{-/-}$IL-21R$^{-/-}$ mice indicating that the "hyper-IgE" phenotype of IL-21R$^{-/-}$ mice was dependent on IL-4 (85). Importantly, these *in vivo* findings were complemented by *in vitro* investigation of the effects of IL-21 on murine B cells. Thus, IL-21 enhanced proliferation of anti-IgM and/or anti-CD40 mAb-stimulated murine B cells and initiated PC differentiation and class switching, as revealed by increased expression of Syndecan-1 (CD138) and surface IgG1 on these cells (86).

These findings provided strong evidence that IL-21 is likely to achieve its potent effect on humoral immune responses *in vivo* by acting directly on B cells. Indeed, this has been verified in a series of studies where IL-21R-sufficient or deficient B cells were adoptively transferred into recipient mice, and the B-cell response to TD Ags or pathogens then tracked. It was generally found that when B cells were unable to respond to IL-21, humoral immunity was compromised with impaired formation of GC, with respect to magnitude and/or kinetics, and of long-lived Ag-specific PC. The mechanism underlying aberrant GC formation was suboptimal induction of Bcl-6 expression in GC B cells, which attenuated affinity maturation and selection of high-affinity variants. Although memory cells were generated in normal numbers from IL-21R-deficient B cells, the IL-21R-deficient memory

cells were unable to respond to secondary challenge with specific Ag, resulting in ineffective recall responses. In contrast to the GC response, the generation of extrafollicular plasmablasts in response to pathogens was unaffected by B-cell specific IL-21R-deficiency (87–92). IL-21 can activate STAT3 (72, 81–84). Intriguingly, analysis of $STAT3^{flox/flox}$ $CD19^{cre}$ mice showed some similarities to mice whose B cells lacked IL-21R. Specifically, $STAT3^{flox/flox}$ $CD19^{cre}$ mice have normal levels of serum IgM, IgA, and IgG, but a large reduction in Ag-specific serum IgG_1 levels and splenic PCs following immunization with TD Ags (93). This established that expression of STAT3 in B cells is important for TD differentiation of B cells into IgG_1-secreting PC (93), with subsequent studies implicating IL-21 as being the key STAT3-activating cytokine potentially involved in this process (87–92). Thus, IL-21/IL-21R signaling, possibly via STAT3, in B cells appears to be required for the generation and maintenance of long-lived PC and humoral memory to TD Ags, but is dispensable for GC-independent Ab responses.

Given the importance of IL-21R expression for normal Ig production *in vivo* (85, 86), a surprising finding was that murine IL-21 could inhibit B-cell proliferation induced by either anti-IgM and IL-4, or TLR ligands such as LPS or CpG (94, 95). Furthermore, although IL-21 impressively promoted proliferation of CD40-activated B cells, the proportion of B cells that was apoptotic in the presence of IL-21 exceeded that observed in its absence (95). Induction of apoptosis by IL-21 in both resting and activated murine B cells correlated with reduced expression of Bcl-x_L and Bcl-2 and elevated expression of Bim (94, 95). Consistent with this, IL-21-induced apoptosis could be prevented by restoring expression of Bcl-x_L or Bcl-2 either by overexpressing these proteins or inducing their expression by activation prior to exposure to IL-21 (94). Increased B-cell apoptosis was also observed *in vivo* in mice either transgenic for IL-21 or that received IL-21 administered via hydrodynamic-based delivery of plasmid DNA. Thus, it appears that IL-21 can differential influence B-cell fate depending on the signaling context (86).

Together, these data show that IL-21 is an important factor for the activation, proliferation, differentiation, Ag production, or death of murine B cells, with the outcome being dependent on the context of co-stimulation. The defect in GC-dependent Ab production in IL-21/IL-21R deficient mice after immunization indicates that differentiation into PCs may be a non-redundant activity of IL-21.

IL-21 AND HUMAN B CELLS

Initial studies into the stimulatory effect of IL-21 revealed that IL-21 potently enhanced the proliferation of CD40-stimulated human B cells, with memory B cells undergoing a much stronger proliferative response than naïve B cells (**Figure 3**; **Table 1**) (96). Despite memory B cells proliferating more than naïve B cells in response to IL-21, the overall effect of IL-21 appeared to be greater on naïve than on memory cells. Thus, naïve B cells stimulated with CD40L/IL-21 exhibited a greater enhancement in their response, as well as a greater reduction in their time to enter cell division, over that induced by CD40L alone than did memory B cells (76). This is probably due to the basal expression of IL-21R on naïve B cells, whereas it is absent from memory cells (**Figure 1**). Although expression of IL-21R increases following activation on naïve and memory B cells, it remained higher on the naive subset (76).

In terms of differentiation, when total $CD19^+$ splenic B cells were stimulated *in vitro* with anti-CD40 mAb in the presence of IL-21, they were induced to secrete IgM and IgG in an IL-21 dose-dependent manner (96). Pene et al. also made the important observation that IL-21 specifically induced production of IgG_1 and IgG_3 by human naïve B cells, demonstrating IL-21 to be a switch factor for these IgG subclasses (96). The findings from this elegant study were confirmed by several groups who also found that IL-21 induced proliferation as well as expression and secretion of IgM, IgG (predominantly IgG_3) as well as IgA (mostly IgA_1), and IgE by CD40L-stimulated naïve B cells that had been isolated from distinct anatomical sites, including umbilical cord blood, spleen, tonsils, and adult peripheral blood (**Figure 3**) (60, 76, 97–100) (**Table 1**). IL-21 also strongly induced Ig secretion from memory and GC B cells isolated from these sites (60, 97, 98, 100). The ability of IL-21 to induce such impressive Ig secretion correlated with the appearance of a substantial proportion of PCs – phenotypically identified as $CD19^{lo}IgD^-CD38^{hi}$ or $CD20^{lo}CD38^{hi}CD27^{hi}$ cells – in cultures of IL-21-stimulated B cells (**Figure 3**) (60, 98). Interestingly, a recent report also found IL-21 could support the survival of and Ig secretion by PCs in secondary lymphoid organs, but not those in the bone marrow (101). This is consistent with the differential expression of IL-21R on PCs from these diverse sites (76, 98, 102) (**Figure 1**), and suggests that IL-21 contributes to humoral immunity not only by inducing PC from naïve, memory, and GC B cells, but also promoting the survival and function of these cells in lymphoid tissues before they alter their requirements for survival within niches in bone marrow (67). The ability of IL-21 to sustain survival of normal PCs is reminiscent of the finding that IL-21 can promote growth and survival of malignant PC in multiple myeloma (103).

When compared to other cytokines that have been characterized as B-cell growth and differentiation factors, the effect of IL-21 was found to exceed that of IL-2, IL-4, IL-13, and IL-10 by up to 100-fold (60, 76, 97, 98). However, the actions of IL-21 could be complemented by these cytokines (**Table 1**; **Figure 3**). For example, IL-4 increased the frequency of IgG^+ cells generated from, and the amount of IgG secreted by, naïve B-cell precursors that had been stimulated with IL-21 (**Figure 3**) (96, 97). Interestingly, while IL-21 favored the induction of $IgG_3{}^+$ B cells, the combination of IL-4 and IL-21 resulted in the preferential generation of $IgG_1{}^+$ switched B cells, which mirrored the effect of IL-4 alone but the magnitude of the response was greater. IL-4 and IL-21 were also capable of acting synergistically to induce 10- to 100-fold higher levels of IgE by CD40L-stimulated naïve B cells over that observed with either cytokine alone (**Figure 3**) (96, 99). In contrast, IL-4 abolished not only IL-21-induced IgM secretion but also switching to and secretion of IgA (97, 98); on the other hand, IgA secretion induced by IL-21 was augmented by IL-10 (97). Lastly, IL-2 could enhance PC differentiation induced by IL-21 (**Figure 3**) (98, 104). This was achieved by IL-21 inducing expression of CD25 – a component of the IL-2R – on activated B cells (104).

The physiological significance of these effects of IL-21 on human B cells has been born from experiments that assessed the relative contribution(s) of $CD4^+$ T cell-derived cytokines to TD

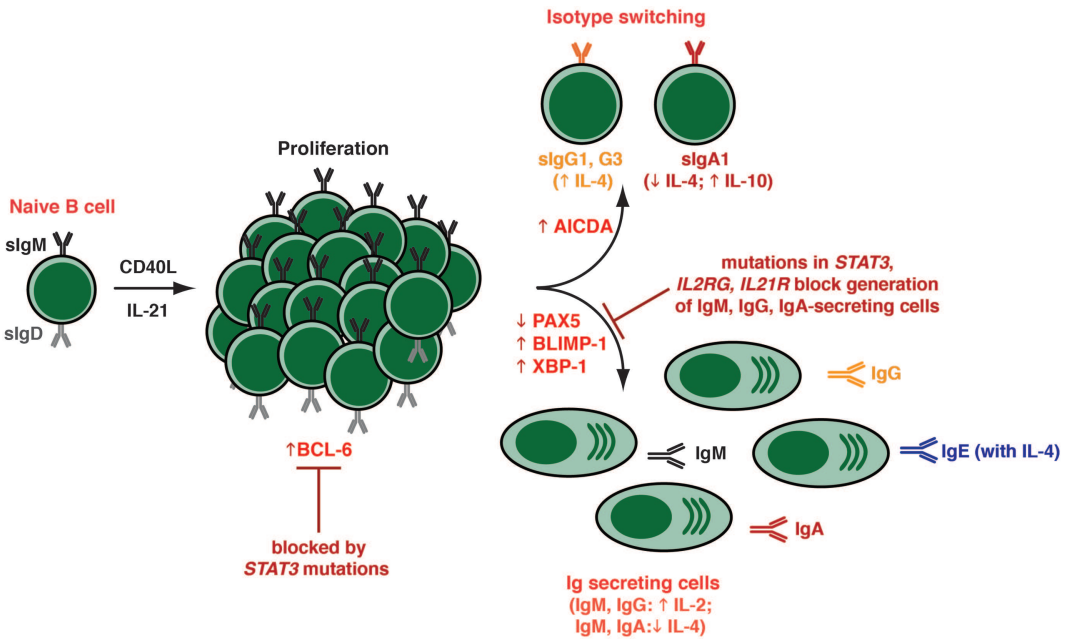

FIGURE 3 | Effects of IL-21 on human naïve B cells *in vitro*. When human naïve B cells are stimulated with CD40L together with IL-21, they were found to undergo intense proliferation. This was followed by induction of Ig class switching – predominantly to IgG_3 and IgG_1; a lesser extent to IgA_1 – as determined by acquisition of expression of switched isotypes or differentiation to plasma-like cells capable of secreting all major Ig isotypes. The B cells that had undergone switching to become IgG^+ or IgA^+ were distinct from those that committed to a plasma cell fate – thus, the secreted Ig detected in these cultures was derived from the *in vitro*-derived plasma cells rather than the surface IgG^+/IgA^+ class switched cells. Both class switching and plasma cell formation induced by IL-21 could be modulated by additional cytokines, such as IL-4 (promoted switching to IgG; inhibited switching to IgA; increased secretion of IgG and IgE; suppressed secretion of IgM, IgA), IL-10 (increased IgA secretion), and IL-2 (increased secretion of IgM, IgG). These differentiation events coincided with the induction in expression of BCL-6, AICDA (required for class switching), and BLIMP-1/XBP-1 (required for plasma cell formation). The ability of IL-21 to induce naïve B cells to differentiate into plasmablasts/plasma cells *in vitro* was abolished by hypomorphic mutations in *STAT3*, as well as null mutations in *IL21R* or *IL2RG*.

B-cell differentiation *in vitro*. Using an *in vitro* system whereby human activated $CD4^+$ T cells can induce Ig production by co-cultured B cells (105), several groups have established that neutralization of IL-21 significantly inhibited T cell-induced B-cell activation, proliferation, differentiation, Ig secretion, and PC survival (60, 101, 106). Delayed blockade of IL-21 also inhibited PC differentiation after initial B-cell expansion, indicating that IL-21 is required for B-cell proliferation and PC differentiation (106). The findings that IL-21 is highly expressed by Tfh cells (78), and the IL-21R is upregulated on GC B cells (76) is consistent with a model of Tfh cells interacting with GC B cells to induce their differentiation to memory cells and PC predominantly via the production and delivery of IL-21 (4–6).

MECHANISM OF ACTION OF IL-21

The ability of IL-21 to guide multiple fates in activated B cells – class switching to express downstream Ig isotypes, commitment to the PC lineage, as well as formation of GCs and memory B cells – reflects the ability of IL-21 to induce the molecular machinery required for these processes. Thus, IL-21 is capable of inducing expression of AICDA, BLIMP1/PRDM1, and XBP-1, as well as reducing expression of PAX5, in both human and murine B cells (**Figure 3**) (60, 83, 84, 86, 98). Collectively, these factors regulate class switching and PC formation (8). Interestingly, the ability of IL-4 to suppress the stimulatory effects of IL-21 on naïve B cells correlated with a reduction in BLIMP-1 expression (60). IL-21 could also induce BCL-6 (83, 86, 89, 92, 98), which would contribute to GC formation *in vivo* (**Figure 3**) (8). Thus, in the setting of TD B-cell activation, Tfh-derived IL-21 can induce B cells to express all of the machinery required to undergo the major fates of differentiation: GC B cells by induction of Bcl-6; PCs following induction of BLIMP-1, and class switched B cells by inducing AICDA. It is likely that IL-21 induces expression of these opposing transcriptional regulators (i.e., BLIMP-1, Bcl-6) in distinct subsets of B cells that will ultimately develop into either PC or memory B cells. However, these outputs will ultimately reflect the balance of signals received and integrated by the B cells, with the effect of IL-21 being influenced by inputs delivered via receptors including the BCR, other complimentary cytokine, and co-stimulatory receptors.

As IL-21 can activate several STATs (73), the relative contribution of individual STAT molecules has been assessed. Diehl et al. demonstrated that constitutive activation of STAT3 in primary human B cells induced BLIMP-1 expression and initiated B cell differentiation, yielding cells with a phenotype ($CD38^{high}CD20^{-}CD19^{low}$HLA-$DR^{low}CD138^{+}$) consistent with PC as well as enhanced Ab-secretion (84). Importantly, up-regulation of BLIMP-1 alone was not sufficient for differentiation

of primary human B cells into PCs; this event also required concomitant down-regulation of BCL-6 (84). This study was the first to propose that STAT3 was the predominant mediator of the differentiation effects that IL-21 has on human B cells. These were largely confirmed by the demonstration that induction of PRDM1, XBP-1, and BCL-6 by IL-21 were abolished in naïve B cells isolated from individuals with hypomorphic mutations in *STAT3*, while these responses were unaffected by loss-of-function mutations in *STAT1* (83, 107). Intriguingly, IL-21-induced expression of *AICDA* in naïve B cells, as well as of *PRDM1* and *XBP1* in memory B cells, still occurred despite the presence of hypomorphic *STAT3* mutations, suggesting that class switching in naïve B cells and PC differentiation from memory B cells requires less STAT3 function than does the generation of PC from naïve B cells (83, 107).

Interestingly, high-affinity signaling through the BCR on immortalized B-cell lines can activate STAT3 (108). Similarly, CD40L enhanced the expression of BLIMP-1 induced by IL-21/STAT3 signaling in a GC B cell-like human cell line, thereby maximizing PC differentiation (109). Thus, it is possible that signals integrated in B cells through receptors such as CD40 and the BCR can amplify the effects of IL-21 by modulating activating or function of STAT3. It is also worth noting that STAT3 activation is important for the survival of multiple myeloma cells (110). As IL-21 is also anti-apoptotic for myeloma cells, it is tempting to speculate that IL-21 could contribute to STAT3 activation *in vivo* in the setting of this malignancy. Collectively, these studies have illuminated the pivotal role of IL-21-mediated STAT3 signaling in guiding key events of human B-cell differentiation.

LESSONS FROM PRIMARY IMMUNODEFICIENCIES

Primary immunodeficiencies (PIDs) result from monogenic mutations that compromise the ability of affected individuals to elicit appropriate immune responses. Consequently, these individuals exhibit susceptibility to infectious diseases and are often unable to respond to vaccination. As the genetic lesion is known in many PIDs, these conditions can reveal the unique functions of specific genes and related signaling pathways in immune cells and the importance of these pathways in productive and protective immune responses. Thus, analysis of PIDs can shed new light on the requirements for lymphocyte development and function. Indeed, several PIDs have confirmed the critical role played by IL-21 in humoral immunity in humans.

Heterozygous mutations in *STAT3* are the major cause of autosomal dominant hyper-IgE syndrome (AD-HIES) (111, 112), a multisystem disease affecting the immune and musculoskeletal systems (113, 114). Immunological defects include skin lesions, recurrent mucocutaneous invasive infections with *S. aureus* and *Candida*. These patients have normal serum levels of IgM, IgG, and IgA but increased levels of IgE (113, 114). Although the frequencies of total peripheral blood B cells are not significantly different between AD-HIES patients and control individuals, STAT3 deficiency impaired the *in vivo* generation of human memory B cells as well as the generation of Ag-specific Ab-secreting B cells and high-affinity serum Abs (**Figure 2**) (83, 107). This reduced number of memory B cells is in line with previously reported defective functional Ab responses in AD-HIES patients (115–118). Cytokines known to be involved in human B cell differentiation are IL-6, IL-10, and IL-21. Consistent with reduced memory B cells and poor induction of Ag-specific Ab responses in AD-HIES, naïve B cells from these patients were unable to respond to the stimulatory effects of IL-10 or IL-21 with respect to differentiation into PC *in vitro* (**Figure 3**). *STAT3* mutations also compromised the ability of IL-21 to prime B cells to the stimulatory effects of IL-2, inasmuch that induction of CD25 – and subsequent responsiveness to IL-2 – was attenuated on IL-21-stimulated STAT3-deficient human naïve B cells (104). These findings revealed that STAT3 plays a non-redundant role in generating Ag-specific memory B cells and Ab-secreting cells *in vivo*. However, it remained to be determined which STAT3-activating cytokine was requisite for these effects. This became clearer by examining patients with mutations in *IL2RG*, encoding γc, or *JAK3*, which associates with γc and delivers signals downstream of γc-containing cytokine receptors (73), that cause X-linked severe combined immunodeficiency (X-SCID) or one type of autosomal recessive (AR) SCID, respectively (73, 119). These PIDs are fatal unless treated by hematopoietic stem cell transplant (HSCT) (119).

X-linked severe combined immunodeficiency and JAK3 deficiency are characterized by a lack of T and NK cells but normal or increased numbers of B cells. However, due to the lack of $CD4^+$ T cell help, B cell responses are impaired (119). While HSCT corrects the humoral defect in ~50% of patients, the remainder still requires ongoing Ig replacement therapy (120). One of the explanations for this is split chimerism, where donor-derived T cells successfully engraft in the recipient, but autologous host-derived B cells persist (120). Thus, despite the presence of functional $CD4^+$ T cells, the *IL2G*/*JAK3* mutant B cells remain unable to respond to T-cell-derived helper signals, rendering the patient immunodeficient with respect to humoral immune responses (119, 120). We took advantage of this chimeric state to examine the B-cell compartment of X-SCID and JAK3 deficient patients who had undergone HSCT (121). Although *IL-2RG*/*JAK3* mutant naïve B cells responded normally to co-stimulatory signals delivered through the BCR, TLRs, and receptors for IL-10, IL-13, and even IL-4 [which can also signal through the IL13R; (73)], these B cells were completely unresponsive to IL-21. Naïve B cells from these individuals also failed to differentiate into memory cells *in vivo* (**Figures 2** and **3**). Thus, despite intact responsiveness to a suite of well-characterized B-cell growth and differentiation factors, the ability to receive signals through a γc-binding/JAK3-activating cytokine is a critical and rate-limiting step for the establishment of humoral immunity in humans (**Figure 2**) (121). Given the potency that IL-21 exerts on human B-cell differentiation, it was highly likely that this was the key γc-binding/JAK3-activating cytokine involved in human B-cell responses *in vivo*.

This was confirmed by the recent identification of individuals with homozygous loss-of-function mutations in *IL21R* that causes a novel PID, features of which include occasionally reduced serum IgG levels, poor Ab responses following vaccination with TD Ags (122), and a paucity of circulating memory B cells, including those expressing class switched Ig isotypes (107, 122). Not surprisingly, IL-21R-deficient naïve B cells exhibited impaired IL-21-induced proliferation, Ig class switching, and PC differentiation *in vitro*. This is consistent with a failure of IL-21 to mediate the acquisition of expression of *AICDA*, *PRDM1,* and *XBP1* in these cells, and

mirrors the humoral immune defects observed in these patients. The cellular and molecular characterization of these patients has definitively established the criticality of IL-21 in establishing long-lived humoral immune responses. Furthermore, the finding that B cells with mutations in *IL2RG*, *JAK3*, or *STAT3* phenocopy IL-21R-deficient B cells, with respect to memory cell formation and responsiveness to IL-21, demonstrates that signaling downstream of the IL-21R/γc complex via JAK3 and STAT3 is essential for the effector function of IL-21 on B-cell differentiation in terms of generating efficient Ag-specific humoral immune responses (**Figures 2** and **3**). However, since serum levels of total IgM, IgG, and IgA are largely normal in most patients with mutations in either STAT3 or IL-21R, it is clear that the production of basal Ig is not dependent on IL-21R/STAT3 signaling. Indeed, as we have previously proposed (83), this is likely achieved by the interplay between ligands that do not signal via STAT3 – these could include many of the cytokines and factors detailed in this review (see **Table 1**), such as IL-4, IL-13, BAFF/APRIL as well as TLR ligands. Despite the availability of these ligands in STAT3- and IL-21R-deficient patients, and their ability to signal normally in IL-21R/STAT3-deficient B cells, these factors are collectively unable to compensate for impaired IL-21R signaling in order to generate a robust, long lasting Ag-specific Ab response.

Intriguingly, IL-21R-deficient individuals also have elevated levels of serum IgE (122), which is obviously also a feature of AD-HIES (113, 114, 116). Thus, it is likely that IL-21 also plays an important role in regulating IgE production by human B cells. However, whether this is due to a direct effect of IL-21 on B cells, or operates through an intermediate cell type [e.g., by inducing production of IFNγ by T cells and NK cells; (40, 100)] remains to be determined.

Lastly, it is worth commenting that prior to the discovery and subsequent characterization of IL-21, IL-10 was considered to be the most efficient cytokine capable of activating human B cells (31, 42, 48, 59). As IL-10 can also activate STAT3 (73), and STAT3-deficient human naïve B cells are unable to respond to the PC-inducing effects of IL-10 (83), it is possible that the humoral defects in AD-HIES patients reflects an inability to respond to not only IL-21 but also IL-10. However, since individuals with mutations in *IL-10* or *IL-10R* have intact specific Ab responses to vaccines (123), it is possible the IL-10 plays only a minor role in regulating human B-cell function *in vivo*. There are caveats to this conclusion, however, as most patients examined were young (<10 years old), and they also suffered from early onset inflammatory bowel disease (123). Thus, it remains plausible that IL-10 does contribute to B-cell function in healthy adults.

IL-21/IL-21R AND SYSTEMIC AUTOIMMUNE DISEASES

Just as impaired signaling via IL-21 manifests as humoral immunodeficiency, aberrant or excessive IL-21-induced B-cell activation has been associated with the development of Ab-mediated autoimmune states in both murine models and human.

The first indication of a potential involvement of IL-21 in autoimmunity was the finding that IL-21 was overexpressed in several strains of mice (e.g., BXSB-*Yaa*, B6.Sle1-*Yaa*, Sanroque, MRL/MpJ-FAS$^{lpr/lpr}$/J) that develop lupus-like disease (86, 124, 125). Furthermore, *in vivo* blockade of IL-21 ameliorated disease progression and severity in some of these settings (126–128), as well as in animal models of rheumatoid arthritis (129) and Sjogren's syndrome (130). This was followed by the demonstration of elevated expression and/or production of IL-21 in human autoimmune conditions including SLE (131–134), rheumatoid arthritis (135), and Sjogren's syndrome (136). Consistent with these findings, as well as with the recognition that IL-21 is predominantly produced by Tfh cells, it was perhaps not surprising that circulating Tfh-like cells have been detected in a broad array of autoimmune conditions including not only SLE, rheumatoid arthritis, and Sjogren's syndrome, but also multiple sclerosis, autoimmune thyroid disease, myasthenia gravis, and juvenile dermatomyositis [reviewed in Ref. (6, 137)]. Importantly, the increases in Tfh cells generally correlated with numerous indices of disease severity, such as titers of autoAb, numbers of Ab-secreting plasmablasts, clinical scores, and even levels of serum IL-21. Furthermore, the expanded population of Tfh cells, as well as clinical features of each of these diseases, could be reduced following initiation and continuation of immunosuppressive treatments [reviewed in Ref. (6, 137)]. Independent confirmation that IL-21/IL-21R may be involved in the development of autoimmune diseases came from genome-wide association studies. Specifically, polymorphisms in either IL-21 or IL-21R genes have been identified that associated with SLE, RA, and primary Sjogren's syndrome (138–141). Collectively, there is convincing evidence that IL-21 – most likely produced by Tfh cells – plays a pathological role in the initiation, development, and/or progression of several human autoimmune diseases caused by the production of autoantibodies.

CONCLUDING COMMENTS AND FUTURE PERSPECTIVES

B cells play myriad fundamental roles in providing protective immunity against infection. However, the most prominent of these is the production of Ag-specific Ab following the terminal differentiation of B cells into long-lived PCs. This event is key to the establishment of long-term humoral immunity and memory, and underlies the success of most currently available vaccines. The criticality of Ab production by B cells to human health is evidenced by the pathological consequences of hypogammaglobulinemia, resulting in immunodeficiency. Conversely, the dysregulated production of excessive quantities of self-reactive Abs can be deleterious in the setting of autoimmunity. The detailed characterization of the effects of cytokines on B cells – from studies in genetically manipulated mice, *in vitro* cultures of human and murine B cells, and analysis of humans with specific PIDs – have revealed the central role that IL-21 has in generating memory B cells and specific Abs following exposure to TD Ags. Remarkably, alternative signals that could be integrated in B cells through other cytokine or co-stimulatory receptors are insufficient to initiate such B-cell responses when the IL-21/IL-21R signaling pathway is compromised. This paves the way for developing directed therapies to improve immune responses to vaccines or in immunocompromised individuals. Supporting this concept is the finding that administration of IL-21 to macaques increased frequencies of memory B cells as well as titers of virus-specific IgG (142). Conversely, therapies aimed at blocking the action of IL-21, either by directly targeting IL-21 itself or indirectly targeting Tfh cells

or appropriate signaling molecules downstream of the IL-21R, so as to restrain the differentiation of rogue, autoreactive B cells into PCs, represents a feasible strategy for the treatment of various autoimmune diseases, as evidenced from numerous murine models (126–129, 143). Hopefully these findings will see successful translation to the clinic, thereby offering new hope for the treatment of these immune dyscrasias.

ACKNOWLEDGMENTS

The Tangye lab is supported by research grants and fellowships awarded by the National Health and Medical Research Council of Australia.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 December 2013; accepted: 05 February 2014; published online: 18 February 2014.
*Citation: Moens L and Tangye SG (2014) Cytokine-mediated regulation of plasma cell generation: IL-21 takes center stage. Front. Immunol. **5**:65. doi: 10.3389/fimmu.2014.00065*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 23 December 2013
doi: 10.3389/fimmu.2013.00467

Apoptotic machinery diversity in multiple myeloma molecular subtypes

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Multiple myeloma (MM) is a plasma-cell (PC) malignancy that is heterogeneous in its clinical presentation and prognosis. Monoclonal gammopathy of undetermined significance (MGUS) consistently preceded development of MM. The presence of primary IgH translocations and the universal overexpression of cyclin D genes led to a molecular classification of MM patients into different disease subtypes. Since Bcl-2 family proteins determine cell fate, we analyzed a publicly available Affymetrix gene expression of 44 MGUS and 414 newly diagnosed MM patients to investigate (1) the global change of Bcl-2 family members in MM versus MGUS (2) whether the four major subtypes defined as hyperdiploid, CyclinD1, MAF, and MMSET, display specific apoptotic machineries. We showed that among the main anti-apoptotic members (Bcl-2, Bcl-x_L, and Mcl-1), Mcl-1 up-regulation discriminated MM from MGUS, in agreement with the prominent role of Mcl-1 in PC differentiation. Surprisingly, the expression of multi-domain pro-apoptotic Bak and Bax were increased during the progression of MGUS to MM. The combined profile of Bcl-2, Bcl-x_L, and Mcl-1 was sufficient to distinguish MM molecular groups. While specific pro-apoptotic members expression was observed for each MM subtypes, CyclinD1 subgroup, was identified as a particular entity characterized by a low expression of BH3-only (Puma, Bik, and Bad) and multi-domain pro-apoptotic members (Bax and Bak). Our analysis supports the notion that MM heterogeneity is extended to the differential expression of the Bcl-2 family content in each MM subgroup. The influence of Bcl-2 family profile in the survival of the different patient groups will be further discussed to establish the potential consequences for therapeutic interventions. Finally, the use of distinct pro-survival members in the different steps of immune responses to antigen raises also the question of whether the different Bcl-2 anti-apoptotic profile could reflect a different origin of MM cells.

Keywords: multiple myeloma, MGUS, Bcl-2 family, *CCND1, MMSET, MAF*

INTRODUCTION

Multiple myeloma (MM) is a plasma-cell (PC) malignancy that is heterogeneous in its clinical presentation and prognosis. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes the development of MM. The presence of primary IgH translocations and the universal overexpression of CCND (cyclinD) genes has led to a molecular classification of MM patients into different disease subtypes (1–3). The main translocations involve the immunoglobulin gene heavy chain locus on 14q32.33 with recurrent chromosome partners. These include t(11;14), t(4;14), t(14;16), and t(14;20) with an overexpression of *CCND1*, *MMSET*, *c-MAF*, and *MAFB*, respectively. Moreover, half of MM patients do not exhibit IgH translocation but present multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 and constitute the hyperdiploid subgroup of MM patients.

Impaired apoptosis is often associated with tumorigenesis and resistance to treatment. Apoptosis is controlled at multiple levels and members of the Bcl-2 family regulate the mitochondrial apoptosis pathway. They can be divided into three functional groups. The anti-apoptotic group comprises Bcl-2, Mcl-1, Bcl-xL, A1, and Bcl-w. These molecules contain four BH-2 homology domains. The pro-apoptotic multi-domain effectors, Bax and Bak, induce mitochondria damage upon activation and constitute a second group (4). Lastly, the BH3-only group, encompasses direct activators of Bax/Bak (Bid, Bim, and Puma) and sensitizers (Noxa, Bik, Bad, Hrk, and Bmf), which bind to anti-apoptotic relatives in order to induce the release of BH3 activators (5).

Individual BH3-only proteins exhibit differential affinities for their pro-survival counterparts. The activators Bim, Puma, and Bid bind all pro-survival members with high affinity, whereas BH3-only sensitizers display more selectivity. For instance, Bad binds with high affinity only to Bcl-2, Bcl-x_L, and Bcl-w, and Noxa only to Mcl-1 and A1. Bax and Bak also differ in their interaction profile. Bak is tightly bound by Mcl-1 and Bcl-x_L but weakly by Bcl-2 whereas Bax seems to be neutralized by all pro-survival members (6).

The cellular content of the Bcl-2 family molecules varies among the different cell types, however it is certain that the interaction

between anti-apoptotic and pro-apoptotic Bcl-2 members dictates whether a cell should die or not (4).

Since Bcl-2 family proteins determine cell fate, we analyzed a publicly available library of Affymetrix gene expression levels from 44 MGUS and 414 newly diagnosed MM patients to investigate (a) the global change of Bcl-2 family members in MM versus MGUS and (b) whether the four major MM subtypes, defined as respectively hyperdiploid (HY) or IgH translocation in 11q13 (CCND1), 16q23 (MAF), and 4p16 (MMSET), display specific apoptotic machineries.

RESULTS

ANALYSIS OF Bcl-2 FAMILY EXPRESSION BETWEEN MGUS AND MM

We found that among anti-apoptotic members, only Mcl-1 was significantly up-regulated in MM compared to MGUS ($p < 0.0001$ Mann–Whitney test; **Figure 1**) whereas *BCL2* and *BCLXL* remained unchanged or slightly decreased in MM versus MGUS (**Figure 1**). Of note, we excluded *BCL2A1* from the study since it is largely expressed in B cells but lost during PC differentiation. The expression of all BH3-only proteins was not modified during the progression from MGUS to MM (**Figure 2**). In contrast, we observed that the expression of multi-domain pro-apoptotic *BAX* and *BAK* was significantly increased in MM ($p = 0.055$ and $p < 0.0001$ respectively Mann–Whitney test; **Figure 1**). Altogether, the major modifications of the Bcl-2 family gene expression during the progression of MGUS to MM mainly affected *MCL1* and *BAK* gene expression (1.27 and 1.54 median fold change, respectively). These modifications between MGUS and MM should be interpreted with caution since PC populations in MGUS include both normal and malignant cells. Accordingly, it has been previously reported that normal PC in MGUS can represent up to 65% whereas in MM the percentage of normal PCs is <2% (7).

COMPARISON OF Bcl-2 FAMILY MEMBERS IN MM SUBGROUPS

Analysis of anti-apoptotic gene expression within the four major subgroups provided evidence that the HY and CCND1 groups can be distinguished from MAF and MMSET groups by a high expression of *BCL2* and a weak expression of *MCL1* (1.3 and 0.7 median fold change, respectively), as already reported (8). Furthermore, *BCLXL* allowed to discriminate HY from CCND1 patients and also MAF from MMSET patients, since CCND1 expressed significantly less *BCLXL* than HY patients ($p < 0.0001$ Mann–Whitney test; **Figure 3**) and MMSET patients expressed significantly less *BCLXL* than MAF patients ($p < 0.0001$ Mann–Whitney test; **Figure 3**). Although *BCLXL* was heterogeneous among the four subtypes, its role in MM physiopathology remained elusive. While we have previously shown that silencing *BCLXL* did not alter the survival of myeloma cell lines (9), other studies have demonstrated that *BCLXL* played a role in chemoresistance (10). Of note, a high expression of *MCL1* was found in the worse prognosis groups (MAF and MMSET) according to the essential function of Mcl-1 in MM cell survival (9, 11, 12). On the other hand, we may question whether the lowest *MCL1* levels present in the CCDN1 group could influence its neutral outcome.

BH3-mimetic small molecules that bind to the BH3 binding sites of anti-apoptotic proteins have been developed. Among them ABT-199 is the newest one, characterized by its high potency to inhibit specifically Bcl-2 (13). In agreement with the high

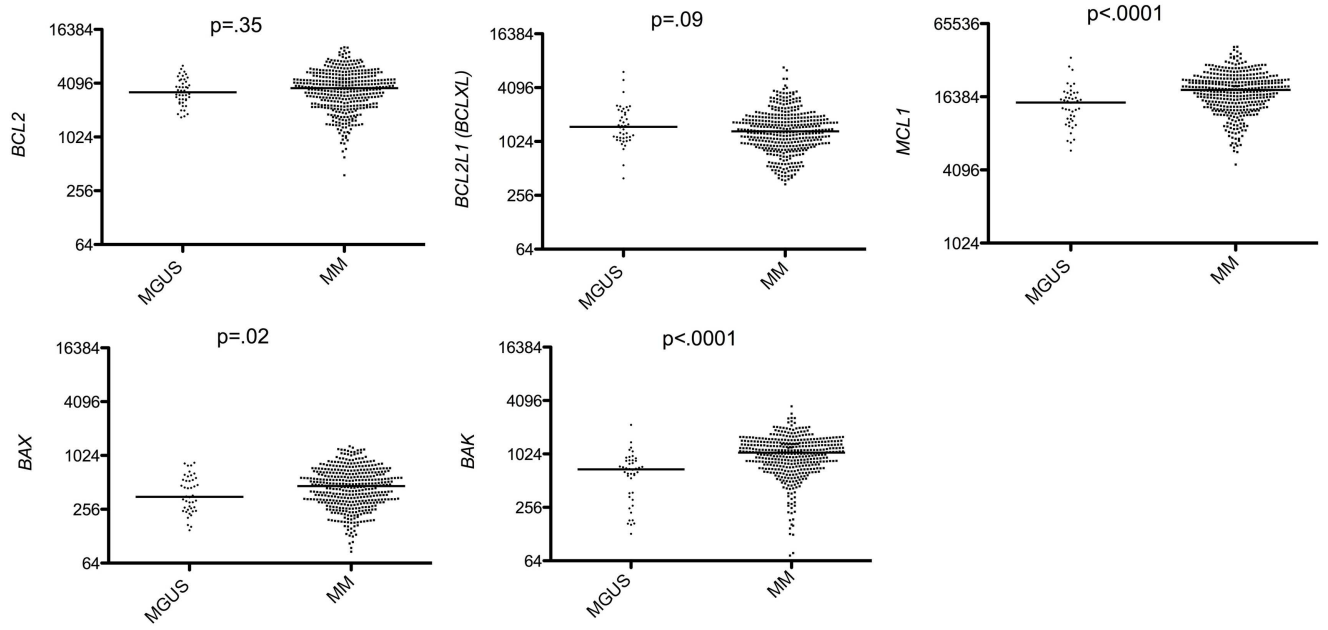

FIGURE 1 | Affymetrix data from a cohort of 44 MGUS and 414 untreated patients from the Arkansas Cancer Research Center were analyzed for *BCL2* (probe set 203685_at), *BCL2L1* (probe set 212312_at), *MCL1* (probe set 200797_s_at), *BAX* (probe set 208478_s_at), and *BAK* (probe set 203728_at) expression using the Amazonia database (http://amazonia.transcriptome.eu/). Median values were indicated. Statistical analyses were done using the Mann–Whitney test.

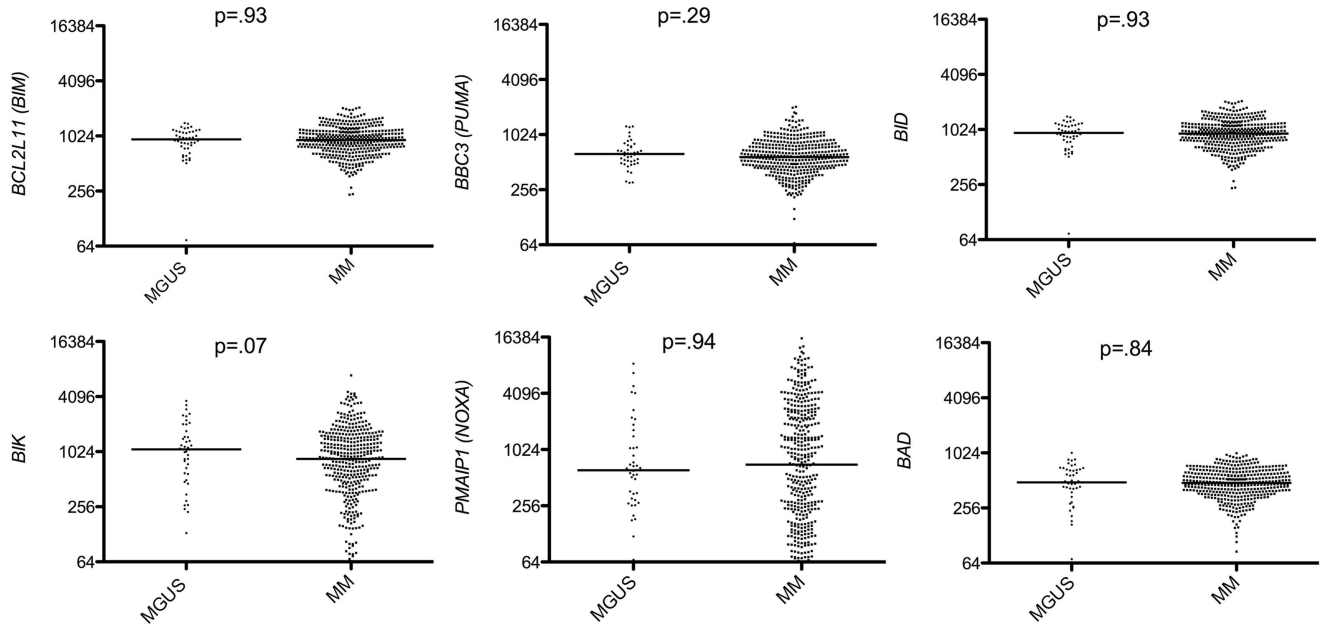

FIGURE 2 | Affymetrix data from a cohort of 44 MGUS and 414 untreated patients from the Arkansas Cancer Research Center were analyzed for *BIM* (probe set 1553088_a_at), *BBC3* (probe set 211692_s_at), *BID* (probe set 211725_s_at), *BIK* (probe set 205780_at), *PMAIP1* (probe set 204285_s_at), and *BAD* (probe set 1861_at), expression using the Amazonia database. Median values were indicated. Statistical analyses were done using the Mann–Whitney test.

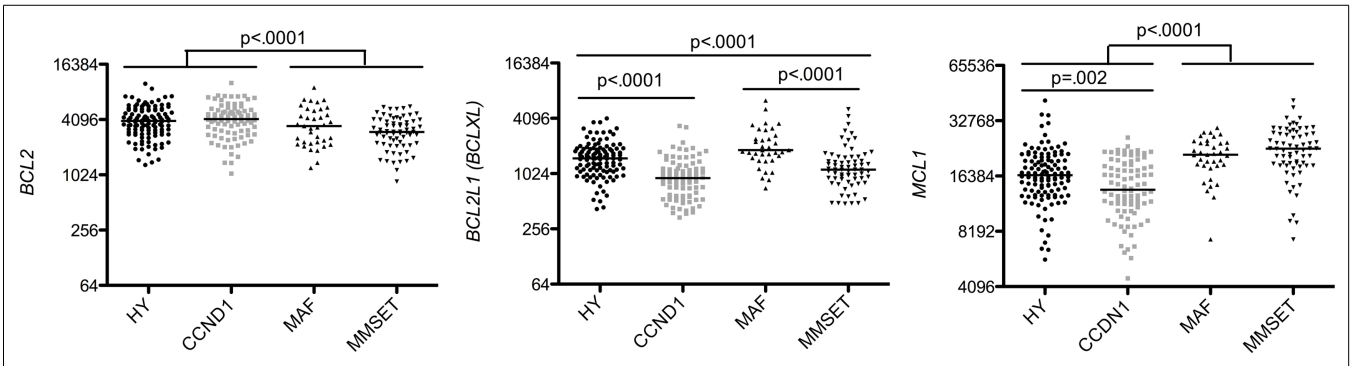

FIGURE 3 | Affymetrix data from untreated patients were analyzed for *BCL2, BCL2L1,* and *MCL1,* expression using the Amazonia database in the different molecular groups classified as follow: hyperdiploid (HY), CCND1 (CD1 + CD2), MAF, and MMSET (3). Probe sets are indicated in **Figure 1**. Median values were indicated. Statistical analyses were conducted using the Kruskall–Wallis test when more than two groups were compared and Mann–Whitney test for the comparison between two groups.

expression of *BCL2* in CCND1 patients, we have recently demonstrated in a small cohort of MM patients that ABT-199 sensitivity was restricted to t(11;14) patients (14). Further analysis of a larger cohort of MM patients for ABT-199 sensitivity could allow identifying HY patients able to respond to ABT-199.

Strikingly, analysis of multi-domain pro-apoptotic members showed that the worse prognosis groups MAF and MMSET displayed higher levels of *BAX* and *BAK* in contrast to the CCDN1 group, which expressed the lowest levels of both effectors (1.38 and 1.85 median fold change, respectively) (**Figure 4**). BH3-only activators (*BIM, PUMA, BID*) were constantly expressed in the four subtypes, suggesting that independently of the subtype, MM cells are primed for death, as already reported (15, 16) (**Figure 5**). Of note, Puma expression was weaker exclusively in CCDN1 group compared to the other subgroups (0.82 median fold change). The promiscuous binding of Bim and Puma to main pro-survival members, associated with the fact that knockout mice for them do not present a particular phenotype, suggest a complementary role for these two members (17). However, based on the constant endogenous expression of *BIM* and *PUMA* found in the four MM subgroups, it would be pertinent to address the question whether they have complementary roles or not in this pathology.

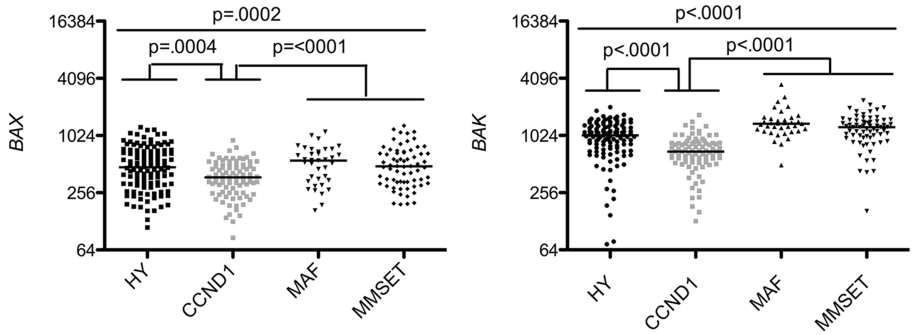

FIGURE 4 | Affymetrix data from untreated patients were analyzed for *BAX* and *BAK* expression using the Amazonia database in the different molecular groups classified as above in Figure 3. Probe sets are indicated in **Figure 1**. Median values were indicated. Statistical analyses were conducted as in **Figure 3**.

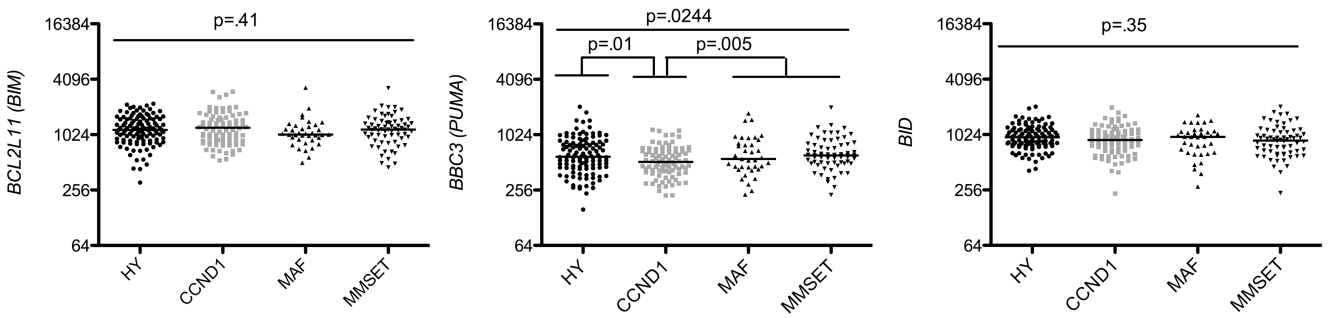

FIGURE 5 | Affymetrix data from untreated patients were analyzed for *BIM, BBC3,* and *BID,* expression using the Amazonia database in the HY, CCND1, MAF, and MMSET molecular groups. Probe sets are indicated in **Figure 2**. Median values were indicated. Statistical analyses were conducted as in **Figure 3**.

In contrast to BH3-only activators, sensitizers (*BIK*, *NOXA*, *BAD*) were heterogeneously expressed in the different MM subtypes. However, each MM subgroup highly expressed at least one sensitizer (**Figure 6**). These results are consistent with the fact that sensitizer BH3-only proteins may have overlapping functions (18). In this respect, it was previously shown that knockout mice for either Bik or Noxa proteins do not develop spontaneous tumors (17, 19). Interestingly, we found that few patients in some subgroups lacked Bik, according to our previous finding showing that some MM cell lines do not express Bik at the protein level (20). Deletions and epigenetic alterations have been shown to contribute to the lack of Bik expression (21, 22). TEF, a PAR-bZIP transcription factor, was identified as a direct activator of BIK promoter (21). We have shown in MM cell lines, that Bik was expressed only in the presence of TEF mRNA (20). However, despite TEF expression, some cell lines did not express Bik. Altogether, these results suggest that lack of Bik might be the result of either an epigenetic alteration or a deletion, as frequently described in other cancers (22).

In addition, *NOXA* allowed discriminating CCND1 from HY patients (5 median fold change) and also MMSET from MAF patients (6.68 median fold change) (**Figure 6**). Although the difference of *NOXA* expression was impressive among the four subtypes, its role in MM physiopathology remained to be determined.

Hyperdiploid patients expressed significantly higher levels of *BAD* than all other subgroups (1.35 median fold change) (Kruskall–Wallis $p < 0.0001$), which may be explained by the localization of *BAD* on chromosome 11q1 (**Figure 6**). Indeed, chromosome 11 trisomy is one the most frequent anomaly in the HY subgroup (23).

CONCLUDING REMARKS

Noteworthy, our analysis demonstrated that the combined profile of the three anti-apoptotic molecules (Bcl-2, Bclx-$_L$, and Mcl-1) was sufficient to discriminate the different MM molecular groups. The CCDN1 subgroup was identified as a particular entity, characterized by a $BCL2^{high}$ $MCL1^{low}$ and a low expression of pro-apoptotic effectors and BH3-only *(PUMA*, *BIK*, and *BAD*) with the exception of high expression of *NOXA*. Since Noxa interacts only with Mcl-1, which is weak in this subtype, we can hypothesize that the anti-apoptotic function of Mcl-1 is totally neutralized by Noxa and that this subtype relies mainly on Bcl-2 for survival. Altogether, the apoptotic machinery of this myeloma subtype is very different from that of other subtypes, suggesting that specific therapeutic approaches should be

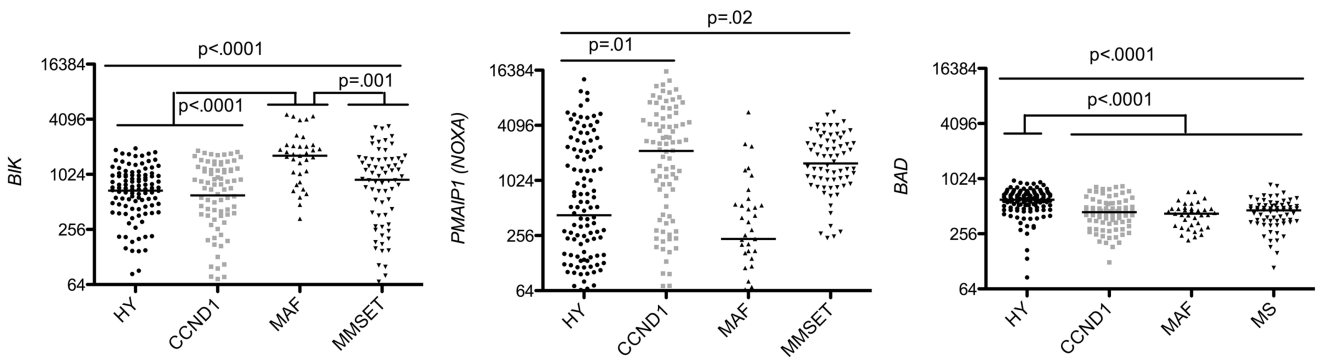

FIGURE 6 | Affymetrix data from untreated patients were analyzed for *BIK, PMAIP1,* and *BAD* expression using the Amazonia database in the HY, CCND1, MAF, and MMSET molecular groups. Probe sets are indicated in **Figure 2**. Median values were indicated. Statistical analyses were conducted as in **Figure 3**.

investigated to target CCDN1 patients. We can also hypothesize that the specificity of this subgroup may reflect a specific origin of malignant PC immortalization. This is also supported by the fact that patients harboring a t(11;14) have a high prevalence of IgM isotype and represent a distinct biological and clinical subgroup (24). Furthermore, recurrent translocations also correlated with particular features (25). Indeed, t(11:14) translocations exhibited a mature lymphoplasmocytoid morphology with a higher incidence of non-secretory MM. In contrast, t(4;14) MM subtype revealed a morphology of immature plasma cells with a significant amount of plasmablasts (25, 26). The HY subgroup shares some similarities with CCND1 in the expression pattern of Bcl-2 family members, particularly high *BCl2* and low *MCL1* levels. However, HY patients expressed higher levels of pro-apoptotic members (*BAX, BAK*, and *BAD*) than those of the CCND1 subgroup (**Figures 5** and **6**). This could favor the apoptotic response to chemotherapy and therefore explain in part the better outcome of these patients. In contrast, MAF and MMSET subgroups differ from CCDN1 and HY in the expression of anti-apoptotic members and were characterized by low *BCL2* and high *MCL1* levels. Surprisingly, MAF and MMSET subgroups expressed high level of effectors, particularly Bak, suggesting their ability to trigger an effective drug response. It will be intriguing to define whether the high expression of effectors in MAF and MMSET subgroups may be related to the fact that bortezomib-containing regimens could overcome the poor prognosis associated with t(4;14) (27). Paradoxically, these subgroups have the poorest outcome, highlighting a potential role of Mcl-1 in chemoresistance. A potential and interesting approach to target efficiently MAF and MMSET patients would be to use pharmacological inhibitors of Mcl-1, which are currently under development (28).

To summarize, our analysis supports the notion that MM heterogeneity extends to the composition of the Bcl-2 family content in each MM subgroup, which should be taken into account for therapeutic intervention in the new approach of personalized therapies. Finally, a better knowledge of Bcl-2 expression patterns may be relevant to address the origin of malignant PC.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2013; accepted: 04 December 2013; published online: 23 December 2013.
*Citation: Gomez-Bougie P and Amiot M (2013) Apoptotic machinery diversity in multiple myeloma molecular subtypes. Front. Immunol. **4**:467. doi: 10.3389/fimmu.2013.00467*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 31 March 2014
doi: 10.3389/fimmu.2014.00137

Immunophenotype of normal and myelomatous plasma-cell subsets

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Plasma cells (PCs) are essentially characterized by the co-expression of CD138 and CD38, which allows their identification in flow cytometry in bone marrow (BM), peripheral blood, or cell suspensions from tissues. These terminally differentiated B-cells may lose the expression of surface CD19 and that of CD20 while retaining CD27. When malignant, they can gain a number of other markers such as CD28, CD33, CD56, or CD117 and lose CD27. Moreover, since each PC is only able to produce a single type of immunoglobulins (Igs), they display isotypic restriction and clonal malignant PCs can be further characterized by their homogeneous expression of either kappa or lambda light chains. In multiple myeloma (MM), such PC clones produce the Ig identified in plasma as an abnormal peak. In the BM where they essentially accumulate, these PCs may however display various immunophenotypes. The latter were explored in a two-way approach. Firstly, the various subsets delineated by the selective or common expression of CD19 together with combined CD56/CD28 were explored in normal and MM BM. Then, other aberrant markers' expression was investigated, i.e., CD20, CD27, CD33, CD56, CD117. These data were compared to literature information. They underline the vast heterogeneity of MM PCs possibly accounting for the various answers to therapy of MM patients.

Keywords: plasma cells, multiple myeloma, flow cytometry, bone marrow, immunophenotype

INTRODUCTION

Plasma cells (PC) represent the terminal differentiation stage of mature effector B-cells. They result from the clonal proliferation of an activated B-cell after antigen recognition, yielding both immunoglobulin (Ig)-producing PC and memory B-cells increasing the individual's ability to generate a humoral immune response upon further encounter of the same antigen. In multiple myeloma (MM), such a PC clone becomes dysregulated and keeps proliferating and secreting Igs, identifiable in the patient's serum as a monoclonal Ig peak. Regular measurements of this peak level allow to appreciate patients' response to therapy. An assessment of the bone marrow (BM) infiltration by malignant PC provides complementary information at diagnosis, in evaluating the importance of the clone. Such an evaluation is especially useful during follow-up and therapy if the serum Ig peak disappears, i.e., allowing to characterize minimal residual disease (MRD) (1). PCs are first identified morphologically on BM smears. Flow cytometry (FCM) is a valuable addition to this observation, allowing to differentially appreciate the subsets of coexisting normal and MM PC. FCM relies on an estimation of the restricted usage of Ig light chains by the clonal population, usefully completed by an assessment of other immunophenotypic features of PC. Choice of the latter, however, is complicated by the diversity of aberrant markers reported to be expressed on clones and subclones of MM PC. Quite a number of studies in the literature have reported on the immunophenotype of both normal and MM PCs (2–5). The most salient feature of normal PC, compared to mature B-cells, is the co-expression of CD138 and CD38, which allows their identification in FCM in BM, peripheral blood (PB), or cell suspensions from tissues (6). These terminally differentiated B-cells may lose surface expression of the pan-B marker CD19 and stop expressing CD20 while retaining the memory-associated antigen CD27. Identification of intracytoplasmic light chains allows to appreciate the polyclonal nature of BM-infiltrating normal PC, which use kappa and lambda light chains at a ratio comprised between 0.76 and 2.21 [(7) and personal data].

When malignant, PC can gain a number of other markers such as CD28, CD33, CD56, or CD117 and lose the memory-associated antigen CD27. However, since each PC is only able to produce a single type of Igs, they display isotypic restriction and clonal malignant PCs can be further characterized by their homogeneous expression of either kappa or lambda light chains.

In 2011, Peceliunas et al. (8) reported on the characterization of different subsets among normal PC, using a two-tubes, six-colors approach combining the investigation of surface CD45, CD19, CD38, CD138, CD20, and CD56. This allowed identifying four different PC subsets based on the expression or not of CD19 and CD56. The latter were dubbed "normal" PC when expressing CD19 in the absence of CD56, "aberrant" when lacking CD19 yet expressing CD56, and respectively "double positive" and "double negative" for the other two. In a series of 11 normal BM samples, all subsets were observed in 5 subjects, 5 were lacking "double positive" PC (median for positive cases, 3%), and 1 was lacking a significant amount of "aberrant" PC (threshold 10^{-3}). The subset of "normal" CD19+/CD56− PC represented between 37 and 72% of BM PC. The median proportions of aberrant and double

negative PC were respectively 10 and 30%. These data obtained in normal BM were compared to those seen in 27 BM samples from relapsed/refractory MM patients. The authors concluded that the presence of these various subsets in normal PC limited the interest of FCM for the identification of MM PC. Of note, light-chain restriction was not investigated by these authors.

We developed along the same lines an FCM strategy separating BM PC in four subgroups, based on the co-expression or not of surface CD19 and of CD28/CD56. In addition, however, light-chain usage restriction was investigated in each of these compartments, allowing to accurately segregate normal and malignant PC. We further explored PC in MM patients by assessing the presence, absence, or partial expression of other differentiation antigens. Data reported here highlight the great heterogeneity of MM PC, as identified by FCM, indicating that subclones, detectable at diagnosis, may drive the evolution of this disease in spite of hopefully efficient therapies.

MATERIALS AND METHODS

A total of 139 samples from MM patients (138 BM and 1 CSF) at diagnosis were stained for FCM after Ficoll density gradient separation (3 samples) or red cell lysis (Versalyse, Beckman Coulter, Miami, FL, USA), then permeabilized (Intraprep, Dako, Glostrup, Denmark) for the detection of intracytoplasmic light-chains usage (7). As shown in **Table 1**, in a first tube, the seven-color combination CD45 (to gate leukocytes), CD19, CD38, CD138, CD28+CD56, for surface staining, completed by intracytoplasmic investigation of kappa and lambda chains was used to explore the four different subsets combining the use of CD19 and CD56+CD28. These subsets were selectively examined among the whole population of CD38+/CD138+ PC. In 132 of these samples, PC expression of CD28 and CD56 was also investigated separately. Additionally, several other PC surface markers were assessed individually in the other combinations shown in **Table 1**: CD20 ($n = 130$), CD27 ($n = 124$), CD33 ($n = 131$), and CD117 ($n = 130$). A series of 26 normal BM samples from BM donors or hospitalized patients without hematological malignancy (mostly ITP) was studied with the same seven-color combination of tube 1.

All samples were incubated using at least 10^6 nucleated cells. Labelings were analyzed on FACSCanto II flow cytometers (BD Biosciences, San Jose, CA, USA) using the Diva (BD Biosciences) software as reported elsewhere (7). Statistical analyses (medians, ranges, partition, graphs) were performed using MedCalc (Marienkirche, Belgium).

RESULTS

In MM patients, light-chain restricted PC represented a median of 97% of total CD38+/CD138+ PC, levels ranging between 76.5 and 100%. Kappa chains were used predominantly (93 cases, 67%). PCs were detected in all MM samples (median 7.5% of nucleated cells, range 0.04–91) and all normal BM samples (median 0.29% of NC, range 0.037–1.2).

Independently of light-chain restriction, the four expression patterns of CD19 and CD56/CD28 were identified as groups (1) CD19+/(CD56/CD28)−, (2) CD19+/(CD56/CD28)+, (3) CD19−/(CD56/CD28)+, and (4) CD19−/(CD56/CD28)−. As shown in **Figure 1**, the predominant immunophenotypic population in MM samples was that of PC lacking CD19 and expressing either CD56 or CD28 (group 3). Medians were respectively 0.3, 0.5, 96.5, and 1.2% of all PC for the four subsets. Of note, PCs with a "normal" immunophenotype (group 1) were at very low levels, with a maximal value of 12.8% except in two patients for whom this was the major monoclonal population with light-chain usage restriction. Light-chain usage was polyclonal in the other samples of this group, as these cells represented the normal component of BM PC, besides the MM population. Only one subset was seen in 50 samples (group 1, $n = 1$, group 3, $n = 48$, group 4, $n = 1$). A combination of groups 2 and 3 was observed in 23 cases, of groups 3 and 4 in 28, and of the three groups 2, 3, and 4 in 32. Finally, in six cases, light-chain restricted cells were seen in all four possible subsets (1, 2, 3, and 4). The size of clonal subsets in one of the groups could be as small as 0.1% of the PC.

By contrast, in normal BM samples, a median of 53.7% of PC was found to retain CD19 expression alone (group 1) and 7% expressed CD19 together with CD28/CD56 (group 2). However, 12% expressed one or both of the latter markers in the absence of CD19 (group 3) and 23% were completely negative for these surface antigens (group 4). Each of these four populations was however clearly polyclonal and displayed a normal partition of kappa and lambda light-chains usage, with a median K/L ratio of 1.27. **Figure 2** displays the polyclonal subsets of normal BM PCs in the four FCM compartments of control BM. By contrast; **Figure 3** shows how the light-chain restricted MM PCs could be retrieved in the various compartments.

Separate exploration of MM PC retrieved CD56 expression in 74% of the cases and of CD28 in 40%. **Figure 4** shows the partition of the various co-expression patterns observed for these two differentiation antigens on MM PC, the most frequent being $CD56^+CD28^-$.

Table 1 | Panels of antibodies used for immunophenotyping of MM PC.

	FITC	PE	PC5	PC7	APC	APC H7	V450
Tube 1	Lambda	CD56+CD28	CD138	CD19	Kappa	CD45	CD38
Tube 2	CD14	CD56	CD138	CD19	CD33	CD45	CD38
Tube 3	CD20	CD22	CD138	Control	CD19	CD45	CD38
Tube 4	Control	CD28	CD138	CD19	CD117	Control	CD38
Tube 4	CD45	Control	CD138	CD27	Control	Control	CD38

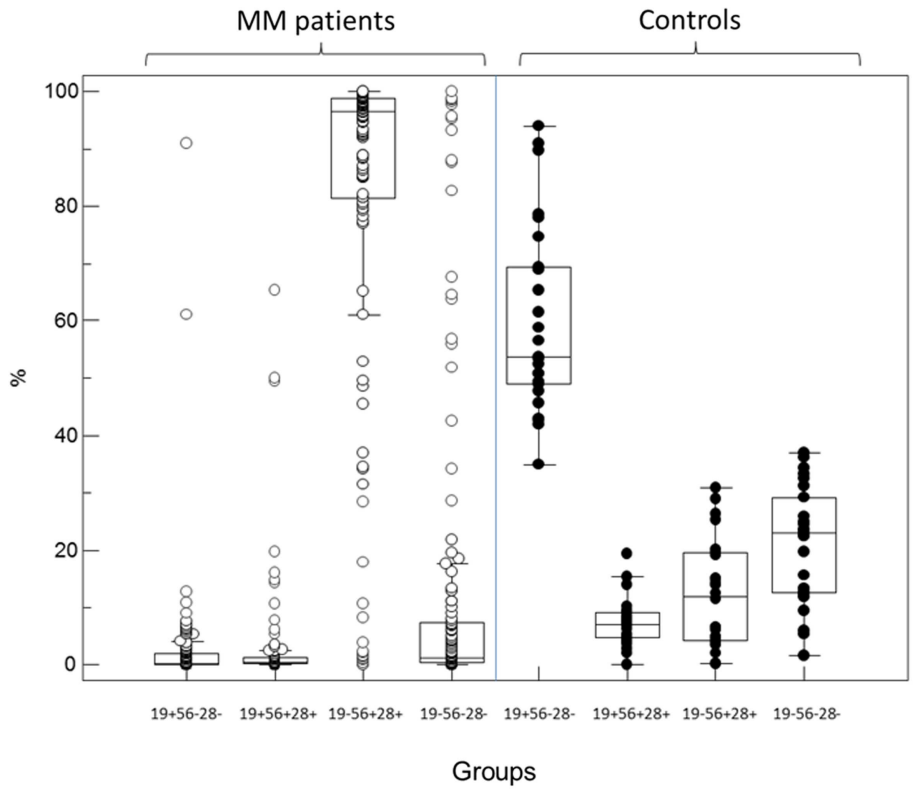

FIGURE 1 | Partition of PC percentages among the four immunophenotypic subgroups defined by the expression or not of CD19 and the combination CD56/CD28 in MM patients (left, open circles) and controls (right, black circles). In diagnosis samples from MM patients, most of the abnormal plasma cells belong to the CD19− CD56+/28+ subgroup 3 while most but not all normal PC in controls retain expression of CD19 in the absence of CD56 and CD28 (subgroup 1).

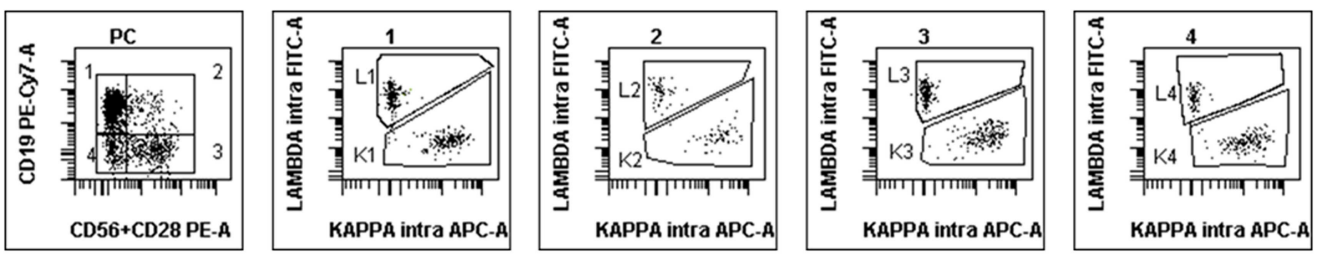

FIGURE 2 | Example of FCM scattergrams of a control BM sample. The left panel shows the partition in four subgroups of CD38+/CD138+ cells (initial gate, not shown) according to the expression or not of CD19 and the combination of CD56/CD28. Most of normal PC are in subgroup 1 but cells can also be seen in the other compartments. Polyclonality is confirmed by the investigation of cytoplasmic light chains in the four subsequent scattergrams gated on groups 1–4 from left to right.

The expression of CD19 was retained on MM PC in eight patients with high levels above 50% in only five. CD20 expression was absent in 73% of the cases. Conversely, expression of CD27 was retained in 77 patients while respectively 16 and 45 cases expressed CD33 or CD117 (**Figure 5**). **Table 2** displays the heterogeneity of immunophenotypic patterns observed in this series of MM PC. Although most samples showed a homogeneous absence or presence of differentiation antigens expression on PC, quite a number showed intermediate levels indicative of putative subclones. Only follow-up would have allowed to see whether any of these subclones ultimately emerged as therapy-resistant, but this heterogeneity highlights the complexity of MM pathophysiology.

Finally, a single weak ($r = 0.16$) negative correlation was observed between CD27 and CD117 expression when comparing differentiation antigens expression.

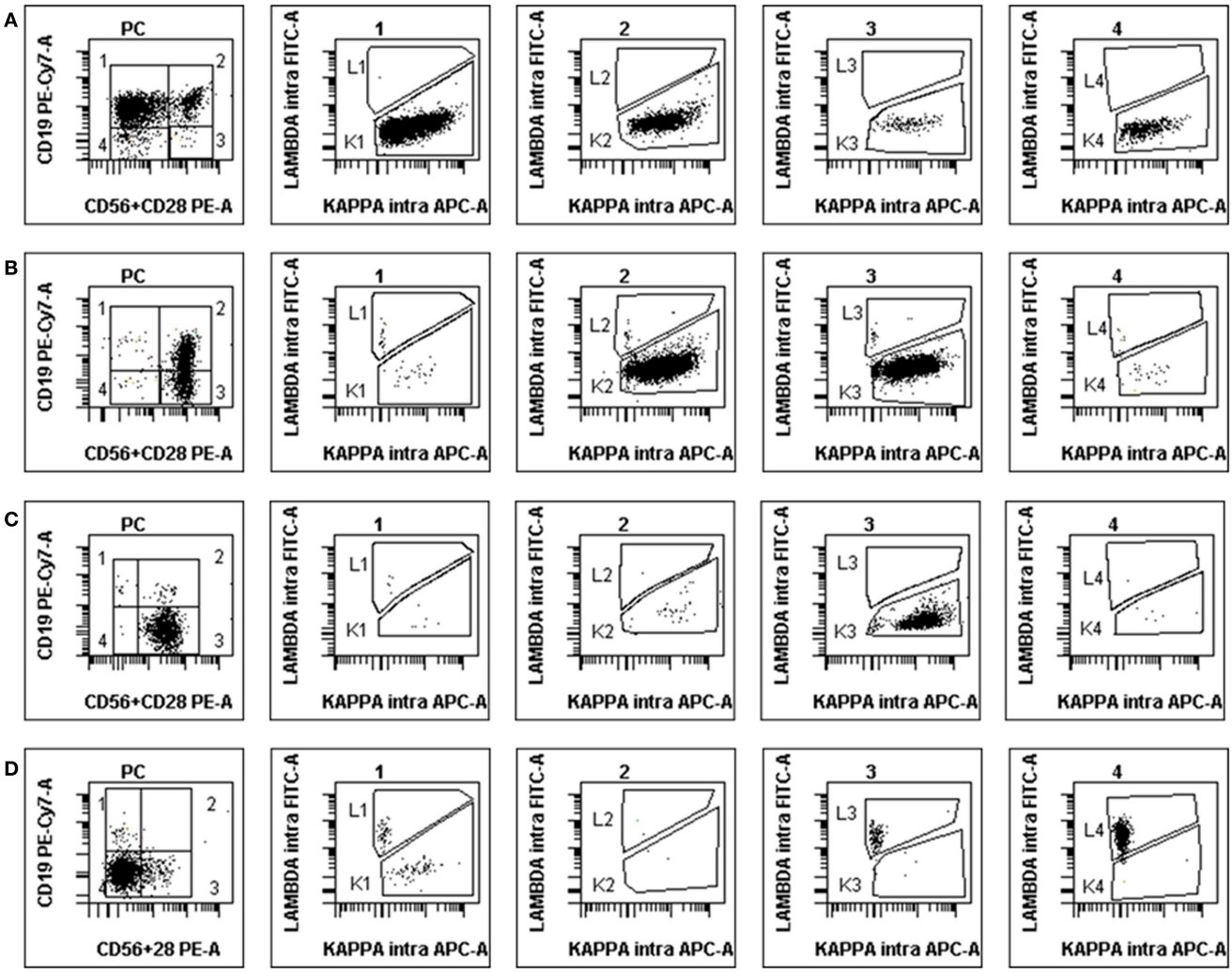

FIGURE 3 | Examples of abnormal PCs in MM BM samples at diagnosis. The disposition is the same as in **Figure 2**, light-chain expression being shown in the four subsets (1–4). **(A)** Patient with κ-restricted abnormal PC presents mostly in subgroups 1 and 2 but also 3 and 4. **(B)** Patient with κ-restricted abnormal PC presents mostly in subgroups 2 and 3, with polyclonal residual normal PC in subgroup 1. **(C)** "Classical" MM patient with the vast majority of κ-restricted abnormal PC present in subgroup 3, i.e., lacking CD19 and expressing either CD56 or CD28 (or both). **(D)** Atypical MM patient with abnormal PC most of which lack all three surface antigens yet clearly display λ-restriction in subsets 4 and 3. Note the small population of residual polyclonal normal PC in subgroup 1.

DISCUSSION

Morphological observation of PC, either the rare normal PC or sheets of abnormal MM PC on BM smears or biopsies, already discloses heterogeneity in the shape and staining of these cells. Although this could be due to the level of differentiation and secreting activity of PC (9), it could have been assumed that the clonal population producing a narrow peak of Ig in MM would be quite homogeneous. The only real level of homogeneity observed is in fact in the consistent isotype restriction of the monoclonal Ig. At first glance, the heavy involvement of patients' BM by light-chain restricted PC (median 97%) could indeed suggest a homogeneous population. However, other immunophenotypic features are quite variant within the clone, possibly denoting the presence of subclones that retained the initial rearrangement of Ig genes, but differ in other molecular features. Several data have underlined the genomic heterogeneity of MM PC (10, 11), yet this has not been straightforwardly correlated to that of their immunophenotype. Indeed, the work of Peceliunas et al. (8) described in the introductive part of this manuscript is one of the first systematic attempts at dissecting the various populations present within a rather easily defined cluster of CD38+CD138+ PC. Here, we demonstrate in a large series of MM patients at diagnosis that this heterogeneity is retrieved by the concomitant presence of PC with the same light-chain restriction in at least two and up to the four subsets defined by the CD19−CD28/CD56 combination in 64% of the patients. Moreover, even in the 50 cases where PC belonged to only one of these four groups, great heterogeneity in the other immunophenotypic markers studied was observed. This is especially true for the

markers with "partial" expression, denoting at minimal a positive and a negative subclone for the marker of interest.

One striking feature of MM PCs is their wide heterogeneity in CD45 expression, which greatly underscores the normally useful backgating of hematopoietic subsets on a CD45/SSC "cartography" (12). This has been reported previously (13, 14) and is at clear variance with the classical very homogeneous bright CD45 staining of low SSC lymphocytes (12).

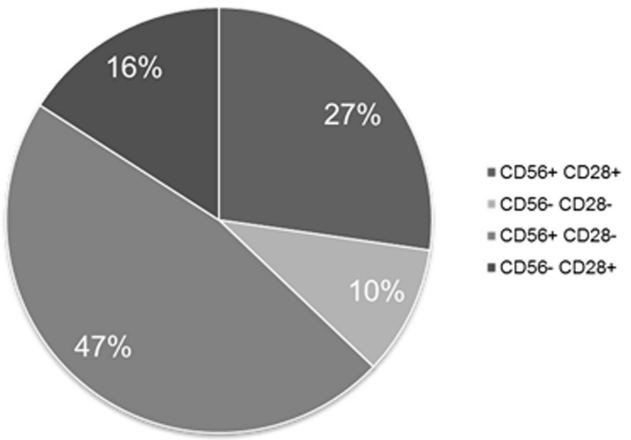

FIGURE 4 | Expression patterns of CD56 and CD28 on MM PCs when tested separately. This figure shows that 90% of MM patients express at least one of these two aberrant markers.

Other abnormal expressions of differentiation markers have been reported on PCs, but more as being useful to discriminate between normal and MM PC than by being representative of clonal heterogeneity (14). Liu et al. (14) explored the features of normal PC and reported on heterogeneous expression of CD45, high but also heterogeneous (range 52–97%) expression of CD19 and loss of CD20. They found that MM PC expressed more aberrancies than normal PC, usually with a stronger expression of such markers as CD28, CD56, or CD117. In this study, they also insisted on the importance of light-chain restriction usage determination to discriminate between polymorphic and MM PC. The review by Kumar et al. (15) and work from Paiva et al. (16) summarize the abnormalities to be expected on MM PC with regard to the major antigens explored here, i.e., CD20, CD27, CD28, CD56, CD33, CD117, with the addition of CD81, positive on normal PC but lost on MM PC. Of note, the ~30% expression of CD117 observed in our series is similar to previously reported data (17–19).

The impact of immunophenotypic variability on prognosis was hinted in 2008 by Mateo et al. (20). These authors identified three risk categories: poor risk (CD28+ CD117−), intermediate (either both markers negative or both positive), and good risk (CD28− CD117+). Here, we observed these four types of immunophenotypes, but without any correlation between CD28 and CD117 expression. Surprisingly, we observed however a negative correlation between the good prognosis markers CD117 and CD27 (21, 22). This might suggest that both these markers should be investigated at diagnosis to assess the possible evolution of the patients, and that those negative for both markers (23% in this series) might deserve special medical attention.

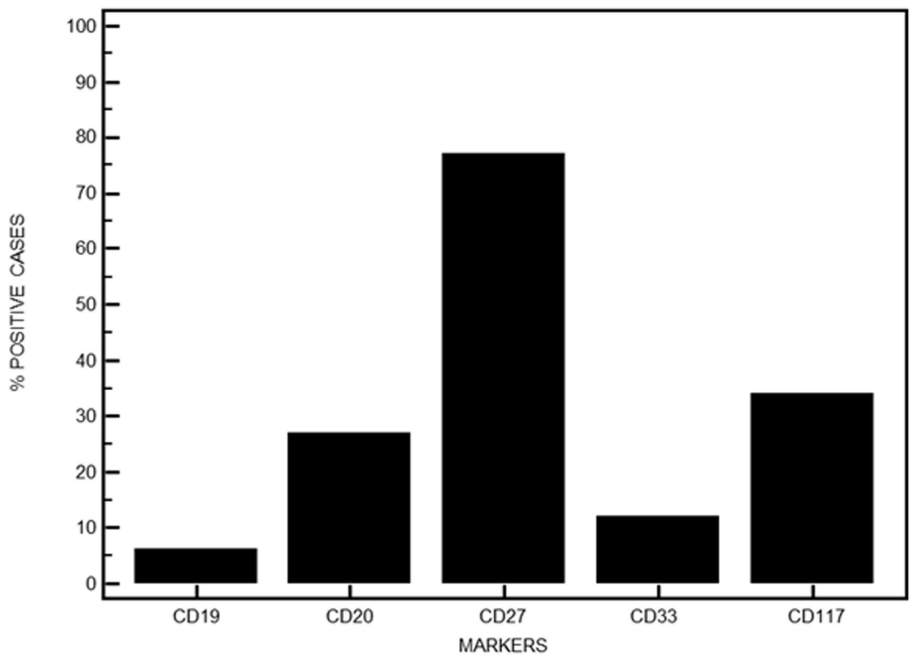

FIGURE 5 | Immunophenotypic features of MM PC when investigating other aberrant surface markers. Data are expressed in percentages for the series presented here.

Table 2 | Expression of aberrant markers on PC from MM patients at diagnosis.

	No expression (*n*)	Expression on all PC (*n*)	Intermediate expression (*n* = % expression range)
CD20	95	22	13 (15–84)
CD27	47	47	30 (12–88)
CD33	114	4	12 (7.5–86)
CD117	86	26	19 (12–91)

The retained expression of CD20, observed here in 27% of the patients is in line with previous reports (23). That this subset of patients could be accessible to therapy with anti-CD20 monoclonal antibodies has however not been confirmed so far (24).

Finally, CD33 expression, which could also potentially be accessible to immunotherapy, is in this series much lower than in an earlier paper from our group (25).

The persistence or disappearance of B-lineage or co-stimulation markers on MM PC can be understood as an evolution of the malignant cells within the B-lineage and could be a reflect of molecular events that occurred in the initial diseased B-lymphocyte. The presence of such more myeloid-restricted antigens as CD117 or CD33 could suggest anomalies developed earlier in the differentiation pattern of the malignant progenitor. CD56 expression, because of its wide promiscuity and major role as adhesion molecule, could be more related to events allowing for MM PC dissemination and contacts with the environment within the BM or in extramedullar localizations. None of these hypotheses has however been verified.

In conclusion, this work provides further information on the intrinsic heterogeneity of normal PC, retained and somewhat exacerbated in MM PC. The most obvious difference is the predominance of CD19+ CD28/56+ in normal PC, a subset clearly minor in MM patients. The most stable feature remains the restricted usage of the light chain rearranged in the initial cell, which can also be found in any of the four subsets delineated by CD19−CD28/56, as well as associated with variable levels of other differentiation antigens. Better definition of an individual patient at diagnosis might help to better track therapy-resistant subsets. Although this has not been fully investigated yet, it might appear that specific immunophenotypic patterns could be associated with a better sensitivity to specific drugs or drugs combinations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 January 2014; accepted: 18 March 2014; published online: 31 March 2014.
*Citation: Robillard N, Wuillème S, Moreau P and Béné MC (2014) Immunophenotype of normal and myelomatous plasma-cell subsets. Front. Immunol. **5**:137. doi: 10.3389/fimmu.2014.00137*
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology.