INNATE IMMUNE CELL THERAPY OF CANCER, 2nd Edition

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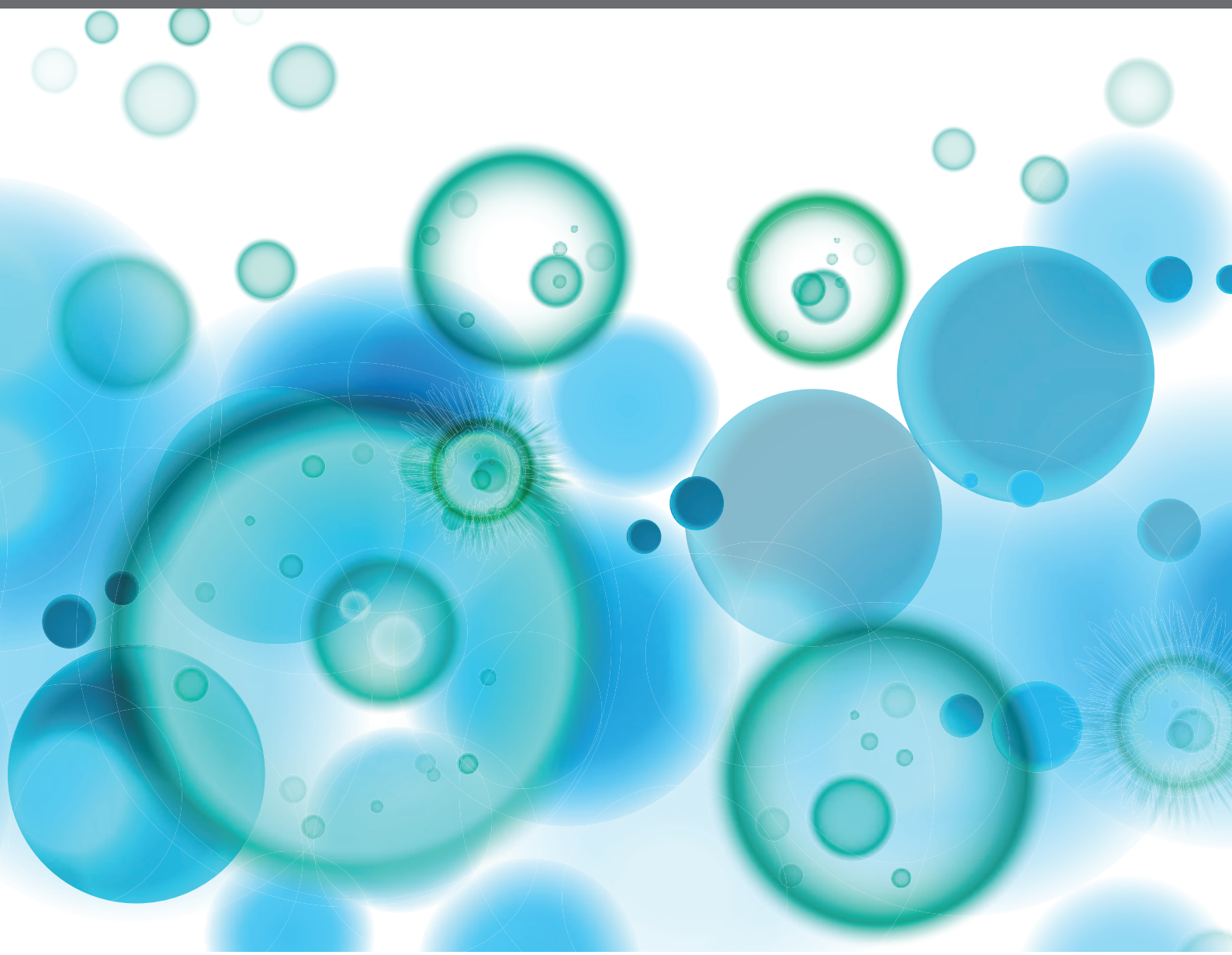

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INNATE IMMUNE CELL THERAPY OF CANCER, 2nd Edition

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Editorial: Innate immune cell therapy of cancer

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Editorial on the Research Topic
Innate immune cell therapy of cancer

According to data from clinicaltrials.gov, the past year has seen a 33% growth in research and development of innate cellular immunotherapies (1). As of April 2022, the majority of cellular therapy trials in oncology involve chimeric antigen receptor-transduced T cells (CAR-T) with roughly 82% of these being pre-clinical and phase I studies and only 0.4% (6 total) FDA-approved (1). Major limitations to these CAR-T approaches include: 1) "off-tumor" toxicities including cytokine release syndromes, 2) potential for graft-versus-host disease (GVHD) associated with allogeneic third-party ("off-the-shelf") T cell sources, 3) suboptimal killing by fatigued or senescent autologous T cells, and 4) poor recovery and expansion of autologous T cells from heavily pre-treated cancer patients.

Owing to their lack of HLA-restriction and ability to regulate allo-responses (1, 2) while addressing several of the above limitations to standard adoptive cellular immunotherapies (ACIs), innate immune cells (NK, NKT, γδT, and myeloid cells) may present a safe, robust, and cost-effective "off-the-shelf" improvement over conventional CAR-T immunotherapies. This Research Topic focuses on innate immune cells as the next frontier in ACI by highlighting some recent pre-clinical and clinical advances in innate ACI platforms against various malignancies. In this regard, it is notable that CAR strategies were originally envisioned and designed by the Campana research group for innate immune targeting and activation (3).

In this edition, Rossi et al. overview key features of NK cells that make them promising ACIs. NK cells kill target cells by perforin-mediated cytolysis, antibody-dependent cell cytotoxicity (ADCC), and/or cytokine/chemokine release. While therapeutic NK cells are easily sourced from peripheral blood (PB), mature PB NK cells can be functionally heterogeneous with low persistence *in vivo* post-adoptive

transfer. To circumvent this limitation, Goldenson et al. use induced pluripotent stem cells (iPSCs) to produce functionally homogenous NK cells that can be reliably engineered for enhanced targeting, anti-tumor function, and persistence. Karvouni et al. overview recent key advances in non-CAR and CAR-engineered NK cells in pre-clinical and clinical settings, including novel combinatorial strategies with anti-angiogenic agents, oncolytic virotherapy, and monoclonal antibodies.

What defines a robust killer cell? Barnes et al. review criteria to consider when selecting optimal NK cell populations for ACI. This article addresses the dual problems of functional heterogeneity across ACI donor sources and differences in NK subset immunobiology within a single donor, highlighting the need to develop a standardized approach to functionally characterize NK cells with robust anti-tumor activity using transcriptomics, epigenomics, and metabolomics. Given the plasticity of innate immune cells, methods are needed to closely and efficiently monitor phenotypic and functional states to identify subsets with robust anti-tumor potential prior to adoptive transfer as well as to track functional changes in the tumor microenvironment (TME) post-transfer. Iyer et al. highlight one method to simultaneously detect up to 60 parameters using Time-Of-Flight (CYTOF®) mass cytometry. The article is intended as a comprehensive overview of the mass cytometry workflow from panel design to data analysis, providing a primer for immunologists with limited expertise in the technique.

NK cells are routinely expanded *ex vivo* using continuous addition of IL-15, a cytokine essential for stimulating NK proliferation and effector functions. However, these cells are found in circulation for only a few weeks post-adoptive transfer. One outstanding question in the field is how to improve *in vivo* persistence of innate ACIs. Mishra et al. show that ADAM-17, a membrane bound metalloprotease that mediates the cleavage or "shedding" of several cell surface proteins, attenuates prolonged IL-15-induced NK cell proliferation. Upon the addition of a well-characterized anti-ADAM-17 blocking human mAb (MEDI3622), IL-15-induced NK cell expansion and proliferation significantly increased *in vitro* and for up to 3 weeks post- *in vivo* adoptive transfer. The authors underscore the potential role of ADAM-17 blockade in enhancing IL-15-induced NK cell *ex vivo* expansion and *in vivo* persistence, without the need for excessive cytokine addition which may lead to overstimulation and exhaustion.

γδT cells have also been recognized for their anti-tumor potential (4, 5). However,several unanswered basic questions limit their translation. Johanna et al. demonstrate the ability to engineer αβ T cells to express a Vγ5Vδ1 TCR (clone FE11), referred to as TEG011. The authors showed that CD4+ αβ T cells displayed enhanced anti-tumor cytotoxicity after being transduced with a CD8α-containing TEG011 construct, called TEG011_CD8α and persisted in the periphery of mice for up to 4 weeks post-transfer.

Finally, one of the major unmet needs in CAR-T based ACI is to effectively target and maintain cytotoxicity within solid tumors. Marofi et al. summarize the major developments in CAR-NK immunotherapies against a variety of solid tumors in both pre-clinical and clinical settings including neuroblastomas, gastrointestinal, breast, and ovarian cancers. Although CAR-NK cells have proven safe and feasible as "off-the-shelf" ACIs, the following barriers have yet to be addressed: (1) tumor heterogeneity; (2) tumor homing; and (3) persistence in the suppressive TME. To overcome these barriers, Sloas et al. proposed CAR-Macrophages (CAR-M). Macrophages and other myeloid cells can effectively home to tumors, navigate through the dense stroma, persist in the harsh TME and recruit various other immune cell populations to help alleviate targeting barriers due to heterogeneity. The success of macrophage and other myeloid-derived immunotherapies will depend on engineering of CAR-Ms, as well as in preventing them from being reprogrammed by the suppressive TME.

The reviews and original articles in this Research Topic are curated with an eye to provide both depth of understanding and breadth of overview of the existing and upcoming landscape of innate cellular therapy platforms for cancer, highlighting some key challenges in each area and novel approaches that may advance future developments in the field. Key areas of need beyond the scope of this Special Edition include the development of pre-therapy guidelines and standardized approaches for patients with specific cancer sub-types or strategies to determine the best personalized ACI approach for individual patients. Successful application of these immunotherapies will also require a more rigorous understanding of tumor immune environments and associated alterations following various ACI approaches. These areas are expected to be the focus of the next generation of preclinical studies and clinical trials.

Author contributions

NK wrote the manuscript. RR and AP wrote and edited the manuscript. AP conceived the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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.

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ORIGINAL RESEARCH
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Activation of ADAM17 by IL-15 Limits Human NK Cell Proliferation

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Natural killer (NK) cells are innate cytotoxic lymphocytes that can recognize assorted determinants on tumor cells and rapidly kill these cells. Due to their anti-tumor effector functions and potential for allogeneic use, various NK cell platforms are being examined for adoptive cell therapies. However, their limited *in vivo* persistence is a current challenge. Cytokine-mediated activation of these cells is under extensive investigation and interleukin-15 (IL-15) is a particular focus since it drives their activation and proliferation. IL-15 efficacy though is limited in part by its induction of regulatory checkpoints. A disintegrin and metalloproteinase-17 (ADAM17) is broadly expressed by leukocytes, including NK cells, and it plays a central role in cleaving cell surface receptors, a process that regulates cell activation and cell-cell interactions. We report that ADAM17 blockade with a monoclonal antibody markedly increased human NK cell proliferation by IL-15 both *in vitro* and in a xenograft mouse model. Blocking ADAM17 resulted in a significant increase in surface levels of the homing receptor CD62L on proliferating NK cells. We show that NK cell proliferation *in vivo* by IL-15 and the augmentation of this process upon blocking ADAM17 are dependent on CD62L. Hence, our findings reveal for the first time that ADAM17 activation in NK cells by IL-15 limits their proliferation, presumably functioning as a feedback system, and that its substrate CD62L has a key role in this process *in vivo*. ADAM17 blockade in combination with IL-15 may provide a new approach to improve NK cell persistence and function in cancer patients.

Keywords: natural killer cell, ADAM17 (a disintegrin and metalloprotease 17), CD62L, proliferation, IL-15

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INTRODUCTION

NK cells interrogate cells in the body for infection and transformation and eliminate these cells by rapidly induced effector activities, including a potent cytolytic process (1). The importance of these innate lymphocytes in cancer immunosurveillance is highlighted in NK cell deficient or depleted animal models where their absence results in failure to reject tumors (2). In addition, NK cell functional abnormalities in humans correlate with an increased risk of certain types of cancer (3). Human peripheral blood NK cells are identified as $CD56^{+}$ $CD3^{-}$, and their effector activities are

rapidly induced by numerous germline-encoded receptors that respond to ligands upregulated and downregulated on tumor cells as well as attached antibodies (1). In consideration of this and their potential for allogeneic use, various NK cell platforms are being evaluated for adoptive cell therapies to treat hematologic malignancies and solid tumors (4, 5).

A limitation of adoptively transferred primary NK cells is their relatively short life span (6). IL-15 is critical for NK cell development, proliferation, and persistence (7). The cytokine binds to a heterotrimeric receptor that consists of the common gamma chain (γc) subunit, the beta chain (βc) subunit (IL-2/IL-15R) shared with the IL-2 receptor, and the IL-15Rα subunit (8). Recombinant human (rh) IL-15, derived IL-15 agonists, and human IL-15 transgene expression have been examined in immunocompromised mouse models and shown to promote the proliferation and effector functions of adoptively transferred human NK cells (9–12). IL-15 is also being examined in various clinical trials (13), and Cooley et al., have recently reported the results of a first-in-human trial of rhIL-15 and allogeneic NK cell therapy for advanced acute myeloid leukemia (14). Challenges for IL-15 immunotherapy, however, include the inhibitory actions of immunological checkpoints that it induces, and thus there is a considerable emphasis on identifying new mechanisms of action that improve the functionality of IL-15 therapy in cancer patients (13).

ADAM17 is a membrane-associated protease that mediates the "cleavage or shedding" of various cell surface proteins (15–17). This process can rapidly reduce the density of various receptors on leukocytes and regulates their activation as well as cell-cell interactions (18). ADAM17 is constitutively expressed by all human peripheral NK cells and its proteolytic activity is rapidly induced by assorted stimuli (19–22), including IL-15 (23). We show that ADAM17 activity regulates IL-15-mediated NK cell proliferation *in vitro* and *in vivo*, and that the homing receptor CD62L is a substrate involved in this process. The impact of these findings is that blocking ADAM17 function in combination with IL-15 stimulation may provide a new therapeutic approach to increase NK cell proliferation and their anti-tumor function in patients.

MATERIALS AND METHODS

Reagents

The anti-ADAM17 monoclonal antibody (mAb) MEDI3622 (human IgG1) has been previously described (22, 24). All commercially available mAbs are listed in **Table 1**. Recombinant human (rh) IL-15 was obtained from the Biological Resources Branch, NCI, NIH and from R&D Systems (Minneapolis, MN).

NK Cell Isolation

Fresh human peripheral blood leukocytes from plateletpheresis were obtained from Innovative Blood Resources (St. Paul, MN). PBMCs were further enriched by Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient and then NK cells were purified by negative depletion using isolation kits from StemCell Technologies (Cambridge, MA) or Miltenyi Biotec (Auburn, CA), as per the manufacturer's instructions, with > 95% viability and ≥ 90% enrichment of $CD56^+$ $CD3^-$ lymphocytes. Viable cell counting was performed using a Countess II automated cell counter (Life Technologies Corporation, Bothell, WA).

In Vitro NK Cell Proliferation

Enriched NK cells were labeled with CellTrace Violet Cell Proliferation Dye (ThermoFisher Scientific) per manufacturer's instructions and incubated for 7 days in media containing or lacking rhIL-15 (R&D Systems), as we have previously described (25). In some experiments, MEDI3622, DREG200, and/or control IgG1 at 5μg/ml each were added to the assay, as indicated. An expansion index was calculated using FlowJo software (FlowJo, Ashland, OR) and represents the fold expansion of the overall culture based on CellTrace Violet dilution.

Human NK Cell Adoptive Transfer

The xenogeneic adoptive transfer model was performed as we have previously described (10). NOD-*scid* IL2Rgamma^{null} (NSG) mice (stock number is 005557 from Jackson Laboratory, Bar

TABLE 1 | Description of the commercial antibodies used in this study.

Antigen	Clone	Catalogue #	Company
CD56	HCD56	318318	BioLegend, San Diego, CA
CD3	HIT3a	300440	BioLegend
CD16	3G8	302038	BioLegend
CD336/NKp44	P44-8	325108	BioLegend
CD335/NKp46	9E2	331914	BioLegend
CD159a/NKG2A	Z199	A60797	Beckman Coulter, Brea, CA
CD314/NKG2D	1D11	320806	BioLegend
CD158a/KIR2DL1	HP-MA4	339504	BioLegend
CD158b1/KIR2DL2/L3	DX27	312612	BioLegend
CD158e1/KIR3DL1	DX9	312714	BioLegend
CD45	HI30	304044	BioLegend
CD62L/L-selectin	DREG56	304810	Biolegend
CD62L/L-selectin	DREG200	HB302	ATCC, Manassas, VA
CD156b/ADAM17	D1(A12)	AB00611-10.0	Absolute Antibody Limited, Oxford, UK
In vivo grade isotype control, human IgG1	CB1	C0001	Crown Bioscience, San Diego, CA
In vivo grade isotype control, mouse IgG1	CB5	C0005	Crown Bioscience

Harbor, ME) were housed in a specific pathogen-free facility. Weight matched (26-30g) female mice were subjected to whole-body preconditioning irradiation (225 cGy using an X RAD 320, Precision X-ray, North Branford, CT, USA) for consistent NK cell engraftment. Freshly, enriched human NK cells underwent initial overnight incubation in B0 media [DMEM, Ham's F12 with 10% human AB serum, Pen/Strep (1%), 2-ME (20 μm), ethanolamine (50 μm), ascorbic Acid (10 μg/ml) and sodium selenite (1.6 ng/ml)] containing 2.5 ng/ml rhIL-15 (NCI), and $4x10^6$ cells were injected *via* tail vein in each mouse. Mice were also administered rhIL-15 (NCI) *ip* at dose of 5 μg. The indicated mAbs were *ip* administered at a dose of 10 mg/kg. A schematic of the treatment schema is provided in **Figure 2A**. Blood was collected *via* retro-orbital route in heparin. Absolute counting of human NK cells in the peripheral blood was performed on a flow cytometer using a bead counting method (AccuCheck, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Flow Cytometric Analyses

NK cells were stained with the indicated antibodies and examined by flow cytometry, as previously described (22). For controls, fluorescence minus one was used as well as appropriate isotype-matched antibodies since NK cells express Fc receptors. An FSC-A/SSC-A plot was used to set an electronic gate on leukocyte populations, and an FSC-A/FSC-H plot was used to set an electronic gate on single cells. To distinguish live vs. dead cells, 7AAD was used as per the manufacturer's instructions (Biolegend, San Diego, CA).

Statistical Analysis

Data were analyzed using Prism Graph Pad software. Student's t-test or one-way ANOVA with multiple comparisons were used to determine statistical significance among groups.

RESULTS

ADAM17 Blockade Increases NK Cell Proliferation by IL-15

We have previously reported that IL-15 stimulation of human NK cells activates ADAM17 in short-term experiments (≤ 24 hours) (23). We examined here if the sheddase had a role in NK cell proliferation during prolonged IL-15 stimulation. ADAM17 was blocked using MEDI3622, a human IgG1 mAb that is well characterized for its inhibitory activity *in vitro* and *in vivo* (24, 26, 27), and also blocks ADAM17 activity in human NK cells (22). Enriched NK cells were labeled with CellTrace dye, cultured for seven days in media containing or lacking rhIL-15 and/or MEDI3622, and then the cells were assessed for dye dilution. In the presence of rhIL-15, NK cells demonstrated increased dye dilution and thus proliferation (**Figure 1A**). We found that in the presence of MEDI3622, but not an isotype-matched control antibody, NK cell proliferation was greatly augmented (**Figure 1A**). NK cells treated with MEDI3622 alone, however, did not undergo a significant increase in proliferation (**Figure 1A**). The same effects on NK cell proliferation were observed when using PBMCs (**Figure 1B**). Moreover, ADAM17 blockade increased the sensitivity of NK cells to IL-15 for proliferation (**Supplementary Figure 1A**).

We next evaluated the effects of blocking ADAM17 on NK cell expansion *in vivo*. Administration of human IL-15 or when expressed by a transgene stimulates the proliferation of transferred human NK cells in immunocompromised mice (9, 10, 12). As shown in the treatment schema in **Figure 2A**, NSG mice were administered enriched NK cells ($4x10^6$) *iv*, rhIL-15 (5μg) *ip*, and/or MEDI3622 (10mg/kg) *ip*, and then circulating NK cells levels were monitored for 3 weeks. The adoptive transfer of NK cells in rhesus macaques revealed that these cells initially accumulated in the lung and by 24 hours they returned to the circulation (28). A similar process occurred for transferred human NK cells in immunocompromised mice (9). Therefore, we initially bled mice two days post-NK cell transfer to determine their baseline recirculating levels. Human NK cells ($CD45^+$ $CD56^+$ $CD3^-$) were identified by a specific cell gating approach (**Supplementary Figure 1B**) and enumerated using cell counting beads. Baseline circulating levels of human NK cells were equivalent in mice treated with or without rhIL-15 (**Figure 2B**), whereas over time, their levels increased when in the presence of rhIL-15 (**Figure 2B**), as expected (10). In a separate experiment, baseline levels of circulating human NK cells from a different donor were again equivalent in mice treated with rhIL-15 in the presence or absence of MEDI3622, but by two weeks post-transfer, NK cell levels were significantly higher in the MEDI3622-treated mice (**Figure 2C**). The levels of NK cell expansion by IL-15 varied between donors (**Figures 2B, C**), and though the enhancement of this process by ADAM17 blockade was consistent, the levels of augmented proliferation also varied considerably between donors (**Figure 2D**, panels 1-6). Similar to the *in vitro* assays, the treatment of mice with control IgG did not enhance IL-15-mediated NK cell expansion (**Figure 2D**, panel 7), and treating mice with MEDI3622 alone did not induce NK cell expansion (**Supplementary Figure 1C**). D1 (A12) is another function-blocking an anti-human ADAM17 mAb (29), and it also increased NK cell expansion by rhIL-15 (**Supplementary Figure 1D**). Collectively, our data reveal that ADAM17 induction in IL-15-stimulated NK cells reduces their proliferation potential.

ADAM17 Regulates the Surface Density of CD62L on Proliferating NK Cells

ADAM17 has a broad array of substrates expressed by diverse cell types (15–17), and a small number of these are expressed by human NK cells (18). One very well described substrate of ADAM17 is CD62L (L-selectin), which is a "homing receptor" that directs most leukocytes from the blood into various tissue locations (30). Essentially all $CD56^{bright}$ NK cells and a subset of $CD56^{dim}$ NK cells in the peripheral blood express CD62L (31), and it undergoes a rapid downregulation in expression following IL-15 stimulation (32). This process was greatly reduced by MEDI3622 treatment (**Supplementary Figure 1E**). MEDI3622 treatment also resulted in markedly higher levels of CD62L on

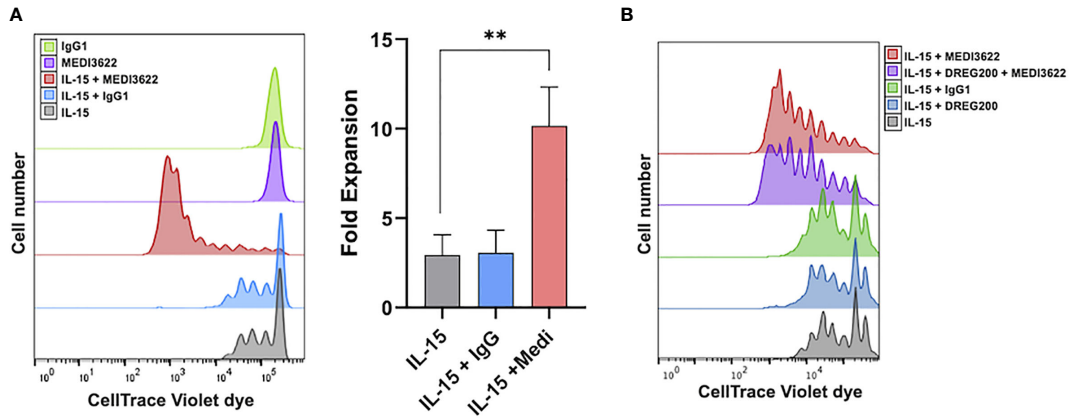

FIGURE 1 | ADAM17 blockade enhances human NK cell proliferation by IL-15 *in vitro*. **(A)** Enriched NK cells were labeled with CellTrace Violet dye and placed in culture for 7 days with rhIL-15 (10ng/ml) and/or MEDI3622 (5μg/ml) and/or control human IgG1 (5μg/ml), as indicated. Cells were then harvested and examined for CellTrace dye dilution by flow cytometry. Data are representative of 3 independent experiments using leukocytes from separate donors. An expansion index was calculated as described in the Methods and is the fold expansion of the overall culture for each condition based on dye dilution. Data are means ± SD of three independent experiments using separate donors. Statistical significance is indicated as **$p < 0.01$. Statistics were calculated using one-way ANOVA. **(B)** Human PBMCs were labeled with CellTrace Violet dye and placed in culture for 7 days with rhIL-15 (10ng/ml), MEDI3622 (5μg/ml), control human IgG1 (5μg/ml), and/or DREG200 (5μg/ml). Cells were then harvested and examined for CellTrace dye dilution by flow cytometry. Data are representative of 3 independent experiments using leukocytes from separate donors.

proliferating NK cells stimulated with IL-15 (**Figure 3A**). This was not entirely expected since prolonged stimulation of T cells has been shown to induce CD62L downregulation mainly by reduced gene transcription (33). ADAM17 also regulated the cell surface density of CD62L on NK cells *in vivo* during their proliferation by IL-15. NSG mice were treated as illustrated in **Figure 2A**, and at three weeks post-NK cell transfer, CD62L levels were evaluated on the circulating NK cells. In the presence of rhIL-15 and MEDI3622, NK cells had significantly higher levels of CD62L than did NK cells from mice treated with rhIL-15 alone (**Figure 3B**). We observed that the expression levels of various other cell surface determinants on proliferating NK were not affected by MEDI3622 treatment, including a sample of inhibitory and activating receptors (**Figure 3C**), though NKG2D expression was modestly increased (**Figure 3C**). The mechanism for this is unclear at this time and we are not aware of any studies showing that NKG2D is a substrate of ADAM17. Taken together, our findings reveal that CD62L is expressed at high levels during NK cell proliferation induced by IL-15, but undergoes considerable shedding by continuous ADAM17 activation.

CD62L Is Required for NK Cell Expansion *In Vivo*

CD62L is involved in the migration of NK cells into lymphoid and peripheral tissues (34–36). The expansion of transferred human NK cells in immunocompromised mice occurs at various locations, including the spleen, liver, and bone marrow (9, 10, 12). We examined the contribution of CD62L to human NK cell proliferation *in vivo*. NSG mice were administered human NK cells alone or NK cells plus rhIL-15 in the presence or absence of DREG200, a well-described anti-human function-blocking mAb previously used in *in vivo* studies (37–39). Baseline levels of circulating NK cells were equivalent in all groups (**Figure 3D**, left panel). In contrast to mice that received NK cells and rhIL-15, very little NK cell expansion was observed in the presence of rhIL-15 and DREG200 (**Figure 3D**, left panel). Moreover, the enhanced expansion of NK cell that occurred in the presence of rhIL-15 and MEDI3622 was also blocked by DREG200 (**Figure 3D**, middle panel). Administration of control IgG instead of DREG200 did not affect NK cell proliferation by rhIL-15 (**Figure 3D**, right panel). These findings reveal that CD62L is critical for IL-15-mediated NK cell expansion *in vivo*, and thus its shedding by ADAM17 would likely impair their accumulation and/or stimulation in expansion niches. However, ADAM17 has numerous substrates that regulate cell activation and cell-cell interactions (17), and therefore substrates in addition to CD62L may also have a role in NK cell expansion. Indeed, we found that blocking CD62L by DREG200 did not affect IL-15-mediated NK cell proliferation *in vitro* (**Figure 1B**).

DISCUSSION

NK cells respond to various ligands and attached antibodies on tumor cells, resulting in natural cytotoxicity and antibody-dependent cell-mediate cytotoxicity (ADCC), respectively. NK cells also release several anti-tumor cytokines and chemokines that modulate other leukocyte subsets of the innate and adaptive immune system (1). Due to their assorted anti-tumor activities, adoptive NK cell therapies are being examined in a number of clinical trials (4). NK cells, however, tend to be shorter-lived cells following adoptive transfer and so cytokine stimulation is being examined to promote their expansion and survival (6, 7). Current strategies are focused on IL-15 and related agonists,

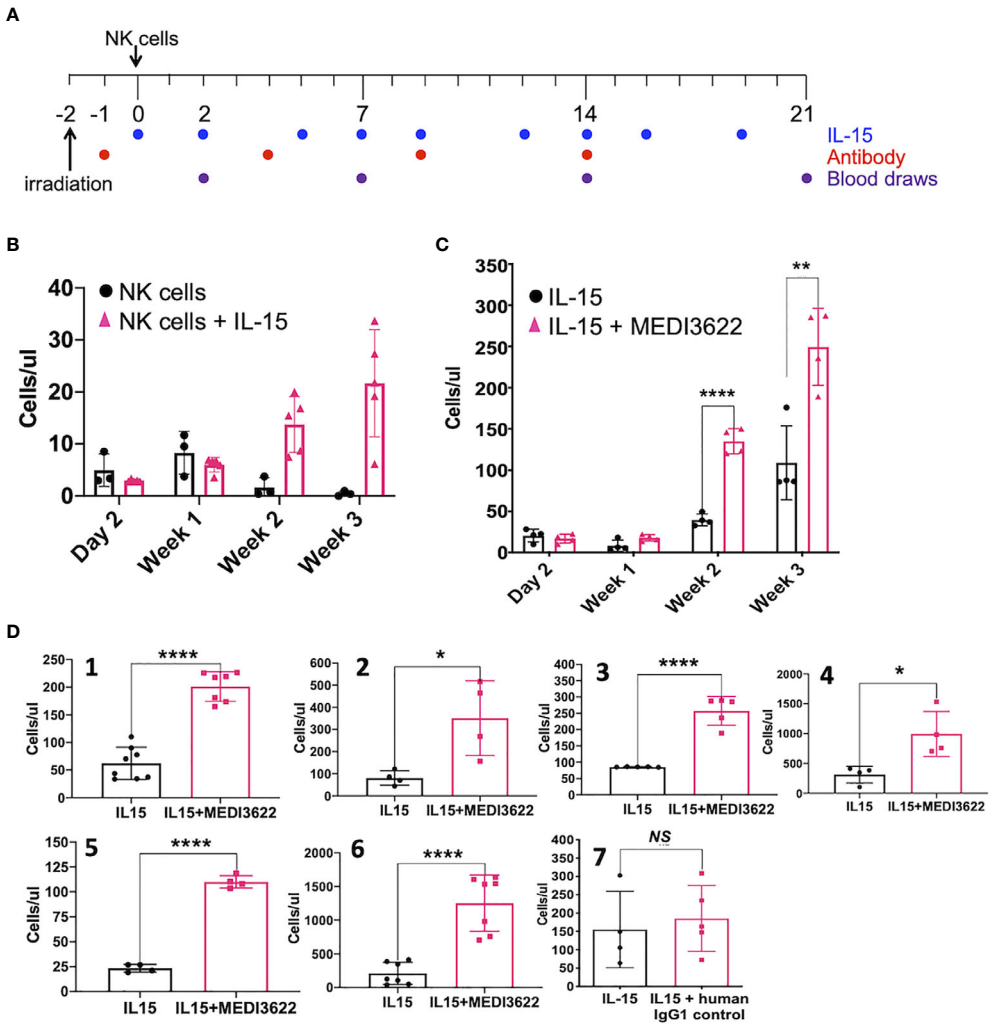

FIGURE 2 | ADAM17 blockade enhances human NK cell proliferation by IL-15 *in vivo*. **(A)** Animal treatment schema. NSG mice were treated as described in the Methods. **(B)** Enriched NK cells were infused in the presence or absence of rhIL-15 (5μg), as indicated. Mouse peripheral blood was collected and the number of human $CD45^+$ $CD56^+$ $CD3^-$ NK cells were enumerated by flow cytometry and are shown as cells/μl. Data are mean ± SD (n = 3 to 5 mice per group). **(C)** Additional mice were administered enriched NK cells from a separate donor plus rhIL-15 (5μg) ± MEDI3622 (10 mg/kg). Data are mean ± SD (n = 4 mice per group). $^{**}p < 0.01$; $^{****}p < 0.0001$. **(D)** The experiment was performed as described in panel **(C)** NK cells were obtained from six separate donors (panels 1-6). Mice were also treated with rhIL-15 in the presence or absence of a human IgG1 control mAb (panel 7). Mouse peripheral blood was collected at day 21 following NK cell adoptive transfer and human $CD45^+$ $CD56^+$ $CD3^-$ NK cells were enumerated by flow cytometry. Group data was tested for normality (Kolmogorov-Smirnov test) and the differences in means were calculated by comparing means ± SD using an unpaired two-sided Student's t-test. n = 3 to 7 mice per group. $^*p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$; ns, not significant.

which induce a potent proliferative signal for NK cells (13). Results from our study show that IL-15 activates ADAM17 upon short-term and prolonged stimulation of human NK cells. We show that blocking this sheddase resulted in a significant increase in NK cell proliferation both *in vitro* and *in vivo*. These findings thus suggest that ADAM17 activity during extended NK cell stimulation functions like a feedback system to modulate their proliferation, presumably through the cleavage of one or more critical substrates.

CD62L is a well characterized substrate of ADAM17 on leukocytes (16, 30), including NK cells (22, 23). CD62L is expressed by $CD56^{bright}$ NK cells and a subset of $CD56^{dim}$ NK cells (31), and of interest is that both NK cell subsets have been reported to be capable of high proliferation following cytokine stimulation (40). We show that cell surface levels of CD62L were markedly higher on proliferating NK cells *in vitro* and *in vivo* when blocking ADAM17. Childs and Berg have reported that *ex vivo* human NK cell proliferation in medium containing IL-15 with nicotinamide resulted in increased CD62L expression and NK cell expansion in immunodeficient mice (41). To directly examine whether CD62L may have a role in the *in vivo* expansion of human NK cells by IL-15, we blocked its function in a xenograft mouse model. This resulted in a dramatic reduction in NK cell expansion in the absence as well as

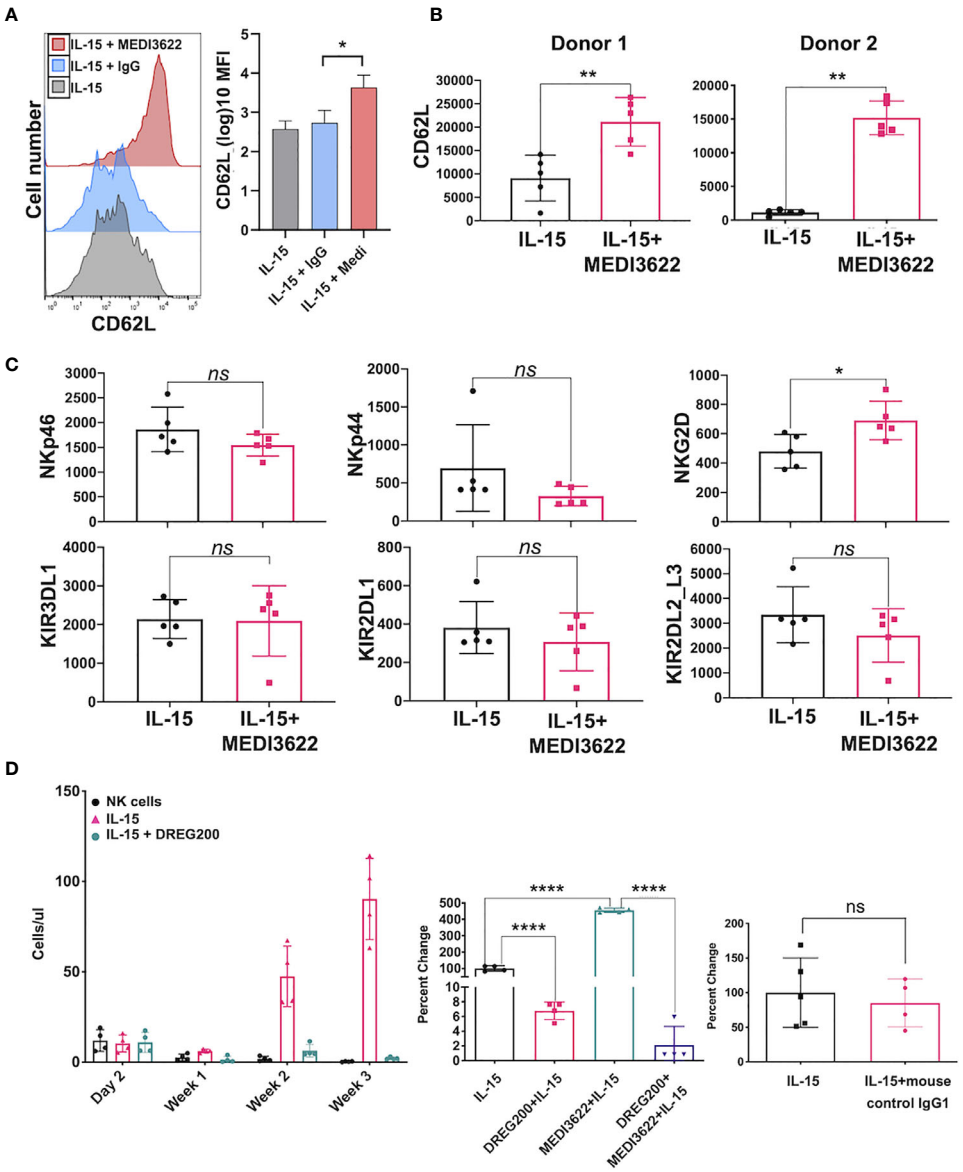

FIGURE 3 | CD62L expression during NK cell proliferation and its role in their expansion in *vivo*. **(A)** NK cells were placed in culture for 7 days in the presence of rhIL-15 (10ng/ml) alone or in the presence of MEDI3622 (5 μg/ml) or an isotype-matched negative control mAb (IgG, 5 μg/ml). CD62L levels were determined by flow cytometry. The histograms show representative data and the bar graph shows mean ± SD of 3 independent experiments using leukocytes from separate donors. *$p < 0.05$. The y-axis on the bar graph indicates mean fluorescence intensity (MFI). Statistics were calculated as described in **Figure 1**. **(B)** Mice were administered enriched NK cells and rhIL-15 (5μg) in the presence or absence of MEDI3622 (10 mg/kg). After 3 weeks, mouse peripheral blood was collected, and relative CD62L expression levels were determined on human $CD45^+$ $CD56^+$ $CD3^-$ NK cells by flow cytometry. Two separate experiments are shown using NK cells from different donors. The y-axis on the bar graphs indicates MFI. Data are mean ± SD (n = 5 mice per group). **$p < 0.01$. **(C)** The experiment was performed as described in panel B and various cell surface markers were evaluated. Data are representative of two separate experiments using NK cells from different donors. Data are means ± SD (n = 5 mice per group). *$p < 0.05$; ns = not significant. Data was analyzed by using unpaired two-tailed student's t-test. **(D)** Mice were administered NK cells and rhIL-15 (5μg) in the presence or absence of DREG200 (10mg/kg) (left panel), DREG200 and/or MEDI3622 (10mg/kg) (middle panel), or mouse IgG isotype-matched mAb (10mg/kg) as a control for DREG200 (right panel). The number of NK cells in the peripheral blood were enumerated by flow cytometry and are shown as cells/μl or as percent change normalized to NK cells plus rhIL-15. Data are means ± SD (n = 4 to 5 per group). ****$p < 0.0001$; ns, not significant. Data was analyzed by using unpaired two-tailed student's t-test.

presence of an ADAM17 function-blocking mAb. The expansion of transferred human NK cells in immunocompromised mice occurs at various locations (9, 10, 12). At this time, it has not been determined whether CD62L might direct NK cells to various expansion niches or primarily to one location, where upon initial proliferation these cells then traffic to other expansion niches. Blocking ADAM17 and CD62L shedding in other innate leukocytes, such as neutrophils, during sterile

inflammation and infection also enhanced their migration to tissue locations (42, 43). Higher CD62L expression levels thus represent a potential underlying mechanism accounting for the increased expansion of NK cells upon blocking ADAM17. Indeed, others have reported that relatively small changes in L-selectin density can have significant effects on leukocyte migration (44, 45).

ADAM17 has numerous substrates and several regulate leukocyte activation (16, 17). Therefore, substrates in addition to CD62L may also play a role in NK cell expansion. Interestingly, we found blocking CD62L did not affect IL-15-mediated NK cell proliferation *in vitro*. Further studies will be required to identify additional mechanisms, direct and/or indirect, by which ADAM17 activation modulates NK cell proliferation. One candidate is IL-15Rα, a component of the trimeric receptor complex that binds IL-15, which has been reported to be cleaved by ADAM17 (46). Abrogation of its shedding could increase IL-15 presentation to IL-2/IL-15Rβ/γc on NK cells and enhance their stimulation and expansion.

Continuous IL-15 stimulation of NK cells has been reported to induce exhaustion (47). Thus, it will be important to assess the effects of ADAM17 blockade on the functional state of IL-15-stimulated NK cells. During NK cell exhaustion, various activating receptors have been shown to undergo downregulation, including NKG2D (48). We observed that NKG2D expression was modestly increased on IL-15-expanded human NK cells in mice treated with MEDI3622, and that other activating and inhibitory receptors did not significantly change in expression. Clinical studies have shown IL-15 administration to also have dose-limiting toxicities (13). Blocking ADAM17 in combination with IL-15 administration might allow for the administration of lower levels of the cytokine or its agonists and still achieve efficacious NK cell expansion, but with less toxicity or NK cell exhaustion. In support of this, we found that the treatment of NK cells with MEDI3622 increased their sensitivity to IL-15. Blocking ADAM17 may have additional anti-tumor effects as well. We have shown that ADAM17 inhibition increased NK cell ADCC and their production of INFγ (22, 49). Blocking ADAM17 activity in tumor cells can also enhance NK cell cytotoxicity. For instance, MHC class I-related chain molecules A and B (MICA and MICB) and natural cytotoxicity triggering receptor 3 ligand 1 (NR3LG1), also referred to B7-H6, are widely expressed by tumor cells (50, 51), and they have been reported to be substrates of ADAM17 (52–55). MICA/B and NR3LG1 are ligands of the NK cell activating receptor NKG2D and NKp30, respectively, and blocking their shedding increased tumor cell killing by NK cells (55, 56). The above findings reveal that ADAM17's impact on NK cells is diverse (e.g., effector functions, proliferation, and trafficking) and multifactorial, thus addressing the effects of its inhibition on their function, especially *in vivo*, is complex.

In summary, our data demonstrates that rapid and prolonged induction of ADAM17 activity occurs in human NK cells stimulated by IL-15 and that this can limit their proliferation. CD62L is shown to play a role in IL-15-mediated NK cell expansion *in vivo* and its shedding by ADAM17 represents a potential underlying mechanism by which the sheddase regulates NK cell proliferation. A potential impact of these studies is that blocking ADAM17 may provide a therapeutic approach to increase NK cell proliferation by IL-15 and their anti-tumor function in patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Minnesota.

AUTHOR CONTRIBUTIONS

HM, KD, and BW collected, assembled, analyzed and interpreted the data, and wrote the manuscript. NP contributed vital reagents. MF and JM analyzed and interpreted the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.711621/full#supplementary-material

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Adding Help to an HLA-A*24:02 Tumor-Reactive γδTCR Increases Tumor Control

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γδT cell receptors (γδTCRs) recognize a broad range of malignantly transformed cells in mainly a major histocompatibility complex (MHC)-independent manner, making them valuable additions to the engineered immune effector cell therapy that currently focuses primarily on αβTCRs and chimeric antigen receptors (CARs). As an exception to the rule, we have previously identified a γδTCR, which exerts antitumor reactivity against HLA-A*24:02-expressing malignant cells, however without the need for defined HLA-restricted peptides, and without exhibiting any sign of off-target toxicity in humanized HLA-A*24:02 transgenic NSG (NSG-A24:02) mouse models. This particular tumor-HLA-A*24:02-specific Vγ5Vδ1TCR required CD8αα co-receptor for its tumor reactive capacity when introduced into αβT cells engineered to express a defined γδTCR (TEG), referred to as TEG011; thus, it was only active in $CD8^+$ TEG011. We subsequently explored the concept of additional redirection of $CD4^+$ T cells through co-expression of the human CD8α gene into $CD4^+$ and $CD8^+$ TEG011 cells, later referred as TEG011_CD8α. Adoptive transfer of TEG011_CD8α cells in humanized HLA-A*24:02 transgenic NSG (NSG-A24:02) mice injected with tumor HLA-A*24:02$^+$ cells showed superior tumor control in comparison to TEG011, and to mock control groups. The total percentage of mice with persisting TEG011_CD8α cells, as well as the total number of TEG011_CD8α cells per mice, was significantly improved over time, mainly due to a dominance of $CD4^+CD8^+$ double-positive TEG011_CD8α, which resulted in higher total counts of functional T cells in spleen and bone marrow. We observed that tumor clearance in the bone marrow of TEG011_CD8α-treated mice associated with better human T cell infiltration, which was not observed in the TEG011-treated group. Overall, introduction of transgenic human CD8α receptor on TEG011 improves antitumor reactivity against HLA-A*24:02$^+$ tumor cells and further enhances *in vivo* tumor control.

Keywords: cancer immunotherapy, TEGs, mouse model, preclinical (*in vivo*) studies, TCR engineering, human leukocyte antigens (HLA), persistence, efficacy

INTRODUCTION

γδT cells share the properties of both innate and adaptive immunity and play an essential role in cancer immunosurveillance (1, 2). Unlike conventional αβT cells, γδT cells recognize their cognate antigens in an MHC-unrestricted manner, targeting stress-induced and malignantly transformed self-antigens (3, 4). As such, γδT cells represent an attractive cell subset to substantiate T cell-based immunotherapeutic strategies that still mainly focus on αβT cells.

Based on their TCRδ chain repertoire, two major subsets of γδT cells can be distinguished: Vδ2$^+$ and Vδ2$^-$ cells. Vδ2$^+$ cells mainly reside in the human peripheral blood, representing up to 5% of total circulating T cells, and sense metabolic changes in tumor cells with intracellular accumulation of phosphoantigens (pAgs) level. Vδ2$^+$ T cell recognition is facilitated by butyrophilin (BTN) family molecules, including BTN2A1 and BTN3A1 (5–10). On the other hand, Vδ2$^-$ cells mainly localize in mucosal and epithelial tissues, but their antitumor properties are scarcely known (4). Vδ2$^-$ cells recognize a broad range of stress-induced ligands, such as the MHC-associated proteins MICA and MICB, foreign lipid antigens presented on CD1c/d molecules in classical HLA-like manner, and CMV-associated UL16-binding protein (ULBP) family members, that are upregulated in stressed or malignant cells (11–15).

Vδ1$^+$ T cells, one of the major Vδ2$^-$ subsets, have been shown to exert antitumor reactivity against leukemia and solid tumors (16–21), indicating their potential in cancer immunotherapy. Adoptive transfer of *in vitro* expanded Vδ2$^+$ cells only showed marginal clinical responses to date (4, 22), while adoptive transfer of Vδ2$^-$ cells is yet to be tested in the clinic (23). Translational efforts using γδT cells and their receptors outside the context of allogeneic stem cell transplantation (24, 25) face substantial hurdles, due to their limited proliferative capacity, underestimated diversity in co-receptors expression and function, as well as scarce information on how γδTCRs interact with their targets.

To bypass these major drawbacks of translating γδT cells-based immune therapies into clinical practice, we developed the concept of TEGs: αβT cells engineered to express a defined γδTCR, allowing the introduction of highly tumor-reactive γδTCR, both Vδ2$^+$ (26, 27) or Vδ2$^-$ (28, 29) subsets, into proliferatively-proficient αβT cells (27, 30, 31). This concept did not only allow to select for highly tumor-reactive γδTCR, but also within the context of Vδ2$^+$ TCRs to reprogram both CD4$^+$ and CD8$^+$ αβT cells (26, 27). Professional help for TCR-engineered CD8$^+$ αβT cells by also functionally engineering CD4$^+$ αβT cells has not only been shown to be important *in vitro* (32) but also to improve clinical responses (33). Within this context, we previously identified an allo-HLA-restricted and tumor-specific Vγ5Vδ1TCR derived from clone FE11, introduced in the TEG concept as TEG011, which was, although not dependent on a defined peptide, selectively targeting HLA-A*24:02$^+$ tumor cells without impairing the healthy tissues (34). Furthermore, we also highlighted that antitumor reactivity of Vγ5Vδ1TCR derived from clone FE11 requires CD8α as costimulatory receptor and showed that both CD8αα on the original clone FE11 and CD8αβ on transduced αβT cells are capable of providing costimulation to the Vγ5Vδ1TCR derived from clone FE11 (34). Thus, for this very particular Vγ5Vδ1TCR, the concept of TEGs would not benefit from reprogramming CD4$^+$ αβT cells when only a Vγ5Vδ1TCR is transferred as CD4-transduced TEG011 cells do not elicit antitumor reactivity.

Human CD8 is a membrane glycoprotein classified in an immunoglobulin-like superfamily consisting of hetero- or homodimer of α and β chains, making up for the CD8αβ or CD8αα co-receptor on the cell surface. CD8αβ predominantly expressed on αβT cells, while CD8αα mainly expressed on the cell membrane of innate immune cells, including macrophages, dendritic cells, natural killer (NK) cells, and γδT cells (35). Transfer of CD8 receptor has been reported for αβTCR engineered αβT cells to functionally reprogram CD4$^+$ αβT cells, when low to intermediate affinity αβTCRs are used for engineering (36). Within this context, we addressed the implication of CD8αα-dependency of FE11 γδTCR in relation to its tumor immunity. Based on this mechanistic basis of antitumor reactivity for TEG011 cells, we hypothesize that the transfer of CD8α receptor can functionally rescue Vγ5Vδ1TCR engineered CD4$^+$ αβT cells. Within this context, we explored now as additional approach to improve the efficacy of TEG011 therapy, the simultaneously co-expressing Vγ5Vδ1TCR derived from clone FE11 together with CD8α receptor in a TEG format, referred to as TEG011_CD8α. Importantly, we demonstrate that introduction of transgenic human CD8α co-receptor into CD4$^+$ TEG011 cells successfully enhanced its antitumor efficacy *in vitro* and *in vivo*, and thus did not require CD8β. Furthermore, we show that the co-expression of CD8α in CD4$^+$ TEG011 provides additional survival signal and facilitates better T-cell persistence and infiltration *in vivo*, both of which are essential to sustain long-term tumor control of adoptively transferred TCR-based immunotherapy.

MATERIALS AND METHODS

Cell Lines

Daudi, SW480, and Phoenix-Ampho cell lines were obtained from ATCC. K562 with HLA-A*24:02-transduced cell line was kindly provided by Fred Falkenburg (Leiden University Medical Centre, Netherlands) and subsequently transduced with luciferase for *in vivo* imaging purposes. EBV-LCL was kindly provided by Phil Greenberg (Seattle, WA, USA). Phoenix-Ampho and SW480 cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco), whereas all other cell lines in RPMI with 1% Pen/Strep and 10% FCS. All cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis and were passaged for a maximum of 2 months, after which new cell line stocks were thawed for experimental use. Furthermore, all cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit (Lonza, Breda, Netherlands). Peripheral blood mononuclear cells (PBMCs) from healthy

donors were isolated by Ficoll-Paque (GE Healthcare, Eindhoven, Netherlands) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, Netherlands).

Cloning of TEG011_CD8α and TEGLM1_CD8α

Clone FE11 was generated as previously described (28). FE11 and LM1 [non-functional γ9δ2TCR with length mutation on the complementary determining region 3 (CDR3) of the δ2-chain (31)] γδTCRs were subcloned to pMP71 retroviral vectors containing both γTCR and δTCR chains, separated by a ribosomal skipping T2A sequence. pU57 constructs containing a ribosomal skipping P2A sequence, followed by full-length human CD8α, were purchased from Baseclear (Leiden, Netherlands). Thereafter, CD8α was subcloned into pMP71 vector using *Xho*I and *Hind*III restriction sites downstream of γ115TCR-T2A-δ115_LM1 sequence to generate a TEGLM1_CD8α (**Supplementary Table 2**) construct that contained *Nco*I and *Xho*I restriction sites up- and downstream of LM1 γδTCR chains. *Nco*I and *Xho*I restriction sites were then inserted up- and downstream of FE11 γδTCR sequences by site-directed mutagenesis PCR, after which this sequence was ligated to P2A-CD8α sequence in pMP71 vector using the introduced *Nco*I and *Xho*I sites, generating a TEG011_CD8α construct (**Supplementary Table 1**). Where indicated, $CD4^+$, $CD8^+$, $CD4^+CD8\alpha\alpha^+$, and $CD4^+CD8\alpha\beta^+$ TCR-transduced T cells were sorted using a FACSAria II (BD) flow cytometry to >99% purity. Expression levels of CD8α mutants were measured by flow cytometry using anti-CD8α antibody (clones RPA-T8).

Functional T-Cell Assays

IFNγ ELISPOT was performed using antihuman IFNγ mAb1-D1K (I) and mAb7-B6–1 (II) (Mabtech) per the manufacturer's protocol. Then 15,000 TEG cells (TEG011, TEGLM1, TEG011_CD8α, or TEGLM1_CD8α) were co-incubated with 50,000 target cells (E:T ratio 1:3) for 18–24 h in nitrocellulose-bottomed 96-well plates (Millipore). IFNγ spots were visualized with TMB substrate (Sanquin), and subsequently the number of spots was quantified using ELISPOT Analysis Software (Aelvis). Where indicated, blocking of CD8α was performed using 10 μg/ml anti-CD8α antibody clone OKT8 (eBioscience) and blocking of CD8β with 10 μg/ml anti-CD8β clone 2ST8.5H7 (Abcam).

Retroviral Transductions of T Cells

TEGs were generated as previously described (30). Briefly, Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV), and pMP71 retroviral constructs containing both γTCR and δTCR chains separated by a ribosomal skipping T2A sequence and followed by CD8α sequence separated by P2A sequence where applicable, using FugeneHD reagent (Promega, Leiden, Netherlands). PBMCs from a healthy donor preactivated with 30 ng/ml anti-CD3 (clone OKT3, Miltenyi Biotec) and 50 IU/ml IL-2 (Proleukin, Novartis, Arnhem, Netherlands) were transduced twice with viral supernatant within 48 h, in the presence of 50 IU/ml IL-2 and 6 μg/ml polybrene (Sigma-Aldrich, Zwijndrecht, Netherlands). TCR-transduced T cells were expanded by stimulation with anti-CD3/CD28 Dynabeads (500,000 beads/10^6 cells; Thermo Fisher Scientific, Breda, Netherlands) and 50 IU/ml IL-2. Thereafter, transduced T cells were depleted of the non-engineered T cells.

Depletion of Non-Engineered T Cells

Non-engineered T cells were depleted as previously described (27). In brief, transduced T cells were incubated with a biotin-labeled anti-αβTCR antibody (clone BW242/412; Miltenyi Biotec, Leiden, Netherlands) and then incubated with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec), most recently reported to preferentially bind to the βTCR chain (37). Thereafter, the cell suspension was loaded onto an LD column, and $\alpha\beta TCR^+$ T cells were depleted by MACS cell separation per the manufacturer's protocol (Miltenyi Biotec). After depletion, TEGs were expanded using a T-cell rapid expansion protocol (REP) (30).

Separation of $CD4^+$ Subsets of TEGs

The separation of $CD4^+$ TEGs was performed using CD4 Microbeads (Miltenyi Biotech) as per the manufacturer's instructions. Briefly, TEGs that were previously expanded on REP were incubated with magnetic microbeads cells and loaded into LS column for MACS cell separation. Thereafter, $CD4^+$ selected or bulk (with CD4:CD8 ratio 50:50) TEGs were expanded separately on the next REP cycle prior to *in vitro* functional assay. TEG expression was monitored prior to functional assays or *in vivo* infusion by flow cytometry using anti-αβTCR-APC (clone IP26, eBioscience), anti-pan-γδTCR-PE (clone IMMU510, Beckman Coulter), anti-CD8-PerCP-Cy5.5 (clone RPA-T8, Biolegend), anti-CD4-PeCy7 (clone TPA-R4, Biolegend), anti-CD4-FITC (clone TPA-R4, Biolegend), and Vδ1-FITC (clone TS8.2, Thermo Fisher Scientific) antibodies.

Animal Model

The NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg(HLA-A24)3Dvs/Sz (NSG-A24:02) mice (38) were bred and housed in the breeding unit of the Central Animal Facility of Utrecht University. Experiments were conducted per institutional guidelines after obtaining permission from the local ethical committee, and performed in accordance with the current Dutch laws on animal experimentation. Mice were housed in individually ventilated cage (IVC) system to maintain sterile conditions and fed with sterile food and water. After irradiation, mice were given the antibiotic ciproxin in the sterile water throughout the duration of the experiment. Both male and female mice were randomized with equal distribution among the different groups, based on age and initial weight (measure on Day −1) into 10 mice/group. Adult NSG-A24:02 mice (11–20 weeks old) received sublethal total body irradiation (1,75 Gy) on day −1 followed by intravenous injection of 1×10^5 K562-HLA-A*24:02 luciferase tumor cells on day 0, and received 2 intravenous injections of TEG011, TEG011_CD8α, or TEGLM1_CD8α cells on days 1 and 6 as previously reported (34). Together with the first TEGs injection, all mice received $0{,}6 \times 10^6$ IU of IL-2 (Proleukin; Novartis) in 100 μl incomplete Freund's adjuvant (IFA)

subcutaneously and subsequently administered every 3 weeks until the end of the experiment. Mice were monitored at least twice a week for any symptoms of disease (sign of paralysis, weakness, and reduced motility), weight loss, and clinical appearance scoring (scoring parameter included hunched appearance, activity, fur texture, and piloerection). The humane endpoint was reached when mice showed the aforementioned symptoms of disease, experienced a 20% weight loss from the initial weight (measured on day −1), developed extramedullary solid tumor masses (if any) reached 2 cm^3 in volume, and when clinical appearance score 2 was reached for an individual parameter or a total score of 4.

Flow Cytometry Analysis

The following antibodies were used for flow cytometry analysis: huCD45-PB (clone HI30; Sony Biotechnology), pan-γδTCR-PE (clone IMMU510; Beckman-Coulter), mCD45-APC (clone 30-F11, Sony Biotechnology), αβTCR-FITC (clone IP26; Biolegend), CD4-PeCy7 (clone RPA-T4, Biolegend), CD8-PerCPCy5.5 (clone RPA-T8, Biolegend), PD-1-BV711 (clone EH12.2H7, Biolegend), and TIM3-BV650 (clone F38-2E2, Biolegend). To exclude non-viable cells from the analysis, Fixable Viability Dye eFluor506 was used (eBioscience). All samples were analyzed on a BD LSRFortessa using FACSDiva Software (BD Biosciences).

Assessment for TEGs Persistence

Mouse peripheral blood samples were obtained *via* cheek vein (max. 50–70 µl/mouse) once a week. Red blood cells were lysed using 1× RBC lysis buffer (Biolegend) and were then stained with a mixture of antibody panels as listed above. The persistence of TEG cells was counted as absolute cell number tumor-reactive TEG cells expressing following cell surface markers $huCD45^+\gamma\delta TCR^+CD8^+$ and $huCD45^+\gamma\delta TCR^+CD4^+CD8^+$ populations or non-reactive TEG cells expressing $huCD45^+\gamma\delta TCR^+CD4^+$ marker observed in mouse peripheral blood using Flow-count Fluorospheres (Beckman Coulter) and measured by flow cytometry.

Preparation of Single-Cell Suspensions

At the end of the study period, bone marrow (mixed from tibia and femur) and spleen sections were isolated and processed into single-cell suspension. Femur and tibia from the hind legs were collected; bone marrow cells were collected by centrifugation of the bones at 10,000 rpm for 15 s and resuspension of the cells in phosphate buffer saline (PBS).

A small section of the spleen was minced and filtered through a 70 µm cell strainer (BD); incubated with 1× RBC lysis buffer cells for maximum 4 min, and subsequently cells were washed and resuspended in PBS.

Absolute cell number of TEG cells were quantified using Flow-count Fluorospheres and measured from a total of 10^6 cells stained for the presence of TEG cells in spleen and bone marrow by flow cytometry analysis (BD LSRFortessa).

Histology Staining and Analysis

Formalin-fixed femur for bone marrow sections were embedded in paraffin and cut into 4 µm sections. Hematoxylin and eosin (H&E) staining was performed for the femur, for bone marrow section. Tissue sections were evaluated to assess for any differences in the presence, distribution, and extension of neoplastic foci indicating tumor tissue. Tissue sections of the femur were evaluated for quantification of tumor tissue by dividing the area covered by the tumor cells by the total area of bone marrow tissue visible in the section using the ImageJ analysis system software (NHI, Bethesda, Maryland, USA) and expressed as a percentage. Images were taken using an Olympus BX45 microscope with the Olympus DP25 camera and analyzed using DP2-BSW (version 2.2) or ImageJ software.

Statistical Analyses

Experimental data were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and shown as mean ± standard deviation (SD) or standard error of mean (SEM) with $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$. Statistical significances between groups were assessed using a non-parametric Kruskal-Wallis test, a two-way ANOVA, and a mixed-effects model with repeated measures where indicated.

RESULTS

Co-Transfer of Transgenic CD8α Receptor Is Sufficient to Re-Establish Tumor Reactivity of $CD4^+$ TEG011 Cells

We previously identified an allo-restricted CD8α-dependent Vγ5Vδ1TCR clone FE11 (28), which showed *in vitro* antitumor reactivity against HLA-A*24:02-expressing tumor cells (34). We therefore investigated whether introduction of CD8αα or CD8αβ along with Vγ5Vδ1TCR derived from clone FE11 could enhance antitumor reactivity of $CD8^+$, and also functionally reprogram $CD4^+$ TEG011 cells. Hence, we co-transduced T cells with the FE11 γδTCR, and with either CD8α alone or CD8α together with CD8β (**Figure S1**). Subsequently, we sorted separate sets of $CD4^+$ TEG011 cells that co-expressed either exogenous CD8αα ($CD4^+CD8\alpha^+$) or CD8αβ ($CD4^+CD8\alpha\beta^+$) as well as TEG011 cells expressing only endogenous CD4 and CD8 as negative and positive controls for tumor recognition, respectively (**Figure 1A**). Thereafter, TEG cells were co-cultured with SW480 and EBV-LCL target cells or healthy PBMCs as mock control. Both $CD4^+CD8\alpha^+$ and $CD4^+CD8\alpha\beta^+$ TEG011 cells secreted significantly higher levels of IFNγ upon exposure to tumor targets than $CD4^+$ TEG011 cells. The acquired antitumor reactivity of $CD4^+CD8\alpha^+$ and $CD4^+CD8\alpha\beta^+$ TEG011 cells could be blocked by CD8α and CD8β blocking antibodies (**Figure 1B**), confirming the strict dependence of FE11 γδTCR on introduced CD8 molecules. Taken together, we showed that introduction of CD8α alone is sufficient to re-establish antitumor reactivity of $CD4^+$ T cells expressing FE11 γδTCR. Introduction of CD8β did not further enhance tumor recognition but was functionally involved in the molecular interaction with its target when present.

For clinical administration, co-expression of both CD8α and the γδTCR in one vector is preferred to allow reproducible and

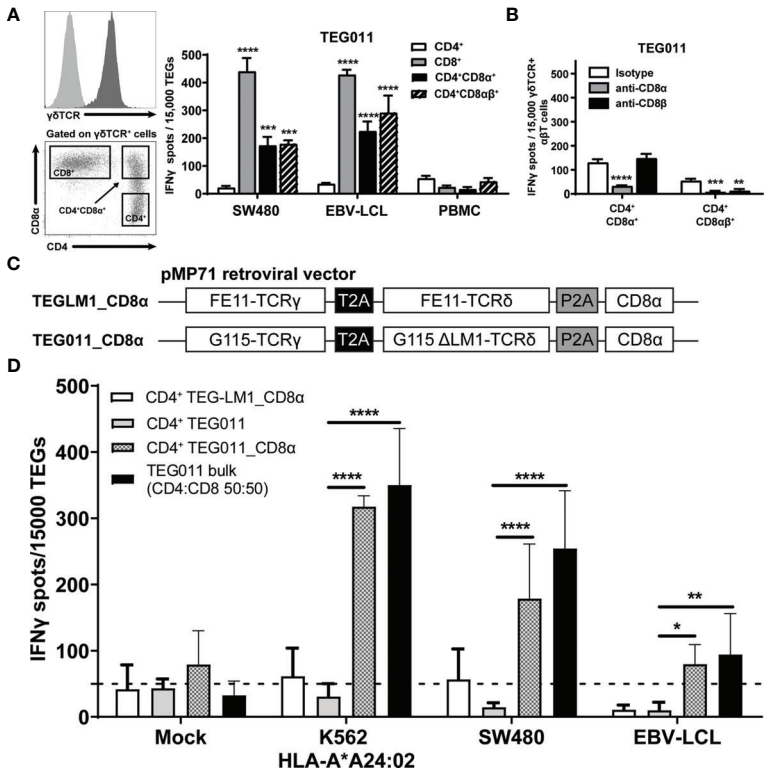

FIGURE 1 | Introduction of transgenic CD8α receptor on TEG011 improves T cell activation. **(A)** TEG011 were retrovirally transduced with either CD8α alone or CD8α in combination with CD8β. $CD4^+$, $CD8^+$, $CD4^+CD8\alpha^+$, and $CD4^+CD8\alpha\beta^+$ subsets of T cells were subsequently sorted (left panel is a representative sorting plot for $CD4^+$, $CD8^+$, and $CD4^+CD8\alpha^+$ cells; $CD4^+CD8\alpha\beta^+$ cells were sorted in a similar manner) and tested for recognition of SW480 and EBV-LCL target cells by IFNγ ELISPOT (right panel). Healthy PBMCs were included as untransformed mock control target cells. Data are of representative of four independent experiments, and error bars represent mean ± SEM (**P < 0.01; ***P < 0.001) calculated by two-way ANOVA. **(B)** CD8α and CD8β blocking on $CD4^+$ T cells were transduced with the FE11 γδTCR and CD8α alone, or CD8α with CD8β. TEG011 was co-incubated with SW480 target cells in the presence of a control antibody, or CD8α or CD8β blocking antibodies. IFNγ production was measured by ELISPOT. Data represent mean ± SD of replicates for each effector (**P < 0.01; ***P < 0.001; ****P < 0.0001) calculated by two-way ANOVA. **(C)** Schematic diagram of pMP71 retroviral vector constructs containing codon-optimized human γδTCR sequences from either clone FE11 (referred as TEG011_CD8α) or non-functional LM1 chains (referred as TEGLM1_CD8α) in combination with full length of human CD8α receptor (top panel). Within the transgene cassettes, individual γTCR and δTCR chains have been linked with a self-cleaving thosea asigna virus 2A (T2A; black box) ribosomal skipping sequence, while the CD8α sequence was connected with a porcine teschovirus-1–derived 2A (P2A; gray box) ribosomal skipping sequence. **(D)** $CD4^+$ αβT cells were transduced with either TEGLM1_CD8α, TEG011, or TEG011_CD8α γδTCR (as effector cells) and subsequently co-cultured with HLA-A*24:02-expressing target cell lines or healthy T cells (E:T ratio is 1:3) for 18–24 h. TEG011 bulk population with 50:50 ratio of both $CD4^+$ and $CD8^+$ TEGs and T cells from healthy donor were used as positive and untransformed mock controls, respectively. Antitumor reactivity was measured by IFNγ ELISPOT, where 50 spots/15,000 cells were considered as a positive antitumor response and indicated by the dashed horizontal line. Data are representative of three independent experiments with replicates for each target, and error bars represent mean ± SD (*P < 0.05; **P < 0.01; ****P < 0.0001) calculated by two-way ANOVA.

cost-effective production processes (26, 27, 39). Moreover, co-expressing both CD8α and the γδTCR in one vector can also overcome the difference in transduction efficiency when they were transduced separately. Therefore, we generated new retroviral constructs carrying either FE11 γδTCR or a non-functional length mutant clone LM1 γδTCR [(31); served as mock control] followed by full-length human CD8α receptor sequences (TEG011_CD8α and TEGLM1_CD8α, **Figure 1C**). The complete sequence of transgenes for these retroviral constructs is listed in **Supplementary Tables 1, 2**, respectively. Subsequently, αβT cells were transduced with either FE11 γδTCR without human CD8α receptor (TEG011), FE11 γδTCR with human CD8α receptor (TEG011_CD8α), or LM1 γδTCR with human CD8α receptor (TEGLM1_CD8α). After TEG expansion, we performed magnetic selection of $CD4^+$ T cells for each TEG constructs. To elucidate whether introduction of transgenic CD8α receptor adequately rescues TEG011 reactivity of non-tumor reactive CD4-transduced cells once delivered by the very same vector, we co-cultured tumor target HLA-A*24:02-transduced CML tumor cells (K562), SW480, and EBV-LVL cells with either $CD4^+$ TEG011_CD8α, $CD4^+$ TEGLM1_CD8α, or $CD4^+$ TEG011 (without introduction of the CD8α receptor). Healthy T cells and TEG011 bulk cells (with CD4:CD8 1:1 ratio) were used as the untransformed mock target and positive effector control, respectively (**Figure 1D**). $CD4^+$ TEG011_CD8α cells produced a significantly higher IFNγ level compared to $CD4^+$ TEG011, which was equivalent to those of TEG011 bulk cells against all tumor targets, without affecting

healthy cells. The equivalent IFNγ level between $CD4^+$ TEG011_CD8α and TEG011 bulk cells comprised of only 50% $CD8^+$ TEG011 implied that reprogrammed $CD4^+$ TEG011_CD8α are surprisingly poorer cytokine secretors. Importantly, enhanced tumor recognition was restricted to $CD4^+$ TEG011_CD8α cells and not $CD4^+$ TEGLM1_CD8α mock cells, highlighting the specific role of CD8α as co-stimulation for the introduced FE11 γδTCR. We concluded that introduction of transgenic CD8α receptor in combination with Vγ5Vδ1TCR derived from clone FE11 allowed reprogramming of $CD4^+$ T cells towards HLA-A*24:02-expressing tumor cells *in vitro*, though activity was lower when compared to $CD8^+$ TEG011.

TEG011_CD8α Improves *In Vivo* Tumor Control and Associates With Higher Persistence of Functional T Cells

In previous studies, we have shown TEG011 efficacy against HLA-A*24:02-expressing tumor cells *in vitro* and an extended *in vivo* safety profile, as well as peripheral persistence of TEG011, where long-term persistence of TEG associated with reduced probability for developing extramedullary solid tumor masses *in vivo* (34, 40). To assess the consequence of the additional expression of TEG011_CD8α, NSG transgenic mice expressing human HLA-A*24:02 (NSG-A24:02) were irradiated, received luciferase-labeled K562 HLA-A*24:02^+ cells, and subsequently received two intravenous injections of either mock control TEGLM1_CD8α, TEG011_CD8α, or TEG011 cells. All infused TEG variants showed comparable γδTCR expression, where the transduced αβT cells expressed $Vδ1^+$ TCR for TEG011 and TEG011_CD8α (**Figure S2**). Mice were monitored for tumor burden assessed by bioluminescent imaging, T cell persistence and infiltration, as well as any other signs of discomfort. Mice were sacrificed when the humane endpoints were reached (experimental outline **Figure 2A**). TEG011_CD8α-treated mice had a significantly lower tumor burden over time compared to the mock control TEGLM1_CD8α and TEG011-treated groups (**Figure 2B**), indicating superior tumor control *in vivo* by TEG011_CD8α. All tumor-bearing mice eventually developed tumor, and measurement of individual mouse indicating tumor growth over time for each treatment group is shown in **Figures 2C, D**. Despite the significant *in vivo* tumor control, we observed only a trend towards an improved overall survival for TEG011_CD8α-treated mice (**Figure S3**). This could be due to limited treatment window of this mouse model contributed by aggressive tumor growth of K562 HLA-A*24:02-transduced cells.

As TEG011 cells carry CD8α-dependent Vγ5Vδ1TCR, we focused our *in vivo* analysis to tumor-reactive CD8-expressing TEG cells (as validated by *in vitro* functional T cell assay in **Figure 1D**) while taking into account the non-tumor reactive $CD4^+$ TEG cells. Therefore, we assessed CD8-expressing TEG cell product properties and persistence by measuring viable $huCD45^+γδTCR^+CD8^+$ single-positive and $huCD45^+γδTCR^+CD4^+CD8^+$ double-positive cells (present in mock control TEGLM1_CD8α and TEG011_CD8α only) in mouse peripheral blood using flow cytometry (gating strategy depicted in **Figure S4**). TEG cells persisted up to 4 weeks after infusion in the mouse peripheral blood with biological variations between mice (**Figure 3A**). To address this interindividual variation in T-cell persistence, we analyzed separately the percentage of mice where $CD4^+$ and $CD8^+$ T cells reached at least 500 cells/ml in the peripheral blood over time, a threshold described previously (41) (**Figure S5A**). We observed a higher percentage of mice with persisting $CD4^+$ and $CD8^+$ T cells in TEG011_CD8α group when compared to mock TEGLM1_CD8α and TEG011 group. Despite some imbalance in the CD4:CD8 ratio with lower numbers for $CD8^+$ TEG011 infused (**Figure S2**), more $CD8^+$ TEG011 persisted over time when compared to $CD8^+$ single-positive TEG011_CD8α. *Vice versa*, endogenous CD4 T cells for TEG011_CD8α were lower before infusion when compared to TEG011 prior to infusion, while $CD4^+CD8^+$ double-positive TEG011_CD8α were higher in numbers over time when compared to both $CD4^+CD8^+$ double-positive TEGLM1_CD8α and $CD4^+$ TEG011 cells (**Figure S5B**). As a net effect, we observed more CD8-expressing T cells for TEG011_CD8α cells when compared to TEG011 (**Figure 3B**). Next, we investigated the expression of PD1 and TIM3 on $CD8^+$ single-positive cells and $CD4^+$ single-positive or $CD4^+CD8^+$ double-positive cells. Higher numbers of T cells expressing PD1 or TIM3 were observed on TEG011_CD8α cells, as compared to mock TEGLM1_CD8α and TEG011 cells (**Figures S6A, B**). $CD8^+$ single-positive TEG011 and TEG011_CD8α showed an increased PD1 expression when compared to $CD8^+$ single-positive TEG_LM1 (**Figure S6A**). A partial decline of TIM3 expression was most pronounced over time in $CD8^+$ single-positive TEG011_CD8α (**Figure S6B**).

Next, we investigated infiltration of TEG cells into spleen and bone marrow on weeks 1 and 2 after infusion. Specifically, we compared the TEG011 and TEG011_CD8α groups to elucidate the contribution of transgenic CD8α co-expression in TEG011 infiltration *in vivo*, and focused on the total sum of CD8-expressing TEG011 cells. We detected a significantly higher number of CD8-expressing TEG cells infiltrating in the spleen and bone marrow of TEG011_CD8α-treated mice at both time points (**Figure 3B**). Importantly, we did not observe rapid clearance of $CD4^+CD8^+$ double-positive TEG011_CD8α cells in these tissues within these time points, whereas $CD8^+$ single-positive TEG011 cells were barely detected. Thus, we conclude that CD8α co-stimulation with TEG011 improves overall *in vivo* tumor control, T cell persistence, and infiltration of CD8-expressing TEG011 cells.

TEG011_CD8α Enhanced T Cell Infiltration and Effectively Cleared Tumor Cells in Bone Marrow

We previously reported an extensive *in vivo* safety profile of TEG011 against healthy tissues that express HLA-A*24:02 molecules, in which no significant histological lesions were observed in major organs, including liver, spleen, and intestine (40). For histopathology analysis, we collected a femur bone marrow section from each treatment group at the end of the study period to further evaluate antitumor efficacy of the new TEG011_CD8α cells (**Figure 4A**). Tissue sections were assessed for the presence and extension of the neoplastic foci composed by round, large, undifferentiated tumor cells. The mock control

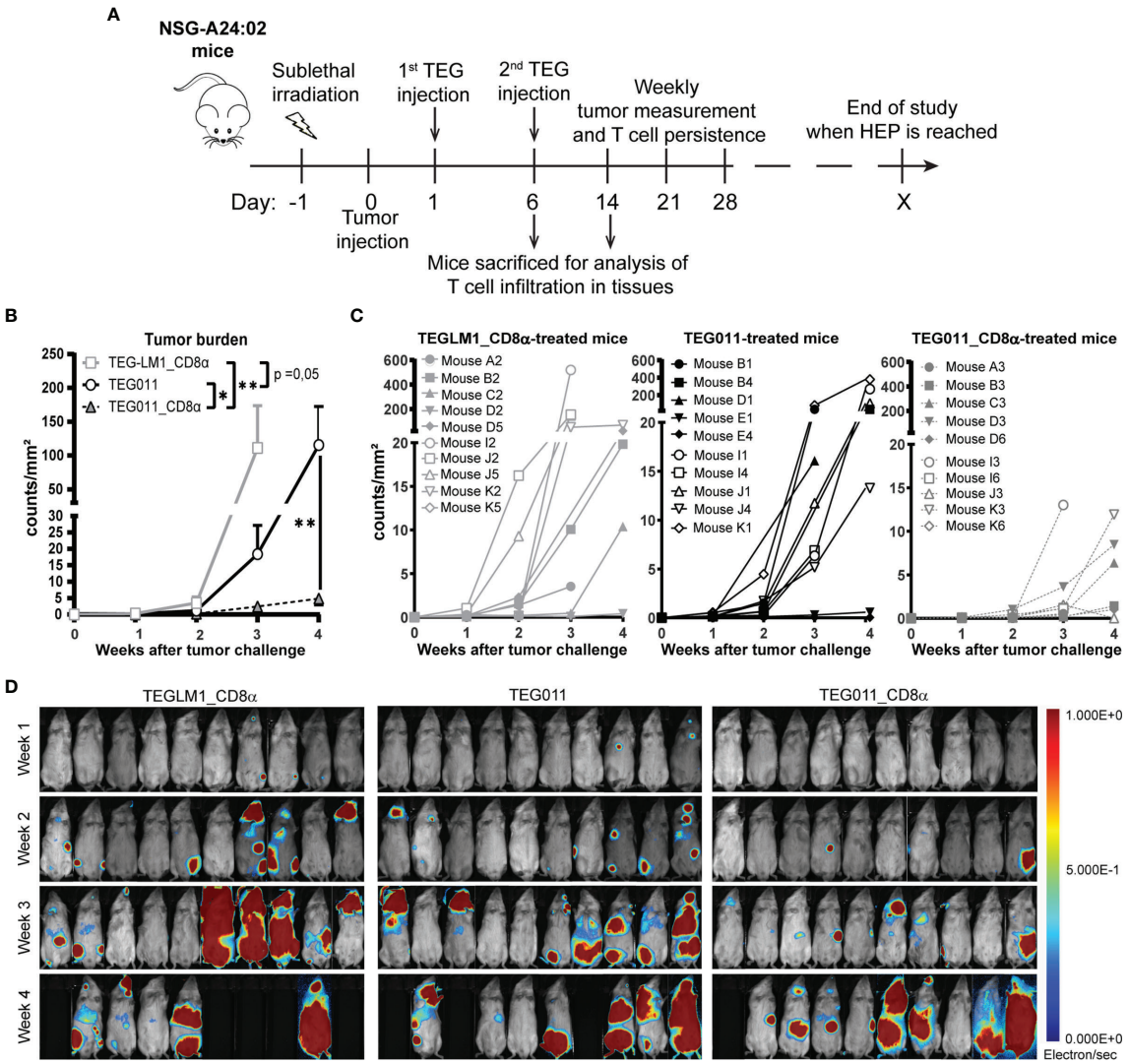

FIGURE 2 | TEG011_CD8α improves *in vivo* tumor control against HLA-A*24:02$^+$ tumor cells. **(A)** Schematic overview of the *in vivo* experiment for NSG-A24:02 tumor-bearing mice. Irradiated mice were intravenously injected with K562-HLA*A24:02-luciferase tumor cells on day 0 followed by two infusions of TEG011, TEG011_CD8α, or TEGLM1_CD8α mock cells on days 1 and 6. Mice were monitored regularly and sacrificed when the humane endpoint (HEP) was reached. **(B)** Tumor burden for K562-HLA*A24:02-luciferase was assessed *in vivo* measuring integrated signal density per total surface area (count/mm^2) by bioluminescence imaging (BLI) with the mouse abdomen facing up. Data are shown only up to week 3 for the TEGLM1_CD8α mock-treated group (open light gray rectangle) due to subsequent mouse dropout >50%, while data for TEG011 (open black circle) and TEG011_CD8α (open black triangle) are shown up to week 4. Data are shown as mean ± SEM of all mice per group (n = 10). Statistical significances were calculated by a mixed-effects model with repeated measure up to week 3 as comparison all treatment group (indicated next to legends) and only between TEG011 and TEG011_CD8α group for week 4 (indicated on the graph); (*P < 0.05; **P < 0.01). **(C)** Tumor burden for individual mouse for each treatment group measured by integrated signal density per total surface area (count/mm^2) using BLI. **(D)** Tumor load for individual mouse was evaluated by bioluminescence imaging on week 1 to week 4 using Milabs Optical Imaging (OI) Acquisition and OI-Post processing software (version 2.0). Anesthetized mice were injected intraperitoneally with 25 mg/ml Beetle-luciferin (Promega). Calibrated units were calculated from integrated density of bioluminescence signal (electron/s) as shown by the right bar. The animals were imaged 10 min after luciferin injection. Black areas indicate loss of mice.

TEGLM1_CD8α-treated group showed evident 19,2% neoplastic infiltration, whereas the TEG011-treated group showed up to 3,4% neoplastic infiltration of a homogeneous population of neoplastic cells in the bone marrow. Interestingly, we did not observe any neoplastic infiltration in the bone marrow of mice in the TEG011_CD8α group, and the appearance of bone marrow cell composition and cellularity was normal (**Figure 4B**). In conclusion, although the number of analyzed bone marrows was limited, our data imply that TEG011_CD8α effectively cleared tumor cells in bone marrow, emphasizing the role of CD8α co-stimulation for better *in vivo* tumor control of TEG011 cells. Overall, our data indicate that introduction of transgenic CD8α on TEG011 cells effectively improves *in vivo* tumor control and better T cell infiltration into bone marrow.

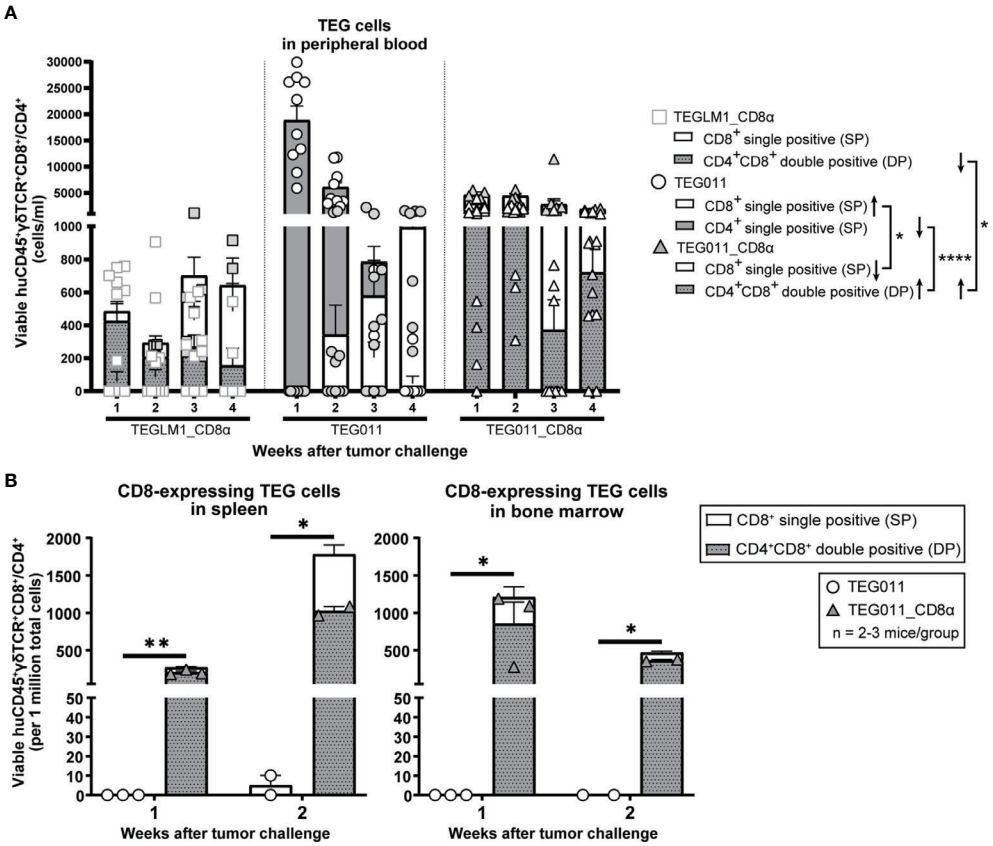

FIGURE 3 | TEG011_CD8α enhances TEG persistence and infiltration. **(A)** TEG cells were measured in peripheral blood using flow cytometry by quantifying the absolute cell numbers of TEGLM1_CD8α mock (open light gray rectangle), TEG011 (open black circle), and TEG011_CD8α (open black triangle) in tumor-bearing mice. TEG cells are distinguished into different cellular compartments: $CD8^+$ single-positive (SP; white stacked bar), $CD4^+$ single-positive (SP; gray stacked bar), and $CD4^+CD8^+$ double-positive (DP; gray dotted stacked bar) cells. Black arrows indicate higher or lower T cell counts observed. Data are shown as mean ± SEM of all mice per group (n = 10 mice). Statistical significances were calculated by a mixed-effects model with repeated measures (*P < 0.05; ****P < 0.0001). **(B)** CD8-expressing TEG cells was assessed in spleen and bone marrow by quantifying the total viable cells of $huCD45^+γδTCR^+CD8^+$ and $huCD45^+γδTCR^+CD4^+CD8^+$ per one million single-cell suspension by flow cytometry. Cell counts of individual mouse per treatment group are represented by each symbol. Functional TEG011 cells consist of two different cellular compartments: $CD8^+$ single-positive (SP; white stacked bar) and $CD4^+CD8^+$ double-positive (DP; gray dotted stacked bar). Data are shown as mean ± SEM (*P < 0.05; **P < 0.01) calculated by a mixed-effects model with repeated measures.

DISCUSSION

TEG011 has been reported to specifically recognize HLA-A*24:02$^+$ malignant cells while sparing the HLA-A*24:02-expressing healthy tissues with the requirement of CD8α co-stimulation (34, 40). While TEG011 has shown a favorable efficacy profile *in vivo*, we only observed in approximately 50% of the mice long-term persistence of $CD8^+$ TEG011 cells, which could be due to the lack of support by antigen-specific $CD4^+$ T cells (29, 40). The presence of both tumor-specific $CD4^+$ and $CD8^+$ αβT cells has been reported to significantly improve clinical responses compared to tumor-specific $CD8^+$ αβT cells alone (33). To further improve the antitumor efficacy of TEG011, we co-expressed a CD8α co-receptor together with the Vγ5Vδ1TCR derived from clone FE11 in TEG format, referred to as TEG011_CD8α cells. Introduction of CD8α receptor is particularly beneficial for TEG011 as this particular γδTCR requires the presence of CD8α as co-receptor for their antitumor reactivity, as we published previously (34, 40). CD8α expression has been reported as common feature of γδTCR after CMV infection (28). These insights imply that also other Vδ1TCR might functionally depend on CD8α, which we could, however, not investigate in a broader context. Thus, when exploring tumor reactivity with selected Vδ1TCR for the development of γδT cell-based immunotherapies (20), the absence of functional reactivity by an introduced Vδ1TCR might not necessarily reflect the absence of binding of the Vδ1TCR to its target but rather the lack of a co-stimulation through, e.g., CD8α or other co-stimulatory molecules. In this study, we reported on the capacity of the introduced CD8α co-receptor to successfully redirect non-tumor reactive $CD4^+$ TEG011 cells *in vivo* and *in vitro* against tumor targets that express HLA-A*24:02 molecules. We now report on more than 80% of mice showing persistence of CD8-expressing T cells after 4 weeks.

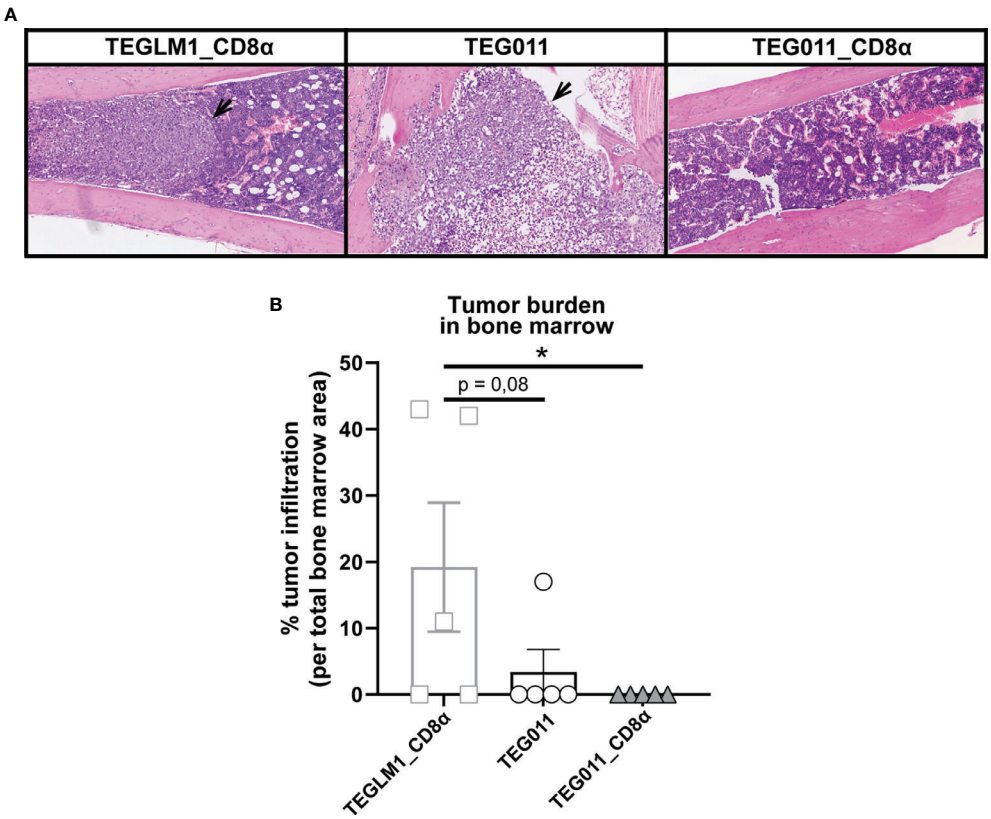

FIGURE 4 | TEG011_CD8α effectively cleared tumor cells in bone marrow, without a significant difference in tumor infiltration observed in other major organs. **(A)** Representative pictures H&E stained of mouse bone marrow with the presence of neoplastic cells (black arrow) from individual mice of each treatment group (n = 5 mice/group). Magnification: 10×. **(B)** Percentage cases of tumor infiltration in mouse bone marrow for each treatment group (n = 5 mice/group). Calculation was performed by dividing the area covered by the tumor cells per the total area of bone marrow tissue visible in the section using ImageJ. Data are shown as mean ± SEM (*P < 0.05) calculated by non-parametric Kruskal-Wallis test.

TEG011_CD8α cells showed also in absolute numbers higher T cell counts and stable peripheral persistence *in vivo*, which was, however, mainly a consequence of the persistence of $CD4^+CD8^+$ double-positive TEG011_CD8α and not an improved persistence of $CD8^+$ single-positive TEG011_CD8α. This finding supports the notions that co-expression of $CD4^+$ and $CD8^+$ T cells provides an additional survival signal for TEG011 cells. This observation is in line with clinical studies for CD19 CAR T cells that reported that a mixture of both $CD4^+$ and $CD8^+$ T cells with 1:1 ratio improved tumor remission in B-ALL patients (42, 43). Regardless of the precise underlying molecular mechanism, for the first time we observed tumor clearance in the bone marrow by TEG011_CD8α, but not by TEG011 alone.

Using humanized transgenic mice expressing human HLA-A*24:02, we could study the implication of CD8α introduction to TEG011, referred to as TEG011_CD8α, elucidating their improved efficacy *in vivo*. We provide evidence that TEG011_CD8α effectively cleared tumor cells in bone marrow and elicited better tumor control against human HLA-A*24:02-expressing tumor cells. We cannot entirely exclude that superior tumor control in TEG011_CD8α may have been caused initially by more CD8 single-positive cells in the TEG011_CD8α product compared to TEG011 product, as $CD4^+/CD8^+$ ratios could not be entirely controlled in the experimental setup prior to infusion. However, our mouse model also allowed us to investigate TEG011_CD8α kinetics in the presence of tumor cells; and we observed sustained long-term TEG persistence mainly for $\gamma\delta TCR^+CD4^+CD8^+$ double-positive and a decline in $\gamma\delta TCR^+CD8^+$ single-positive TEG011_CD8α cells. Importantly, the sustained peripheral TEG persistence was only observed for TEG011_CD8α but not TEGLM1_CD8α, highlighting the key role of a functional tumor-reactive γδTCR. This observation rather argues against the classical helper function of $\gamma\delta TCR^+CD4^+CD8^+$ double-positive TEG011_CD8α cells within the context of TEG011_CD8α. Hence, the concurrent expression of $CD4^+$ and $CD8^+$ co-receptor most likely provided additional survival signal for tumor-specific $CD4^+$ T cells, which did not, however, translate into classical helper functions towards $CD8^+$ T cells (40, 44, 45). $CD4^+$ T cells have been reported to avoid expression of inhibitory receptors on $CD8^+$ T cells (46) and as an important cell subset to induce memory T cell formation (47). Along this line we observed over time reduced expression of TIM3 in $CD8^+$ single-positive

TEG011_CD8α cells compared to mock and TEG011 group. $CD4^+CD8^+$ double-positive TEG011_CD8α cells had lower levels of TIM3 when compared to $CD8^+$ single-positive TEG011_CD8α cells. These data remain difficult to interpret, and most likely simply reflect different regulation and activation of non-tumor reactive $CD4^+$ and tumor-reactive $CD8^+$ TEG011 cells, respectively. We also acknowledge that xenograft mouse models do not allow to completely mimic all potential helper roles of human $CD4^+$ T cells, due to the lack of human professional antigen-presenting cells.

Reprogramming $CD4^+$ T cells by genetic engineering has been reported to clinically impact efficacy and toxicity by high affinity receptors, like CARs (48). Vγ9Vδ2TCR (30) and CD8αβ-independent αβTCRs (32) have been also reported to reprogram $CD4^+$ T cells, which not only have the ability to exert tumor cell killing but also induce maturation of professional antigen-presenting cells. Transfer of CD8αβ in combination with intermediate affinity tumor reactive αβTCR has been reported to support tumor control *in vitro* and *in vivo* (49, 50), and for high affinity αβTCR with artificial signaling domains adding CD8α alone has been shown to reprogram $CD4^+$ T cells (36). Within this context, our data show that CD8αα in combination with a natural γδTCR serves as costimulatory receptor, as opposed to the well-described inhibitory function of CD8αα on αβT cells within the context of a natural αβTCR. Expression of that CD8αα on activated $CD4^+$ and $CD8\alpha\beta^+$ αβT cells has been reported to act as corepressor by competing with $CD8\alpha\beta^+$ cells for $p56^{lck}$ signaling molecule (51). Though we investigated the role of CD8αα in the TEG concept, our data support the notion that CD8αα in combination with a γδTCR is synergistic on natural γδT cells, as activated $CD8\alpha\alpha^+$ γδT cells were reported in supporting control of HIV infection (52). We have also previously reported significant increases in circulating $CD8\alpha\alpha^+$ γδT cells in CMV-positive population (28). Thus, CD8αα appears to have opposing functions on innate and adaptive immune cells, where it acts as costimulatory receptor in the context of a γδTCR.

The precise molecular interaction between CD8αα and its specific ligand in our context remains yet to be unraveled. The CD8αα receptor has been shown to bind to MHC Class I molecules, including HLA-A*02:01, HLA-A*11:01, HLA-B*35:01, HLA-C*07:02, *via* protruding α3 domain loop of MHC molecules with lower affinity than the binding of a TCR-pMHC complex (53–56). Polymorphisms in the MHC α3 domain contributes to a binding variation of CD8αα to different HLA molecules, such as HLA-A*24:02. In this context, HLA-A*24:02 is one of the possible ligands for CD8αα on TEG011, in line with an earlier study that reported CD8αα interaction with HLA-A*24:02 in a similar way with HLA-A*02:01, involving binding to the α2 and α3 domains, as well as to the β2m domain of pMHC complex, but with different conformation that suggests CD8αα plasticity (57). The non-classical MHC molecules are also reported to interact with CD8α, such as HLA-G and HLA-E (58). HLA-G is a known ligand for CD8αα, which is expressed on some colorectal cancer (59–61), while HLA-E is mainly expressed in human endothelial cells and is highly expressed in tumor cells (58). Other studies also demonstrated the interaction between CD8 and CEACAM5, which support the possibility of CEACAM5 as CD8α ligands (62).

Overall, we demonstrate that TEG011 equipped with human CD8α coreceptor elicits superior tumor control and long-term persistence, which mainly impacted numbers of $\gamma\delta TCR^+$ $CD4^+CD8^+$ double-positive TEG011_CD8α cells, and associated with better T-cell infiltration. In addition, TEG011_CD8α cells successfully cleared tumor cells in the bone marrow. In contrast to currently emerging immunotherapy approach using CAR T cells, our strategy allows tumor-specific targeting of HLA-A*24:02-positive cancer patients, irrespective of antigen-specific expression on cell surface and the type of cancer, and thus TEG011_CD8α therapy has broader applicability towards a substantial amount of cancer patients with HLA-A*24:02-positive haplotype highlighting its therapeutic potential for further clinical application.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Utrecht Animal Welfare Body (IvD) and Central Authority for Scientific Procedures on Animals (CCD). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

IJ, TS, ZS, and JK conceptualized, designed, and developed the *in vivo* models. IJ, PH, WS, and SH performed the *in vitro* and *in vivo* experiments. LB and AB performed the histopathology examination of the mouse tissues. DB and RO contributed vital components. IJ analyzed all *in vitro* and *in vivo* data and was a major contributor in writing the manuscript. IJ, ZS, and JK interpreted all *in vitro* and *in vivo* data. IJ and JK wrote the manuscript. All authors read, reviewed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.752699/full#supplementary-material

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Making a Killer: Selecting the Optimal Natural Killer Cells for Improved Immunotherapies

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Over the past 20 years natural killer (NK) cell-based immunotherapies have emerged as a safe and effective treatment option for patients with relapsed or refractory leukemia. Unlike T cell-based therapies, NK cells harbor an innate capacity to eliminate malignant cells without prior sensitization and can be adoptively transferred between individuals without the need for extensive HLA matching. A wide variety of therapeutic NK cell sources are currently being investigated clinically, including allogeneic donor-derived NK cells, stem cell-derived NK cells and NK cell lines. However, it is becoming increasingly clear that not all NK cells are endowed with the same antitumor potential. Despite advances in techniques to enhance NK cell cytotoxicity and persistence, the initial identification and utilization of highly functional NK cells remains essential to ensure the future success of adoptive NK cell therapies. Indeed, little consideration has been given to the identification and selection of donors who harbor NK cells with potent antitumor activity. In this regard, there is currently no standard donor selection criteria for adoptive NK cell therapy. Here, we review our current understanding of the factors which govern NK cell functional fate, and propose a paradigm shift away from traditional phenotypic characterization of NK cell subsets towards a functional profile based on molecular and metabolic characteristics. We also discuss previous selection models for NK cell-based immunotherapies and highlight important considerations for the selection of optimal NK cell donors for future adoptive cell therapies.

Keywords: natural killer cells, cancer immunotherapy, donor selection, cell metabolism, phenotype, epigenetics, transcriptomics

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INTRODUCTION

Natural Killer (NK) cells were first characterized in the 1970s by their ability to detect and eliminate tumor cells without prior antigen sensitization (1). Following observations in the transplantation clinic almost 30 years later, NK cells were identified as one of the first populations to reconstitute following hematopoietic stem cell transplantation (HSCT) and were found to exhibit direct cytotoxicity against malignant cells (2). Indeed, this natural potency against tumor cells has sparked a great deal of interest in exploiting the NK cell platform to treat cancer. Although NK cell-based therapies have not yet achieved the same clinical success as adoptive T cell therapies, early

successes in pre-clinical and clinical trials over the past decade have generated enthusiasm for maximizing their therapeutic potential. Several studies have sought to optimize the source from which therapeutic NK cells are derived and the *ex vivo* activation and expansion strategies by which their activity and persistence *in vivo* can be enhanced. However, although the NK cell repertoire is highly heterogeneous both between and within individuals, relatively little attention has been given to the initial selection of NK cells which harbor the greatest antitumor activity. Here, we review the current state of donor selection for peripheral blood NK (pb-NK) cell-based immunotherapies and discuss the factors which drive NK cell effector function along with the challenges associated with identifying highly potent NK cell populations for immunotherapy.

NK CELL ACTIVATION AND ANTITUMOR IMMUNITY

NK cells are a cytotoxic subset of innate lymphoid cells (ILCs) with marked potency against malignant cells. NK cells and other ILCs originate from the same bone marrow-derived common lymphoid progenitor cells (CLPs) as B and T lymphocytes (3). Although details of human NK cell development remain largely unknown, bone marrow (BM)-derived $CD34^+CD45RA^+$ CLPs are thought to migrate to various anatomical sites where they subsequently undergo interleukin-15 (IL-15) mediated differentiation along the NK cell lineage (4).

In humans, mature pb-NK cells can be divided into two major functional subsets traditionally characterized based on the relative cell-surface density of the CD56 molecule and expression of the low-affinity IgG Fc region receptor III (FcγRIII; CD16): $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$. $CD56^{bright}$ cells represent approximately 10% of the pb-NK cell population and primarily act as potent producers of pro-inflammatory cytokines such as interferon gamma (IFNγ) following cytokine stimulation (5). In contrast, $CD56^{dim}$ cells comprise approximately 90% of the pb-NK cell population and produce IFNγ in response to direct interactions with target cells rather than *via* cytokine activation (6). In addition, $CD56^{dim}$ cells are more strongly cytotoxic towards malignant cells than their $CD56^{bright}$ counterparts and harbor high baseline levels of cytotoxic molecules such as perforin and granzyme B (7). The $CD56^{dim}$ population can be further divided into $CD57^-$ and $CD57^+$ cells, the latter of which represent terminally differentiated NK cells typically considered to harbor the highest cytotoxic potential.

Unlike T and B lymphocytes, NK cell receptors do not undergo somatic rearrangement to generate antigen specificity. Rather, NK cells rely on the stochastic expression of germline-encoded activating and inhibitory receptors, with the complex integration and hierarchy of signals generated through these receptors tightly controlling NK cell function. NK cells express a suite of activating receptors which detect various molecules upregulated by malignant cells. Simultaneous engagement of multiple activating receptors is typically required to overcome an NK cell's intrinsic activation threshold and trigger effector function (8). A notable exception is CD16, which is the only receptor that can activate NK cells in the absence of other activating signals (9). CD16 is the most potent activating receptor, with crosslinking of CD16 molecules by the Fc region of IgG antibody-opsonized target cells resulting in NK cell activation through a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). Other activating receptors include: the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, which directly bind to a wide variety tumor-associated ligands (10); NKG2D, which recognizes the cell-stress induced major histocompatibility complex class I-related molecules MICA and MICB and the UL16-binding proteins (ULBP-1-6) (11); 2B4, which binds to CD48 (12, 13); DNAM-1, which recognizes two protein markers of cellular stress CD112 and CD155 (14); NKp80, which binds to activation-induced C-type lectin (AICL) (15); and the self-associating CD2-like receptor activating cytotoxic cells (CRACC/CD319/CS1/SLAMF7) (16).

NK cells also express a diverse repertoire of inhibitory receptors which recognize human leukocyte antigen (HLA) molecules and regulate self-tolerance to healthy tissues by dominantly inhibiting NK cell activation (17). Two major families of NK cell receptors recognize HLA molecules: the killer immunoglobulin-like receptor (KIR) family and the CD94/NKG2 family of C-type lectin receptors. Up to 15 genes are encoded within the *KIR* locus on chromosome 19 (18), resulting in 14 functional receptors comprising seven inhibitory KIR (-2DL1-2DL3, -2DL5, and -3DL1-3DL3), six activating KIR (-2DS1-2DS5 and -3DS1), and KIR-2DL4 which carries out both activating and inhibitory functions. These KIR genes are highly polymorphic and cluster into haplotypes that differ between individuals, creating at least 40-50 possible *KIR* genotypes and more than 20 haplotypes (18, 19). Haplotypes are divided into two groups characterized by their enrichment for inhibitory (Haplotype A) and activating (Haplotype B) KIRs. Although individual KIR recognize distinct allelic epitopes present in certain HLA-A, HLA-B or HLA-C allotypes, also referred to as KIR ligands, inhibitory KIR have higher avidity for their cognate ligands than activating KIR (20). Similarly, the non-classical HLA molecule, HLA-E, is recognized by both the inhibitory CD94/NKG2A and activating CD94/NKG2C receptors, though the CD94/NKG2A heterodimer binds with higher affinity (21).

Interaction between these major inhibitory receptors and their specific HLA ligand is critical for NK cells to achieve functional maturation through a process known as "licensing" or NK cell education (22). Educated NK cells exhibit the highest reactive potential against target cells that have lost or downregulated HLA expression through a process known as "missing self" recognition, but are susceptible to inhibition by tumor cells that have retained HLA expression (23, 24). Interestingly, as any given inhibitory receptor is present on only a fraction of the NK cell repertoire, both uneducated and educated NK cell populations may coexist within an individual (23). Although the mechanisms underlying NK cell education remain poorly defined, several models have been proposed which debate the relative contributions of

inhibitory and activating receptors towards this ongoing process of functional maturation [reviewed (25)].

Following activation, NK cells carry out a range of antitumor effector functions including the direct lysis of target cells and indirect modulation of both innate and adaptive antitumor immunity through the production of various immunomodulatory cytokines and chemokines (**Figure 1**). Given their potent antitumor activity, relatively low likelihood of severe adverse effects such as graft-*versus*-host disease, and potential for combination with other treatment strategies, NK cell-based therapies have emerged as promising candidates for the treatment of a variety of hematological malignancies and solid tumors (26).

DEVELOPMENT OF NK CELL THERAPIES

The ongoing development of NK cell-based therapies has typically focused on optimizing two major factors: the source from which therapeutic NK cells are derived and the methods by which their activity can be enhanced. NK cells can be obtained from several allogeneic sources including the peripheral blood of related or unrelated donors, umbilical cord blood (UCB), induced pluripotent stem cells (iPSC), and immortalized NK cell lines, with each source harboring intrinsic advantages and disadvantages that must be considered when designing an optimal cellular therapy [reviewed (27, 28)]. Similarly, numerous *ex vivo* enhancements have been developed with the aim of increasing the *in vivo* activity, persistence, and tumor-targeting of the isolated NK cells following infusion. Common strategies include *in vitro* cytokine and feeder cell expansion, cytokine activation in the absence of NK cell expansion, and genetic modification to express a manufactured chimeric antigen receptor (CAR) directed against specific tumor-associated antigens [reviewed (26–28)]. Although several clinical trials are currently evaluating the efficacy of alternative NK cell sources [reviewed (28)], pb-NK cells have remained the most widely utilized source of therapeutic NK cells in clinical trials to date as they are relatively easy to source, have a mature phenotype, and harbor strong cytotoxic activity that can be further enhanced through cytokine stimulation prior to infusion (26). Indeed, pb-NK cells harbor stronger cytolytic activity and greater expression of activating receptors such as CD2 and CD16 than their UCB counterparts (29–31). Furthermore, whilst NK cell lines such as NK-92 represent a robust and renewable source of therapeutic NK cells with strong cytotoxic activity, the requirement for irradiation limits the *in vivo* persistence of the infused cells to a maximum of 48 hours and thus prevents the generation of a long-lasting clinical effect (32). More recently, studies have focused on the development of readily available "off-the-shelf" cellular therapies utilizing unrelated third-party donor-derived pb-NK cells or iPSC-derived NK cells. Indeed, several clinical trials are currently investigating the safety and efficacy of various "off-the-shelf" adoptive NK cell therapy strategies for the treatment of hematological malignancies (**Table 1**).

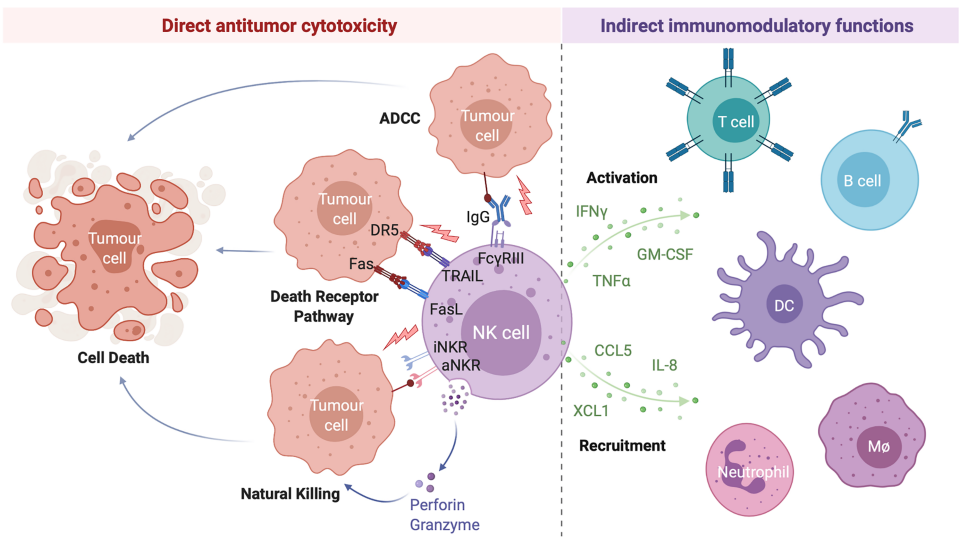

FIGURE 1 | NK Cells Exert a Range of Direct and Indirect Antitumor Effects. NK cell activation is governed by the net balance between signals received through various activating and inhibitory NK cell receptors. When the balance is tipped towards activation, NK cells can directly lyse target cells through release of the preformed cytotoxic granules granzyme B and perforin (natural killing) or by the engagement of target cell death receptors by NK cell-expressed death receptor ligands TRAIL and FasL (death receptor pathway). Antibody opsonized target cells may also be directly lysed through engagement of the NK cell IgG Fc region receptor III (FcγRIII; CD16) in a process of antibody dependent cell-mediated cytotoxicity (ADCC). Activated NK cells are also potent producers of immunomodulatory cytokines (such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and chemokines (including interleukin-8 (IL-8), CCL5, and XCL1) which activate and recruit other immune cells to the tumor microenvironment, indirectly driving a multifaceted antitumor response. Created with (Biorender.com).

TABLE 1 | Clinical trials of "off-the-shelf" adoptive NK cell therapies for the treatment of hematological malignancies.

Trial Identifier	Therapeutic Agent (source)	Malignancy	Age (years)	Treatment Approach	Study Phase (status)
NCT04808115	KDS-1001 (third-party)	CML	All	In combination with TKI therapy	Phase I (not recruiting)
NCT04848064	IL-21 expanded "off-the-shelf" NK cells (third-party)	R/R cutaneous T cell lymphoma or T cell leukaemia/lymphoma	18+	In combination with Mogamulizumab	Phase I (not recruiting)
NCT04632316	oNKord® (third-party)	AML	18+	In combination with chemotherapy	Phase I/II (recruiting)
NCT04220684	mbIL-21 expanded "off-the-shelf" NK cells (third-party)	R/R AML or MDS	1-80	In combination with chemotherapy	Phase I (recruiting)
NCT04623944	NKX101 (related donor or third-party)	R/R AML or MDS	18+	In combination with chemotherapy	Phase I (recruiting)
NCT04310592	CYNK-001 (third-party iPSC)	AML	18-80	In combination with chemotherapy	Phase I (recruiting)

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; iPSC, induced pluripotent stem cell; NK, natural killer; mbIL-21, membrane-bound interleukin-21; MDS, myelodysplastic syndrome; R/R, relapsed and/or refractory; TKI, tyrosine kinase inhibitors.

DONOR SELECTION FOR IMPROVED NK CELL THERAPIES

Despite the existence of a plethora of strategies to enhance the cytotoxicity and persistence of therapeutic NK cells *in vivo*, the initial selection of highly functional cells with strong innate potency is essential for the widespread success of NK cell therapies. Over the past decade it has become increasingly evident that not all NK cells have the same baseline capacity to eradicate leukaemic cells (33–37). In addition to the functional differences between traditional NK cell subsets (33–35), variability also exists in the functional capacity of NK cells derived from different individuals (36, 37). However, relatively little attention has been given to optimizing the particular subset or donor from which these cells are derived. We have recently reported that resting donor-derived pb-NK cells display marked variability in their capacity to mount an effector response against leukaemic target cells (36). Intriguingly, we identified a pool of donors with strong activity against multiple leukaemic cells, representing ideal candidates for the development of efficacious "off-the-shelf" NK cellular therapies. However, there is currently no standardized criteria by which NK cell donors are selected to improve clinical efficacy. We believe the intrinsic diversity in NK cell activity between individuals is an important consideration when developing and optimizing pb-NK cell-based therapies. Specifically, the selection of donors who harbor NK cells with high baseline anti-tumor activity may provide the opportunity to further improve the success of future NK cell therapies.

Donor Selection for HSCT

In the context of HSCT, donor selection is critical for preventing graft rejection, graft *versus* host disease and reducing the risk of relapse. When selecting a donor for allogeneic HSCT the goal is to find the closest HLA match to the recipient, typically a sibling or unrelated donor with genetically identical HLA. While this can be difficult to identify for many recipients, a half-matched donor, known as a haploidentical donor, can be found for most individuals. This scenario presents an opportunity for NK cell alloreactivity, in which a mismatch between the donor and patient KIR and/or HLA leads to NK cell activation and results in the elimination of residual leukaemic cells following HSCT (**Figure 2**). This strategy was first exploited by the Perugia group nearly two decades ago (2). In this seminal study, acute myeloid leukaemia (AML) patients receiving haploidentical HSCT experienced enhanced engraftment success, decreased rates of graft rejection, decreased risk of relapse, and increased overall survival compared to those without predicted alloreactivity (2). Strikingly, the five-year overall survival rate for patients receiving HSCT in which NK cell alloreactivity was predicted in the graft-*versus*-host (GvH) direction was 65% compared to 5% in patients without predicted alloreactivity (2). This observation formed the KIR ligand model of donor selection, in which favorable donors were selected by predicting NK cell alloreactivity based on the HLA genotype of the donor and recipient. Ignited by these drastic increases survival, multiple groups used this KIR ligand model of predicted NK cell alloreactivity to investigate its effect on overall survival rates in historic HSCT datasets [reviewed (38)]. Farag and colleagues reported on over 1500 unrelated transplants for AML, chronic myeloid leukaemia (CML), and myelodysplastic syndrome (MDS) and found no association with NK cell alloreactivity and reduced risk of relapse in these diseases (39). In contrast, Hsu and colleagues reported a beneficial effect of NK cell alloreactivity in a cohort of 1770 patients receiving fully ablative T cell replete HSCT for a range of diseases (40). Similar beneficial effects of NK cell alloreactivity were reported in a cohort of over 2000 patients with AML, CML or MDS (41). However, as these observations arose from retrospective analyses of historic datasets spanning a variety of diseases and treatment regimes, it remained difficult to establish the true impact this model of NK cell alloreactivity had on HSCT outcomes.

To enable better prediction of NK cell alloreactivity, Leung and colleagues (42) described a more refined model of donor selection that involved assessing incompatibilities between the recipient's HLA and the donor's inhibitory KIR repertoire. This receptor-ligand model of donor selection was suggested to be better at predicting the risk of relapse following HSCT, particularly in patients with lymphoid disease (42). However, using this receptor-ligand model of donor selection, Cook and colleagues

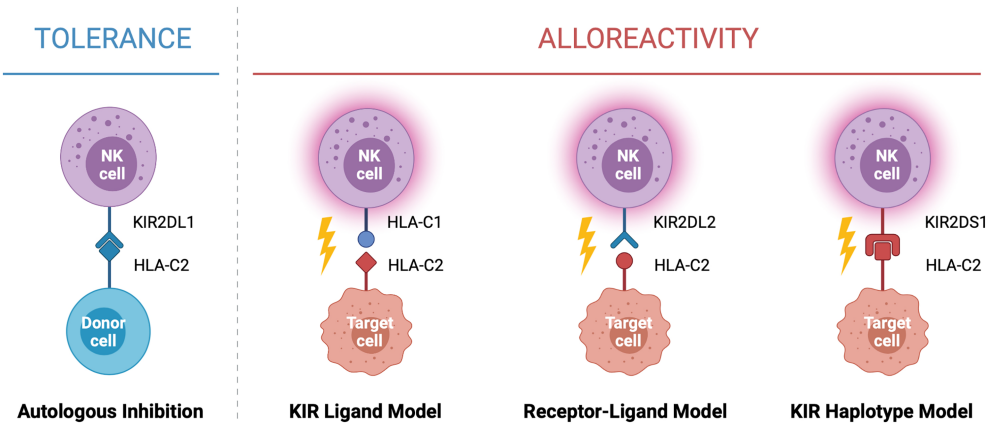

FIGURE 2 | Models of NK Cell Alloreactivity Based on KIR and HLA Expression. Donor NK cells maintain tolerance to self through interactions between inhibitory killer immunoglobulin-like receptors (KIRs) and their cognate human leukocyte antigen (HLA) ligands expressed on healthy autologous cells. In the context of haploidentical HSCT, interactions between donor and recipient KIR and HLA mediate NK cell alloreactivity against target cells. Three models have been described which predict NK cell alloreactivity: mismatch between the donor and recipient HLA (KIR ligand model), mismatch between the donor KIR and recipient HLA (receptor-ligand model), and expression of specific donor KIR haplotypes enriched for activating KIR (KIR haplotype model). When alloreactivity is predicted in the graft-*versus*-host direction, donor NK cells become activated and carry out cytotoxic effector functions against the recipient's tumor cells. Created with (Biorender.com).

reported worse overall survival in transplants between donors carrying the activating *KIR* gene KIR2DS2 and HLA-C2 homozygous recipients when compared to recipients with the HLA-C1 allele (43). This led to more intensive focus on the presence or absence of certain KIR genes and their association with risk of relapse and overall survival. Following analysis of a cohort of over 1400 HSCT recipients, Cooley and colleagues reported that donor *KIR* genotype influenced transplantation outcomes for patients with myeloid but not lymphoid disease (44). Favorable outcomes were observed if the recipient received a transplant from a donor with at least one KIR B haplotype, with the greatest outcomes observed if the donor was homozygous for *KIR* genes within the centromeric region of haplotype B (Cen-B). These observations formed the basis of the KIR haplotype model of donor selection (44). Other studies have also reported similar associations with the Cen-B haplotype, especially with KIR2DS2 [reviewed (38)]. Intriguingly, this contradicts the aforementioned study by Cook et al. which reported a negative association between KIR2DS2 and patient outcomes (43). In a large cohort of over 1200 patients, Venstrom and colleagues reported an association between donor KIR2DS1 (Tel-B gene) and protection against relapse, however this was only observed for donors with HLA-C1 ligands and not those homozygous for HLA-C2 (45). Venstrom et al. also reported reduced recipient mortality with the presence of donor KIR3DS1 (Tel-B gene). Recent studies continue to report associations between activating KIR and disease outcomes, including in children and lymphoid diseases (46–49). Interestingly, similar hierarchical responses have been reported for the inhibitory KIR, KIR3DL1, and its corresponding ligand HLA-Bw4 (50). Indeed, KIR3DL1 and HLA-B combinations resulting in weak or no inhibition towards the recipient's cells were associated with significantly lower rates of relapse in a study of over 1300 patients with AML (50).

While there doesn't appear to be a consensus on using KIR haplotypes to select donors, a comprehensive database of *KIR* sequences exists to allow clinicians to assess presence of *KIR* genes and their content to aid in donor selection if desired (51). However, major discrepancies in the literature regarding the association of HLA and/or KIR with beneficial or detrimental outcomes following HSCT makes it difficult to understand the extent to which predicted NK cell alloreactivity actually contributes towards the elimination of leukaemic cells post-transplant (38). Many factors likely contribute to differences reported between studies and transplant centers around the world, including donor source, method of T cell depletion, preparative regimens (fully-ablative or reduced conditioning), and the inclusion and/or type of prophylaxis strategy to mitigate graft-*versus*-host disease. Nevertheless, two main factors contribute to the success of HSCT: the function of the NK cells post-transplant and the ability of these NK cells to target and eliminate tumor cells. If NK cell function is poor and they fail to recognize the tumor, then donor selection based on genetics has little influence on disease outcomes. Thus, a greater appreciation for the underlying functional state of the NK cells should form an important consideration when attempting to describe associations between HSCT strategies and improved patient outcomes. Furthermore, while NK cells are well-known to be the first lymphocyte to reconstitute following transplantation, engrafting NK cells are developmentally immature and exhibit lower effector function compared to healthy donor NK cells (52–57). Interestingly, we demonstrated that CMV reactivation post-HSCT has a significant impact on the reconstituting NK cell repertoire, enhancing NK cell effector function (54). Moreover, presence of CMV-expanded NK cells (now collectively referred to as adaptive NK cells) in HSCT recipients has been correlated with improved outcomes (34). Yet it remains unclear how these adaptive NK cells are involved in better

clinical outcomes. Furthermore, additional research is required to better understand the associations between activating KIR and improved outcomes post-HSCT. While KIR2DS1 has been shown to recognize HLA-C2 and mediate alloreactivity against cancer cells (58–61), a direct role for KIR2DS2 is harder to decipher (62). KIR2DS2 can recognize HLA-C in combination with specific peptides (63, 64) and may potentially interact with certain HLA-A alleles (36, 65, 66) or non-HLA ligands (48, 67) suggesting many mechanisms for KIR2DS2$^+$ NK cells to target cancer cells post-HSCT. Gaining a better understanding of how associations with activating KIR result in improved responses and this information will allow us to not only better select donors for HSCT, but also to consider additional ways we may be able to exploit these findings to enhance NK cell anti-leukaemic responses.

Donor Selection for Adoptive Cell Therapy

The success of adoptive NK cell therapy also relies upon alloreactivity between the donor NK cells and the recipient's tumor cells. In the landmark study conducted by Miller and colleagues, significantly higher rates of complete remission were achieved when graft-*versus*-leukaemia alloreactivity was predicted based on the KIR ligand model, in which 3 out of 4 patients (75%) achieved complete remission compared to 2 out of 15 patients (15%) without predicted alloreactivity (68). However, these findings were not replicated in larger patient cohorts with no reported correlation between complete remission and KIR ligand mismatch (69). Although freshly isolated and activated NK cells have been investigated in several clinical trials to date, the use of *ex vivo* expanded NK cells has become the focus of many ongoing and upcoming trials [reviewed (26)]. However, there is currently no standard criteria by which donors are selected to generate these expanded NK cell therapies. Expanded NK cells display greater expression levels of activating receptors such as NKG2D and NCRs and exhibit significantly greater levels of cytotoxicity against tumor targets compared to resting NK cells (70, 71). Interestingly, KIR/KIR ligand interactions and prior *in vivo* education have been reported to influence NK cell activity following *ex vivo* activation and expansion strategies (72). Specifically, expanded NK cells were found to be more potent when they expressed one or more "licensed" KIR, for which the donor had the corresponding KIR ligand genotype, reflecting the *in vivo* process of education that had occurred prior to isolation and expansion (72). Based on these *in vitro* findings, Wang and colleagues have proposed the licensed receptor-ligand mismatch model of donor selection for adoptive NK cell therapy in which the patient is missing a KIR ligand for which the donor has a licensed KIR (73). Based on the frequency of each HLA genotype in the population, the probability of finding a suitable donor for patients missing at least one KIR ligand is high, requiring a screen of between 3 to 8 unrelated donors (73). However, no suitable donor would exist for patients with all three KIR ligands present. Furthermore, whilst the broad groupings of HLA-C1 and KIR2DL2/3, HLA-C2 and KIR2DL1/S1, and HLA-Bw4 and KIR3DL1 are good indicators of which NK cells may be educated in an individual and thus have the capacity to mediate alloreactivity following transfer, not all ligands bind with the same affinity to their KIR receptor, thus resulting in differing functional potentials. Indeed, early after the identification of the ligands for KIR3DL1 (74), hierarchical responses were described between Bw4 alleles that harbor an isoleucine at position 80 *versus* a threonine at the same position (75). This is further complicated by not all HLA-Bw4 alleles binding to KIR3DL1 as predicted (76) and as at the end of 2020 there are 183 reported alleles of KIR3DL1, some of which differ in their expression and interaction with HLA-Bw4 (50, 77, 78). Similar hierarchical responses have also been identified for KIR2DL1, KIR2DL2 and KIR2DL3 and their respective HLA-C ligands (62, 79–81). These differences in binding affinities have been attributed to differing capacity to educate NK cells and form the basis of the tuning or rheostat model of NK cell education where the level of HLA stimulation influences the functional capacity of the NK cell (82–84). Adding to the complexity is the ability of NKG2A to educate NK cells though its ligand HLA-E, with individuals harboring a methionine at position -21 (-21M) of HLA-B more likely to have NK cells educated strongly through NKG2A (85). This is due to HLA-B alleles with -21M generating peptides that can bind to HLA-E whereas other HLA-B alleles with threonine at -21 cannot. Growing evidence from studies of murine NK cells has also highlighted a potential mechanism of MHC class I (MHC-I)-independent education involving non-MHC-I molecules such as CD48 (2B4 ligand), SLAM family member 6 (SLAM6, self-ligand), C-type lectin-related ligand (Clr-b, NKRP1-B ligand), and poliovirus receptor CD155 (TIGIT ligand) [reviewed (86)]. Collectively, this makes it challenging to rely on classic models of predicated educational status when selecting NK cell donors for enhanced antitumor activity. Furthermore, there are also reports of non-educated NK cells mounting effective responses against cancer and virally infected cells (87–90). A greater understanding of how educated and non-educated NK cells respond within the tumor microenvironment *in vivo* is therefore required to accurately select NK cell donors based on educational status for enhanced antitumor activity.

A distinct advantage of using *ex vivo* expanded NK cells for adoptive cell therapy is the ability to generate large numbers of cells from relatively small starting populations. As such, this process presents the unique opportunity to select for specific NK cell populations which may otherwise represent only a small portion of a donor's circulating NK cell repertoire. A notable example is FATE-NK100, an NK cell immunotherapy product pharmacologically enriched for NK cells with a CMV-driven adaptive phenotype. Specifically, pb-NK cells are isolated from a related CMV-seropositive donor, depleted of CD3$^+$ and CD19$^+$ lymphocytes, and cultured *ex vivo* for 7 days in the presence of IL-15 and CHIR99021, a small molecule inhibitor of glycogen synthase kinase 3-beta (GSK3β), to generate the final CD3$^-$CD19$^-$ CD57$^+$NKG2C$^+$ NK cell product (91). Three phase I clinical trials of FATE-NK100 have been undertaken: DIMENSION for the treatment of advanced solid tumors (NCT03319459; ongoing), APOLLO for the treatment of recurrent ovarian cancer (NCT03213964), and VOYAGE for treatment of relapsed or refractory AML (NCT03081780). In the APOLLO trial FATE-NK100 cells were observed to persist and exhibit enhanced cytotoxic function compared to the patient's endogenous NK cells for up to 21 days, with clinical benefit reported in three of the nine

patients recruited (92, 93). The VOYAGE trial has also reported early success, with all refractory AML patients in dose cohort 2 achieving a morphologic leukaemia free state at day 14 (93). Despite the existence of various other NK cell populations with diverse functional outputs, selection of other subsets for enhanced therapeutic potential remains relatively unexplored. To ensure the continued success of adoptive NK cell therapies, new efforts should seek to identify additional populations of NK cells which harbor high baseline antitumor activity.

DON'T JUDGE A BOOK BY ITS COVER: DISCREPANCIES BETWEEN NK CELL PHENOTYPE AND FUNCTION

For several decades phenotypic analysis has played an integral role in inferring the identity, maturation state, and functional capacity of NK cell populations. For example, the classic model of NK cell maturation describes a gradual downregulation of CD56 expression and acquisition of CD16 and CD57 expression as pb-NK cells progress from an immunoregulatory $CD56^{bright}CD16^{-}$ phenotype towards the cytotoxic $CD56^{dim}CD16^{+}$ phenotype, before eventually transitioning into the terminally differentiated $CD56^{dim}CD16^{+}$ $CD57^{+}$ population with the highest cytotoxic activity. Several other phenotypically and functionally distinct subsets have been described in both healthy and diseased states. For example, infection with cytomegalovirus (CMV) drives the expansion of the $CD57^{+}NKG2C^{+}KIR^{+}$ adaptive NK cell population with an increased capacity for ADCC (35). In contrast, a population of $CD56^{-}$ NK cells with impaired cytotoxicity and ADCC have been described at low frequencies within healthy individuals but are expanded following both acute (94) and chronic (95, 96) viral infections.

Although phenotyping remains an accessible means by which the heterogeneity of the NK cell repertoire can be explored, increasing evidence suggests these phenotypic classifications of maturity and functional state are inherently flawed. For example, the relative expression level of CD56 does not necessarily inform on maturation state, as $CD56^{dim}CD16^{+}$ cells can up-regulate CD56 expression upon cytokine stimulation to become $CD56^{bright}CD16^{+}$ (97). Similarly, a proportion of $NKG2A^{-}$ clones have been reported to regain NKG2A expression and $CD56^{dim}CD57^{+}$ clones lost CD57 expression following expansion with K562 feeder cells (98). Although typically considered a signature of NK cell functional maturation, KIR expression has also been observed on both $CD56^{dim}$ and $CD56^{bright}$ cells (99). Recent studies have raised further discrepancies in the classic functional roles assigned to the $CD56^{bright}$ and $CD56^{dim}$ populations. Although $CD56^{dim}CD16^{+}$ cells are traditionally considered the cytotoxic subset, these cells have also been observed to carry out regulatory functions. Following culture with TGF-B, IL-15, and IL-18, $CD56^{dim}CD16^{+}$ pb-NK cells demonstrated reduced cytotoxicity and pro-inflammatory cytokine production, but increased secretion of the immunoregulatory protein VEGF-A (100). Similarly, the traditionally "regulatory" $CD56^{bright}$ NK cell subset is also capable of potent anti-tumor activity. Following priming with IL-15, Wagner and colleagues reported that $CD56^{bright}CD16^{-}$ pb-NK cells displayed greater cytokine production, degranulation and killing of leukaemic targets than their $CD56^{dim}CD16^{+}$ counterparts (101). A population of highly cytotoxic $CD56^{superbright}$ NK cells have also recently been described following expansion of patient-derived NK cells with K562 feeder cells (102). In this study it was reported that NK cell degranulation, cytotoxicity, and IFNγ production increased alongside increasing expression of CD56. Furthermore, these expanded $CD56^{superbright}$ NK cells were able to eliminate autologous ovarian tumors *in vivo* in patient-derived xenograft models (102). Consideration of other NK cell markers is similarly unable to address these discrepancies between NK cell phenotype and functional output. For example, both regulatory and cytotoxic NK cells can express high levels of activating receptors such as the NCRs and NKG2D, though stimulation through these receptors elicits distinct functional programs in each subset (100, 103, 104). Furthermore, both NK cell populations can express either high or low levels of inhibitory receptors such as NKG2A and KIRs (101, 102, 105, 106). Collectively, these studies suggest there is no specific combination of markers that can consistently distinguish regulatory and cytotoxic NK cell subsets. In a clinically relevant example, traditional phenotypic markers cannot be used to delineate the potency of a donor's NK cell response against leukaemic cells (36). Indeed, methods to distinguish NK cells with highly potent antitumor activity remain elusive.

The discovery of NK cell populations with a capacity for "memory-like" effector function has also stretched our understanding of how phenotype relates to functional potential. In a unique immunological phenomenon, CMV shapes the phenotypic and functional properties of the NK cell repertoire by driving the expansion of a subset of $CD56^{dim}NKG2C^{+}$ adaptive NK cells with memory-like properties [reviewed (107)]. Although predominantly defined by expression of NKG2C, adaptive NK cells are also considered to have a mature phenotype as they typically lack NKG2A expression, express low levels of NKp30 and NKp46, and have high levels of KIR and CD57 expression (34, 108). Functionally, CMV-driven adaptive NK cells are specialized for enhanced ADCC, producing greater levels of IFNγ following activation through CD16 (109). Intriguingly, expansion of this $NKG2C^{+}$ NK cell population is only observed in approximately one-third of CMV seropositive individuals (35). Whilst it has been revealed that the infecting strain of CMV impacts the degree of adaptive NK cell expansion through peptide-specific interactions between the UL40 peptide-HLA-E complex and the activating NKG2C receptor (110), recent findings have challenged the requirement for NKG2C in generating this memory-like functional fate. Indeed, a population of NK cells with a similar memory-like response to CMV has been described in $NKG2C^{-/-}$ individuals (111). Moreover, adaptive NK cell responses have also been reported following CMV reactivation in patients that had received HSCT from $NKG2C^{-/-}$ donors (112). Adaptive NK cells may actually be defined by an array of phenotypes including the loss of all, some, or none of following signaling proteins: FcεRIγ, Eat-2, and Syk (35, 113, 114). Whilst it is quite possible that other viruses or environmental exposures may similarly impact upon the

NK cell repertoire *in vivo*, most observations are confounded by donor CMV seropositivity (115). Furthermore, as many studies have focused solely on this pre-defined phenotypic classification of $NKG2C^+$ adaptive NK cells, other markers that may delineate memory-like populations have not yet been identified. Indeed, not all memory-like NK cells display a classically mature phenotype. For example, a population of cytokine-induced memory-like (CIML) NK cells have been described following *in vitro* stimulation with IL-12/IL-15/IL-18 (116, 117). Although this CIML NK cell population is primarily composed of $CD56^{dim}$ cells, the expression of NKG2A, CD25, CD69, CD94, and lack of KIR and CD57 expression suggests a more immature phenotype (116, 117). Strikingly, CIML NK cells display significantly enhanced IFNγ production following cytokine restimulation or target cell activation compared to conventional NK cells, and have since gone on to achieve preliminary success in a phase I clinical trial for the treatment of AML (118). A subset of tissue-resident NK cells with memory-like activity has also recently been described in the context of pregnancy (119). This population of pregnancy-trained decidual NK cells harbored a unique $CD56^{bright}CD16^-NKG2C^+$ phenotype and displayed enhanced production of IFNγ and VEGF-A compared to conventional decidual NK cells (119). Taken together, these studies emphasize the vast heterogeneity of memory and memory-like NK cell responses. Moreover, the capacity for these stimuli to shape the functional fate of the NK cell repertoire, and drive a broad spectrum of different phenotypes, highlights the difficulty in assigning functional properties based on phenotypic analysis.

Further discrepancies between NK cell phenotype and function are encountered when attempting to describe dysfunctional NK cell populations. In cancer patients or the setting of chronic viral infection, dysfunctional NK cells are typically characterized by their reduced functional capacity including decreased cytotoxicity in response to target cell stimulation and reduced expression of IFNγ and granzyme B [reviewed (120, 121)]. However, as these are general markers of dysfunction, specific dysfunctional states such as exhaustion, anergy or senescence are difficult to define. Whilst several studies have reported that functional exhaustion of NK cells in tumors or chronic infections is accompanied by the downregulation of activating receptors such as NKG2D, CD16, NCRs, DNAM-1, and 2B4, or the upregulation of markers of T cell exhaustion such as PD-1, TIGIT, TIM-3 and LAG-3, it remains controversial whether NK cells even undergo exhaustion (120). As such, phenotypic markers of NK cell dysfunction remain ill-defined. Indeed, there is currently no established phenotype that can consistently distinguish dysfunctional from functional NK cell populations.

Collectively, these studies demonstrate that there remains no unifying phenotype by which NK cell maturation, functional state, or capacity for memory can be defined, which has important implications for the generation of NK cell products to achieve maximum therapeutic benefit. Indeed, if these classic phenotypes are truly unable to predict NK cell activity, then what other measures can be used to inform on an NK cell's functional potential?

ADDING FUEL TO THE FIRE: CAN METABOLISM INFORM NK CELL FUNCTION?

It is now apparent that cellular metabolism is not only a means by which cells generate energy and biochemical precursors required for homeostasis, but it is intrinsically tied to immune cell function. Recent studies have reported that immunometabolism plays a critical role in regulating NK cell development, education, activation, and memory response [reviewed (122, 123)]. As metabolism plays such an integral role in dictating NK cell biology, a new paradigm has emerged in which distinct "metabolic fingerprints" underpin NK cell functional fate (122). Specifically, differences in the capacity for and regulation of glucose-driven metabolic pathways may identify NK cells with enhanced cytotoxic potential (**Figure 3**).

Dynamic changes in the glucose-driven metabolic pathways glycolysis and oxidative phosphorylation (OxPhos) coincide with NK cell development and effector function. In mice, developing NK cells utilize both glycolysis and OxPhos to fuel the energy-intensive process of proliferation (124), whereas mature NK cells are considered metabolically quiescent at steady-state and preferentially use OxPhos to meet their homeostatic needs (125). Although less is known about the metabolic requirements of developing NK cells in humans, mature pb-NK cells also demonstrate low rates of glycolysis and OxPhos at resting state (126). Interestingly, metabolic differences have been reported between the $CD56^{bright}$ and $CD56^{dim}$ NK cell compartments. Resting $CD56^{dim}$ NK cells have a greater mitochondrial mass and demonstrate higher rates of glycolysis and OxPhos compared to the $CD56^{bright}$ subset (127). $CD56^{dim}NKG2C^+$ adaptive NK cells isolated from CMV seropositive individuals also exhibit an increased capacity for glycolysis and OxPhos compared to donor-matched $CD56^{dim}NKG2C^-$ canonical NK cells (128). However, basal levels of these pathways were comparable between adaptive and canonical NK cells, suggesting that adaptive NK cells may have a greater capacity to upregulate these metabolic pathways following activation (128). As NK cells are poised to respond rapidly following activation it is perhaps unsurprising that the rates of glycolysis and OxPhos remain unchanged during short-term cytokine stimulation (4 hours with IL-15 and/or IL-12 and/or IL-18) or receptor ligation (6 hours with anti-NK1.1 or anti-Ly49D) (125). However, inhibition of either pathway was shown to significantly impair IFNγ production, especially following receptor ligation. Taken together these findings suggest that the low basal metabolic rate of resting NK cells is sufficient to fuel acute NK cell effector responses. Interestingly, several studies have also reported that prolonged NK cell activation drives robust changes in cellular metabolism. Indeed, overnight stimulation with cytokines significantly increases the rates of glycolysis and OxPhos in both human and murine NK cells (124, 126, 129, 130). Accumulating evidence suggests that this increased level of glucose-driven metabolic fitness drives enhanced NK cell cytotoxicity. For example, NK cells stimulated with IL-15 for 3-5 days demonstrate higher rates

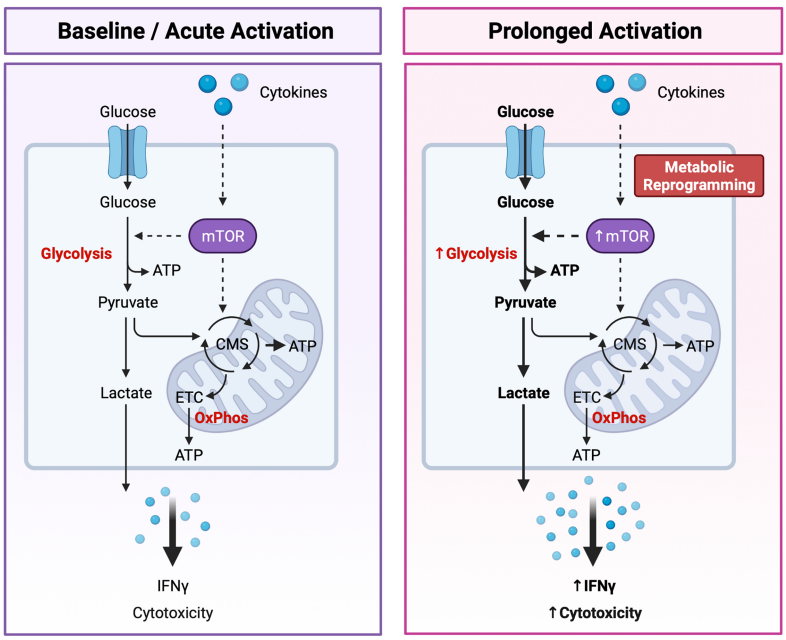

FIGURE 3 | Dynamic Changes in Glucose Metabolism Underly NK Cell Effector Potential. NK cells are primarily fueled by glucose. Following uptake into the cytoplasm, glucose is first converted to pyruvate through glycolysis, generating two molecules of adenosine triphosphate (ATP) per glucose molecule. Pyruvate is then either converted to lactate and expelled from the cell or transported to the mitochondria where it is further metabolized through the citrate malate shuttle (CMS) to fuel the electron transport chain (ETC) and oxidative phosphorylation (OxPhos), driving efficient production of ATP. Basal rates of glycolysis and OxPhos are sufficient to fuel the homeostatic needs and acute effector functions of resting NK cells. Following prolonged cytokine stimulation NK cells experience an increase in the rates of glycolysis and OxPhos to support their increased capacity for IFNγ production and cytotoxic activity. Highly cytotoxic NK cells can also undergo cytokine-induced metabolic reprogramming towards glycolysis through the mechanistic target of rapamycin (mTOR), a master regulator of cellular metabolism. An increased capacity for glucose-driven metabolism and more robust activation of the mTOR pathway identifies NK cells with the greatest cytotoxic potential. Created with (Biorender.com).

and overall capacity for glycolysis and OxPhos and exhibit greater levels of IFNγ production in response to receptor-mediated activation compared to those that receive short-term stimulation of 4-24 hours (124, 125). Furthermore, this prolonged IL-15 stimulation eliminated the metabolic requirement for NK cell activation, with IFNγ production sustained following OxPhos inhibition (125). Metabolic reprogramming may also underpin the enhanced cytotoxic capacity of educated NK cells. Schafer and colleagues reported that following activation and expansion using IL-21-expressing K562 feeder cells, educated NK cells were metabolically reprogrammed towards glycolysis and mitochondrial-dependent glutaminolysis to support their increased cytolytic activity, whereas the uneducated subset relied solely upon OxPhos (131). In addition, metabolic fitness plays an important role in governing NK cell effector function within the tumor microenvironment (TME) [reviewed (132, 133)]. Limited nutrient availability within the TME may restrict NK cell metabolism, thus impairing effector function (134–136). Accumulation of tumor-derived metabolites within the TME has been reported to dampen NK cell activity through impairing key metabolic pathways. For example, adenosine has been shown to inhibit the metabolic activity of human NK cells by inhibiting OxPhos and reducing their glycolytic capacity (137) and uptake of lactic acid by NK cells leads to intracellular acidification and impaired energy production (138). More recently, Poznanski and colleagues reported that NK cell dysfunction within the TME is due to suppression of glucose-driven metabolic pathways *via* lipid peroxidation-associated oxidative stress (139). Strikingly, expanded NK cells reprogrammed towards complete metabolic substrate flexibility demonstrated greater metabolic fitness and enhanced antitumor activity against ovarian tumors *in vivo* (139). These findings suggest that an increased capacity for glucose-driven metabolism and high metabolic fitness may identify NK cells with enhanced cytotoxic activity and thus greater therapeutic potential.

Tight regulation of the mechanistic target of rapamycin (mTOR) is also critical for NK cell development and activation. mTOR is a highly evolutionarily conserved serine/threonine kinase comprised of two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Together, these complexes act as master regulators of cellular metabolism and integrate signals for nutrient availability, growth, and activation to adjust the rates of glycolytic metabolism and biosynthesis accordingly (140). Several studies have reported that mTOR signaling plays a crucial role in the early stages of NK cell development (124, 141, 142). For example, IL-15-induced activation of the mTOR pathway is required for E4BP4

expression in developing BM-NK cells, which in turn promotes transcription of Eomes and drives commitment towards the NK cell lineage (141). More recently, crosstalk between mTORC1 and mTORC2 was found to promote NK cell maturation through controlling the expression of transcription factors Tbx21 and Eomes in a cooperative and non-redundant manner (143). Interestingly, mTORC1 and mTORC2 were also reported to regulate NK cell metabolism and anti-tumor activity in opposing ways. NK cells from mTORC2 deficient mice displayed greater cytolytic activity and increased metabolic rate compared to their wild-type counterparts, whilst cytotoxicity and cellular metabolism were significantly diminished in mTORC1 deficient NK cells (143). Other studies have also demonstrated that mTOR plays a critical role in controlling NK cell activation in both mice and humans, with IL-15 induced activation of the mTOR pathway required for priming of cytotoxicity in the periphery (124, 144). Marçais and colleagues reported that mTOR activity downstream of the IL-15 receptor increased granzyme B expression in both murine and human NK cells, whereas inhibition of mTOR by rapamycin abrogated NK cell cytotoxicity (124). Interestingly, IL-15 stimulation has been reported to activate the PI3K/Akt/mTOR pathway more robustly in $CD56^{bright}$ NK cells compared to the $CD56^{dim}$ population, corresponding with their potent increase in cytotoxicity following prolonged stimulation (101). Transforming growth factor-β (TGF-β), a major immunosuppressive cytokine well-known for its role in inhibiting NK cell cytotoxicity, has also been reported to directly suppress NK cell activation through inhibition of mTORC1 and through mTORC1-independent inhibition of mitochondrial metabolism (130, 145). Indeed, *in vitro* treatment with TGF-β reduced the metabolic activity, cytotoxicity, and abundance of various NK cell receptors in both murine and human NK cells (130). Furthermore, deletion of the TGF-β receptor subunit TGF-βRII in murine NK cells enhanced mTOR activity and NK cell cytotoxicity *in vivo* (130). Together, these findings highlight the importance of mTOR activity in regulating NK cell antitumor function both *in vitro* and in the tumor microenvironment. Differences in mTOR activity, or in the relative activity of the mTORC1 and mTORC2 pathways, may therefore be useful in delineating NK cell cytotoxic potential. Moreover, boosting the metabolic activity of NK cells through targeting the mTOR pathway may be an effective strategy for enhancing the antitumor activity of NK cell-based therapies and thus warrants further investigation.

It is now apparent that metabolism plays an essential role in dictating NK cell functional fate. As metabolism is intrinsically tied to NK cell survival and antitumor activity, the potential to manipulate NK cells *ex vivo* for enhanced metabolic fitness holds promise for enhancing the efficacy of NK cell-based therapies. Indeed, several strategies have been proposed to genetically or pharmacologically "rewire" NK cell metabolism to promote *in vivo* longevity, improve tumor recognition, sustain antitumor function, increase trafficking to the tumor site, and protect the adoptively transferred NK cells from the tumor microenvironment itself (146). As more tools than ever before are now available to interrogate NK cell metabolism [reviewed (147)], future investigations should also seek to define specific "metabolic fingerprints" which can be used to identify NK cells with the highest therapeutic potential. Although our understanding of NK cell metabolism is currently in its infancy, a deeper appreciation of the interplay between metabolism and molecular regulators of NK cell functional fate, as discussed below, holds promise for unlocking the full potential of NK cell therapies.

PROGRAMMED TO KILL: MOLECULAR DETERMINANTS OF NK CELL FUNCTION

The fields of transcriptomics and epigenomics are rapidly advancing. Recent breakthroughs in the development of high-resolution and high-throughput sequencing technologies have enabled researchers to explore the transcriptional and epigenetic landscape of NK cells in more depth than ever before. However, our understanding of the molecular regulation of NK cell functional fate is still in its infancy. Relatively little is known about the molecular pathways and regulatory programs that underly NK cell development, effector function, and memory response. As a central goal of genetic and epigenetic studies involves understanding the factors that drive individual variation, a deeper understanding of NK cell biology at the molecular level may also aid in identifying optimal donors for NK cell immunotherapy.

The development of high-resolution transcriptomic analyses such as RNA sequencing (RNA-seq) and single cell RNA-seq (scRNA-seq) has provided researchers with unprecedented insight into the developmental and functional plasticity within the NK cell compartment. A particular interest has arisen in unravelling the developmental trajectory of human NK cells. Based on phenotypic analyses, the current model of NK cell differentiation describes a linear relationship between the immature $CD56^{bright}$ and terminally differentiated $CD57^{+}$ NK cell populations. However, the well-established loss of $CD56^{bright}$ but not $CD56^{dim}$ NK cells in GATA2-deficient individuals challenges this current dogma of NK cell development (148). Using scRNA-seq to analyze NK-lineage cells derived from a donor with the $GATA2^{T354M}$ mutation, Yang et al. confirmed the loss of $CD56^{bright}$ cells in this donor due in part to a higher rate of apoptosis compared to GATA2-sufficient cells (149). Furthermore, whilst the heterogeneity of this donor's NK cell repertoire was mostly intact compared to healthy controls, defects in steady-state activation were also observed (149). Although the developmental trajectory of these GATA2-deficient NK cells remains unclear, transcriptomic analyses of healthy donor-derived NK cells supports the linear model of differentiation, suggesting that the $CD56^{bright}$ subset is a precursor to the $CD56^{dim}$ population with $CD57^{+}$ cells representing the terminal stage of NK cell differentiation (149, 150). Interestingly, analysis of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell populations derived from various tissues suggests that NK cell developmental and functional fate is shaped by the tissue site from which they are derived (151). Indeed, tissue-

specific transcriptional patterns of maturation, distribution, and function were largely maintained across donor age, sex and CMV serostatus (151). Yang et al. have also described the shared presence of five distinct NK cell clusters across the BM and peripheral blood derived from healthy donors (149). Interestingly, two of these five clusters ("Mature NK" and "Terminal NK") were predicted to form the $CD56^{dim}CD57^{+}$ population together, suggesting that this classically terminally differentiated NK cell subset is not as homogenous as previously thought (149). An additional three novel subsets of pb-NK cells have been described using scRNA-seq, including type I IFN-responding $CD56^{neg}$ NK cells, CIML NK cells, and a small population of NK cells with reduced ribosomal expression, decreased OxPhos and markers of cellular activation (152). Heterogeneity within the $CD56^{dim}CD16^{+}CD57^{-}$ subset was also observed, with two distinct subpopulations distinguished based on the relative abundance of chemokine mRNA and frequency of KIR-like receptor expression (152). In addition to this high level of variation within an individual's NK cell repertoire, several studies have also reported the presence of strong donor phenotypes in scRNA-seq datasets (149, 153, 154), likely reflecting the unique genetic profile and immunological history of each donor. As these donor phenotypes are present under physiological conditions, exploration of the transcriptomic differences between donors may uncover an even deeper level of NK cell functional heterogeneity than previously described by phenotypic analysis alone. Indeed, several studies have reported the presence of adaptive NK cell clusters in CMV seropositive individuals (149, 153), and an "inflamed" NK cell cluster specific to one BM donor (149). However, the development of new annotation tools and more robust sequencing technologies may be required to interrogate the full heterogeneity of the NK cell repertoire between healthy donors. More recently, Crinier and colleagues have reported strong donor phenotypes in BM-NK cells derived from AML patients at diagnosis (153). Remarkably, the extent of transcriptomic heterogeneity between AML patients was so high that traditional annotation tools were unable to identify conserved NK cell subsets, even when considering patients at the same classification of AML (153). Interestingly, the overall transcriptomic profile of BM-NK cells from AML patients was enriched for genes involved in cytokine and type I IFN signaling pathways, whereas healthy BM-NK cells displayed a transcriptomic profile enriched for genes involved in NK cell cytotoxicity (153). These findings highlight the strong donor-specific effects AML carries out on the endogenous NK cell repertoire. However, the relationship between these distinct transcriptomic profiles and patient outcomes has not yet been elucidated. Similarly, the extent to which other hematological malignancies impact on the endogenous NK cell compartment, or on adoptively transferred NK cells, remains unexplored. Indeed, further investigation is needed to fully appreciate the role transcriptomic regulation plays in controlling NK cell activity in health and disease.

Epigenetic alterations are reversible and heritable changes to the genome that do not alter the DNA sequence itself, but have profound impacts on gene expression, cell phenotype, and functional fate. Broadly, NK cell development and effector function is regulated through various epigenetic alterations including DNA methylation, histone modification, transcription factor (TF) changes, and microRNA (miRNA) expression [reviewed (155)]. NK cells undergo profound epigenetic remodeling throughout their development. For example, gradual demethylation of gene promoters at the *KIR* and *IFNG* loci during NK cell differentiation corresponds with acquisition of KIR expression (156) and the ability to produce IFNγ (157), respectively. The dynamic interplay between chromatin accessibility and gene expression levels throughout NK cell development has recently been described (158). Using the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to assess changes in genome-wide chromatin accessibility levels of different developmental stages of *in vitro*-derived NK cells, Li and colleagues revealed the presence of two distinct TF clusters that regulate NK cell differentiation (158). Additionally, two novel TFs were identified (FOSL2 and EGR2) and found to be essential for controlling NK cell maturation and function (158). However, as this study utilized an *in vitro* model of NK cell differentiation it is unclear how these findings will translate to the natural *in vivo* process of NK cell development. Indeed, Li and colleagues have reported differences in chromatin accessibility between these *in vitro*-derived NK cells and their naturally occurring counterparts (158). Nevertheless, the identification and characterization of TFs and transcriptional regulatory networks involved in NK cell differentiation presents an opportunity to artificially drive NK cell functional fate along a desired pathway *in vitro*. Indeed, pharmacological inhibition of GSK3 kinase during *ex vivo* NK cell expansion with IL-15 is currently used to drive late-stage maturation and enhanced effector function of FATE-NK100 cells through upregulating the expression of TFs such as T-BET, ZEB2, and BLIMP-1 (91). Chromatin dynamics also play an important role in regulating the function of the mature NK cell repertoire. For example, target cell recognition drives alterations in the NK cell histone methylation state that correspond with changes in gene expression levels (159). Interestingly, small-molecule compounds targeting H3K4 and H3K27 methyltransferases and demethylases were able to mimic these activation-induced histone modification states in the NK92MI cell line, inducing significantly greater levels of degranulation (UNC1999) and expression of IFNγ and TNFα (OG-L002 and MM102) compared to untreated controls (159). Entinostat, a histone deacetylase inhibitor, also modulates NK cell effector function through modifying chromatin accessibility (160). Mechanistically, treatment with entinostat was reported to increase chromatin accessibility of the *IFIT1* gene promoter region, driving the epigenetic upregulation of the IFIT1-mediated IRF1, STAT4 and STING pathways, and resulting in increased NK cell cytotoxicity against tumor targets (160). Although entinostat was found to enhance NK cell cytotoxicity, it remains unclear how other epigenetic modifying drugs (several of which are currently in clinical trials for the treatment of various cancers) may directly or indirectly impact upon NK cell function (155). Furthermore, the breadth of other exposures which can imprint upon the NK cell epigenetic landscape and thus modulate NK cell activity is currently unknown.

Perhaps the most notable example of an environmental exposure driving epigenetic remodeling of the NK cell repertoire is the CMV-driven expansion of adaptive NK cells.

The unique phenotypic and functional characteristics of this "memory-like" adaptive NK cell population corresponds with epigenetic imprinting at the regulatory regions of genes encoding IFNγ, FcεRIγ, EAT-2, and PLZF (35, 113, 161). For example, demethylation of the *IFNG* locus increases the accessibility of the CNS1 region and drives the characteristic increase in IFNγ expression displayed by adaptive NK cells (161). Conversely, hypermethylation of the *FCER1G* and *SH2D1B* (encoding EAT-2) loci corresponds with the reduced expression of these signaling proteins (35). Hypermethylation of an intronic region of the *ZBTB16* locus (encoding PLZF) was also observed, corresponding with a striking 77% downregulation of this transcript in adaptive NK cells compared to conventional NK cell populations (35). Demethylation of the *IFNG* locus at CNS1 has also been reported in NKG2C$^{-/-}$ adaptive NK cells, further highlighting the crucial role epigenetic remodeling plays in driving this unique functional state. Although described only in the context of CMV to date, it remains possible that a broader range of exposures to other viral, bacterial, or even eukaryotic pathogens may also contribute towards a memory-like functional fate (162). However, as many studies have focused solely on known adaptive NK cell phenotypes, distinct functional or epigenetic profiles driven by other environmental stimuli have not been identified. Indeed, it remains unclear how a lifetime of exposures may imprint upon the epigenetic landscape of an individual's NK cell repertoire. It is also unclear whether *ex vivo* expansion methods or the tumor microenvironment itself may drive epigenetic changes in adoptively transferred NK cells, potentially affecting their persistence and antitumor activity *in vivo*. Nevertheless, as epigenetic alterations are both reversible and druggable, identification of the specific epigenetic signatures underlying enhanced antitumor immunity will yield novel targets that can be exploited to further improve NK cell-based therapies. More broadly, a deeper appreciation of the epigenetic determinants of NK cell functional potential may also aid in identifying and selecting optimal NK cell populations or donors for immunotherapy.

Transcriptomic and epigenomic analyses have uncovered a greater level of heterogeneity, both within and between the NK cell repertoires of different individuals, than previously observed by phenotype alone. As emerging evidence continues to highlight the high level of complexity and plasticity within the NK cell compartment, new studies into the molecular regulation of NK cell functional fate hold great promise for revolutionizing the field of NK cell immunotherapy. Indeed, a comprehensive understanding of the transcriptional and epigenetic programs underlying enhanced NK cell activity or longevity *in vivo* may reveal a plethora of molecular phenotypes and targets that can be exploited to improve future NK cell-based therapies.

CONCLUSION AND PERSPECTIVES

NK cells have tremendous potential to revolutionize the field of cancer immunotherapy. Coupled with their innate potency against cancer and ability to be transferred between donors and patients without mediating severe adverse effects, NK cells have emerged as ideal candidates for the development of readily available "off-the-shelf" therapies. Whilst a range of NK cell sources and *ex vivo* manipulation strategies have been extensively investigated over the past two decades, there remains no standard criteria by which NK cells with enhanced therapeutic potential can be identified and selected for immunotherapy. Indeed, it is now apparent that NK cells displaying similar functions can express a wide variety of phenotypic markers, and individual NK cells within a defined phenotypic population can fulfil a range of distinct functional roles. As accumulating evidence continues to expose discrepancies between the NK cell phenotype and functional output, there is a need to develop new strategies by which NK cell donors or populations with enhanced antitumor potency can be identified.

Advancements in the fields immunometabolism, transcriptomics, and epigenomics have led to an exciting new era for NK cell research, highlighting a deeper level of complexity and plasticity within the NK cell compartment than previously described. Recent studies have leveraged these multi-omics technologies to describe novel determinants of enhanced NK cell activity, including increased rates of glycolytic metabolism, greater metabolic fitness, and epigenetic remodeling towards a "poised" effector state. However, it remains unclear how these metabolic and molecular "fingerprints" can be used to select NK cell donors or populations for greater antitumor activity *in vivo*. Whilst several measures of metabolic fitness and mTOR activity can be assessed simultaneously using flow cytometric analyses [reviewed (147)], efficient profiling of NK cells based on epigenomic or transcriptomic signatures remains unachievable. However, these unbiased approaches are indispensable for gaining a greater appreciation for the heterogeneity in the NK cell repertoire both within and between donors. A comprehensive understanding of how these molecular regulatory programs interact with cellular metabolism and drive NK cell functional fate will aid in developing new strategies for profiling NK cells based on functional potential. Furthermore, a deeper knowledge of these important regulatory pathways will uncover new targets that can be exploited to enhance the efficacy of future NK cell-based therapies.

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Engineered CAR-Macrophages as Adoptive Immunotherapies for Solid Tumors

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Cellular immunotherapies represent a promising approach for the treatment of cancer. Engineered adoptive cell therapies redirect and augment a leukocyte's inherent ability to mount an immune response by introducing novel anti-tumor capabilities and targeting moieties. A prominent example of this approach is the use of T cells engineered to express chimeric antigen receptors (CARs), which have demonstrated significant efficacy against some hematologic malignancies. Despite increasingly sophisticated strategies to harness immune cell function, efficacy against solid tumors has remained elusive for adoptive cell therapies. Amongst cell types used in immunotherapies, however, macrophages have recently emerged as prominent candidates for the treatment of solid tumors. In this review, we discuss the use of monocytes and macrophages as adoptive cell therapies. Macrophages are innate immune cells that are intrinsically equipped with broad therapeutic effector functions, including active trafficking to tumor sites, direct tumor phagocytosis, activation of the tumor microenvironment and professional antigen presentation. We focus on engineering strategies for manipulating macrophages, with a specific focus on CAR macrophages (CAR-M). We highlight CAR design for macrophages, the production of CAR-M for adoptive cell transfer, and clinical considerations for their use in treating solid malignancies. We then outline recent progress and results in applying CAR-M as immunotherapies. The recent development of engineered macrophage-based therapies holds promise as a key weapon in the immune cell therapy armamentarium.

Keywords: CAR (chimeric antigen receptor), solid tumor, adoptive cell immunotherapy, synthetic biology, macrophage/monocyte

INTRODUCTION

In recent years, cellular immunotherapy has emerged as a promising approach for treating cancer. These therapies harness the immune system's capacity to clear foreign pathogens and redirect the response towards tumor associated antigens (TAAs). Cells expressing chimeric antigen receptors (CARs) represent a major class of cellular immunotherapy that program immune cells to recognize TAAs and initiate a targeted antitumor response (1). T cells equipped with CAR (CAR-T) have shown

clinical efficacy in numerous hematological malignancies, leading to the approval of CD19 and BCMA targeted CAR-T products (2).

Although some hematological malignancies have been readily treated by CAR-T, solid tumors present distinct challenges that limit anti-tumor activity. Unlike hematologic malignancies – which allow for disease access in the peripheral blood, bone marrow, lymph nodes, or spleen – solid tumors require active trafficking, extravasation, and penetration into often immunologically cold and dense fibrotic masses. Developing tumors limit T cell recruitment and infiltration, activate broad suppressive pathways to limit T cell activation, and demonstrate heterogenous TAA expression (3–5). Highlighting the potential of CAR-T against solid tumor targets and the barrier of tumor infiltration, a recent case report demonstrated that anti-HER2 CAR-T were able to clear HER2+ sarcoma that metastasized to the bone marrow – a niche to which CAR-T have access (6). Overwhelmingly, systemic therapy with CAR-T have led to minimal efficacy or transient responses. Numerous efforts have therefore been made to create improved iterations of CAR therapies that overcome solid TME challenges. One approach has been to better equip T cells for the TME using synthetic biology – optimization of CAR framework and signaling domains, deletion of inhibitory receptors with CRISPR, and overexpression of accessory genes such as cytokines, immune ligands, and/or transcription factors (7, 8). Combination therapies with checkpoint inhibitors have also improved CAR-T efficacy, as demonstrated with mesothelin-targeting CAR-T and programmed cell death protein 1 (PD-1) blockade (9).

More recently, significant progress has been made in extending the CAR platform from T cells to alternative leukocytes, such as CAR-expressing NK and gamma-delta (γδ) T cells, whose biological functions may offer improved safety profiles or off-the-shelf potential (10, 11). Compared to conventional CAR-T, these lymphocytes offer reduced risk of alloreactivity, distinct modes of cytotoxicity, and reduced likelihood of cytokine release syndrome (CRS) (11). The success of these novel CAR-lymphocytes raises the question: which immune cells are the best chassis for adoptive CAR immunotherapies? An ideal CAR-immune cell would localize to and persist within the TME while coordinating a broad and robust immune response. The careful choice of immune cell could provide the critical foundation for efficacious CAR therapies, building upon the extensive body of work that has been achieved with CAR-T. Given that CARs have only been tested in a subset of immune cells, continued exploration is warranted to identify the optimum cell type for targeting solid tumors.

Macrophages and other cells of the myeloid lineage could potentially overcome the barriers to treating solid tumors that have hindered CAR-T thus far (12–16). Macrophages are phagocytic cells of the innate immune system that are critical for clearing foreign pathogens (13). Unlike lymphocyte-based therapies, macrophages readily localize to and persist within the TME (14). Macrophages can influence surrounding immune cells in both pro- and anti-inflammatory manners and are adept at remodeling the extracellular matrix (ECM) (13, 15). Macrophages are innate immune cells with potent phagocytic and cytotoxic capabilities that can initiate and potentiate an adaptive immune response *via* T cell recruitment, antigen presentation, co-stimulation, and cytokine secretion (13, 16). Taken together, these effector functions enable epitope spreading and alleviate challenges from target antigen heterogeneity. In this review, we discuss the application of macrophages as cell therapies for targeting solid tumors. We outline strategies and challenges for engineering antitumor functions in adoptively transferred macrophages. We particularly focus on the design of CAR-Macrophages (CAR-M) and provide a current perspective on the field.

MACROPHAGES FOR TARGETING SOLID TUMORS

Macrophages are capable of numerous effector functions that could support tumor clearance. Their phenotype is highly plastic and exists across a spectrum of pro- and anti-inflammatory states. Several reviews have comprehensively summarized the dichotomous nature of macrophage polarization (17, 18); here, we provide a brief overview of macrophage phenotype for the context of solid tumor therapies. "Classically activated" (M1) macrophages feature a proinflammatory phenotype that is typically induced by IFN-γ from T helper cells Type 1 (Th1). M1 macrophages secrete pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-1β which can coordinate an immune response and generate reactive oxygen species to facilitate killing of pathogens (19, 20). Through such mechanisms, M1 macrophages have been shown to exhibit increased tumoricidal activities *in vitro* (21). Activated macrophages upregulate expression of antigen presentation machinery, such as major histocompatibility complex class II (MHC-II), CD80 and CD86, and can thereby serve as antigen presenting cells (APCs) that activate the adaptive immune response by cross-presenting phagocytosed antigens (22–24). Macrophages can thus remove pathogens either directly or by educating the surrounding immune system, both of which would be invaluable for eradicating solid tumors.

In cancer, macrophages often adopt an anti-inflammatory or "alternatively activated" (sometimes referred to as M2) phenotype. Alternatively activated macrophages mediate tissue repair and secrete immunoregulatory cytokines such as IL-4, IL-10, IL-13 and TGF-β, which many solid tumors exploit to support their own growth (25–27). Monocytes are actively recruited to the TME *via* chemoattractants such as CCL2, where they differentiate into tumor-associated macrophages (TAMs) (28). Within the TME, hypoxia and elevated T helper cells Type 2 (Th2) cytokine levels bias TAMs to express tumor-favoring genes (29–31). TAMs support angiogenesis and increased vascular density, thereby promoting tumorigenesis (32). Furthermore, TAMs favor regulatory T cell responses and suppress effector T cell functions through mechanisms including immunosuppressive cytokine secretion, upregulation of programmed death ligand-1 (PDL-1), and enzymatic depletion of L-arginine (33, 34). TAM enrichment in the TME is thus correlated with poor overall prognosis during natural tumor

progression (35). While M1 and M2 macrophage categorization is a significant simplification of the intratumoral phenotypic spectrum, macrophages have a dynamic relationship with the TME, supporting the notion that using synthetic biology to control macrophage phenotype and function has significant potential to drive anti-tumor immunity.

REPROGRAMMING MACROPHAGES FOR TUMOR SUPPRESSION WITH CELLULAR ENGINEERING

A crucial challenge when generating macrophage-based cancer therapies is enabling proinflammatory effector functions that persist despite the immunosuppressive TME. Efforts to do so broadly fall into two camps – *in situ* reprogramming of TAMs, or *ex vivo* priming of macrophages for adoptive cell transfer. Extensive work has been done on the former to repolarize or deplete TAMs *in situ*, and this work has recently been reviewed elsewhere (36, 37). Here, we focus on *ex vivo* manipulations used in adoptive therapies, including pre-treatment with recombinant proteins, expression of therapeutic transgenes, and gene editing with CRISPR-Cas9.

Historically, adoptive macrophage therapies have used recombinant proteins or small molecules to prime immune responses *ex vivo* (38–42). Earlier studies have shown that IFN-γ treatment enhances macrophage cytotoxicity *in vitro* (21). The first dose-escalation studies in humans therefore isolated peripheral blood monocytes from patients, cultured and differentiated them into macrophages over the course of 7 days, and primed them with IFN-γ for 18 hours prior to infusion (38, 39). However, IFN-γ-primed macrophages had minimal clinical efficacy and failed to induce significant tumoricidal activity. The adoptive transfer of M1-activated macrophages was well-tolerated by patients, with clinical side effects primarily limited to fever and flu-like symptoms (41). Follow-up studies further showed that radiolabeled macrophages were detected at sites of metastasis for more than 7 days following infusion (43). Collectively, these trials demonstrated the feasibility of manufacturing and safety of delivering billions of autologous macrophages through intravenous administration. Results from these early trials thus provided a critical foundation for adoptive myeloid cell therapies.

Recent approaches have used genetic engineering to design macrophages that express proinflammatory transgenes of interest (12, 44–49). These strategies leverage the tumor-homing tendences of macrophages to locally deliver therapeutic cargo and induce cytotoxic activity within the tumor niche. For example, IL-12 is a pro-inflammatory cytokine that activates T and NK cells, but its clinical application is hindered by a narrow therapeutic window that precludes safe systemic administration (50). Multiple groups have attempted to overcome the limitations of IL-12 cytokine therapy by recombinantly expressing the cytokine within genetically engineered macrophages (GEMs) or myeloid cells (GEMys) (44, 45). Preclinical models demonstrated that GEMs and GEMys were able to activate a T cell response and prolong survival without inducing systemic toxicity. Similarly, studies have used GEMs to locally deliver interferon α (IFN-α) or IL-21, which promote immune cell activation, or soluble transforming growth factor receptor II (TGFβR2), which impedes TGFβ-mediated immunosuppression (46, 47). Whereas these approaches stimulate the immune system in a constitutive manner, other studies have focused on confining cytotoxicity to antigen-specific contexts. Gardell et al. engineered antigen-specific killing using GEMs that secrete a bispecific T cell engager (BiTE), which creates a functional bridge between T cell receptors and mutated epidermal growth factor receptor variant III (EGFRvIII) on glioblastoma cells (48). BiTE-secreting GEMs facilitated antigen-specific killing by T cells, which was further augmented by the groups work on IL-12 GEMs (44). Cha et al. similarly targeted EGFR by encoding a secreted single-chain variable fragment (scFv) fused to a Fc moiety, which opsonized tumor cells and induced antibody-dependent cellular phagocytosis (ADCP) by macrophages (49). Notably, engineered macrophages can deliver cargo other than genetically encoded proteins; for example, Huang and colleagues used nanoparticles to engineer macrophages that carry photo-sensitive cytotoxic agents, which are released and induce immunogenic cell death upon exposure to near infrared light (51).

Rather than overexpressing transgenes, inhibiting gene expression using CRISPR-Cas9, zinc finger nucleases, and TALENs have been utilized to augment CAR-T and NK cell function (52–54). Recently, there has been increasing interest in gene editing human myeloid cells, and several nucleofection-based methods for transiently delivering CRISPR-Cas9 ribonucleoproteins (RNPs) to primary myeloid cells have been employed (55, 56), as well as specialized methods using nanoparticles to deliver Cas9 plasmid or RNPs (57, 58). Attractive targets for gene editing include regulatory proteins that block anti-tumor functions, such as signal regulatory protein-α (SIRPα). Cancer cells expressing CD47 stimulate macrophage SIRPα to generate a "don't eat me" signal to evade phagocytosis (59), and the SIRPα/CD47 signaling axis is now a well-established checkpoint in tumor immunity (60). Ray et al. therefore performed a SIRPα knockout (KO) in the murine monocyte/macrophage cell line RAW264.7 using CRISPR-Cas9 and demonstrated that SIRPα-KO macrophages in this system exhibited enhanced phagocytic ability against cancer cells *in vitro* (58). A subsequent study by Bian et al. demonstrated the therapeutic potential of SIRPα-KO macrophages using syngeneic *in vivo* models and SIRPα-deficient mice (61). The authors in this study demonstrated that SIRPα-deficient macrophages gained potent anti-tumor properties and coordinated a robust immune response when delivered in combination with radiotherapy (61). Similarly promising results were generated by Myers et al. upon targeting the tyrosine phosphatase Shp1, which signals downstream of SIRPα to propagate anti-phagocytic signals (62). Instead of irreversibly editing genes, numerous CRISPR-based technologies regulate gene transcription using a catalytically dead Cas9 (dCas9) and chromatin remodeling factors (63). For example, Liu et al. silenced *CD45*, *CD209* and *TICAM1* genes in primary human monocytes using CRISPR interference (CRISPRi), wherein dCas9 is fused to a KRAB domain (64). Dong et al. used dCas9 fused to a histone methylase to

epigenetically silence hypoxia inducible factor 1 subunit alpha (*Hif1α*), which mediates TAM immunosuppressive functions (65). When tested in a murine melanoma model, their *Hif1α* Epigenetically Repressed Macrophage ("HERM") was able to reprogram the tumor's immunosuppressive microenvironment and prolong survival (65).

CAR-M: MACROPHAGES TAKE THE WHEEL

CARs provide a flexible platform for directing immune cell effector functions towards antigen-expressing tumor cells and can promote macrophage antitumor capabilities. Initial studies demonstrating the success translating the synthetic receptors to macrophages are summarized in **Figure 1**.

Designed, Sealed, and Delivered; Producing CARs for Expression in Macrophages

Current efforts to engineer CAR-M have found that basic CAR design principles from the T cell field hold true for macrophage biology. Traditional CARs are modular transmembrane proteins consisting of an extracellular antigen-recognition domain, a hinge domain, and one or more cytoplasmic signaling domains (1, 66). We have demonstrated that CARs comprising an scFv against broadly representative targets CD19, HER2, and mesothelin, a CD8 hinge and transmembrane domain, and the CD3ζ intracellular domain efficiently redirect macrophages, guiding antigen dependent phagocytosis, cytokine release, and anti-tumor activity (67). Macrophages expressing CARs with CD3ζ, but not with CD3ζ deletions/tyrosine mutations, killed and phagocytosed tumor cells in an antigen-specific manner. Although CD3ζ is canonically used in CARs due to its role in T cell activation, its cytosolic domain bears significant homology with the macrophage-native Fc receptor common gamma chain (FcRγ) that drives ADCP, though with 3 ITAM domains. We confirmed that CAR-M constructed with either the CD3ζ or FcRγ activating domain were functionally similar in phagocytosis assays, conversely complementing earlier findings showing that CD3ζ- and FcRγ-based chimeric receptors were comparably capable of activating T cells (66).

Indeed, the choice of signaling domain is of particular interest when designing CAR-M, and several groups have explored alternative domains. Morrissey et al. designed CAR-M by screening cytoplasmic domains from murine phagocytic receptors including multiple EGF-like-domains protein 10 (Megf10), FcRγ, adhesion G protein-coupled receptor B1 (Bai1) and tyrosine-protein kinase Mer (MerTK) (68). Primary murine macrophages expressing the FcRγ- or Megf10-based CAR exhibited antigen-specific phagocytic capabilities. Niu et al. designed anti-C-C chemokine receptor type 7 (CCR7) CAR-M to target a newly identified $LD^{hi}CCR7^{hi}$ immunosuppressive cell population (69). Their design utilized CCL19, the natural ligand of CCR7, as the receptor's antigen-recognition domain, rather than an scFv. For the intracellular domain, they evaluated activation domains from MerTK, toll-like receptor 2 (TLR2), TLR4, TLR6 and the CAR-T second-generation 4-1BB-CD3ζ. When screened

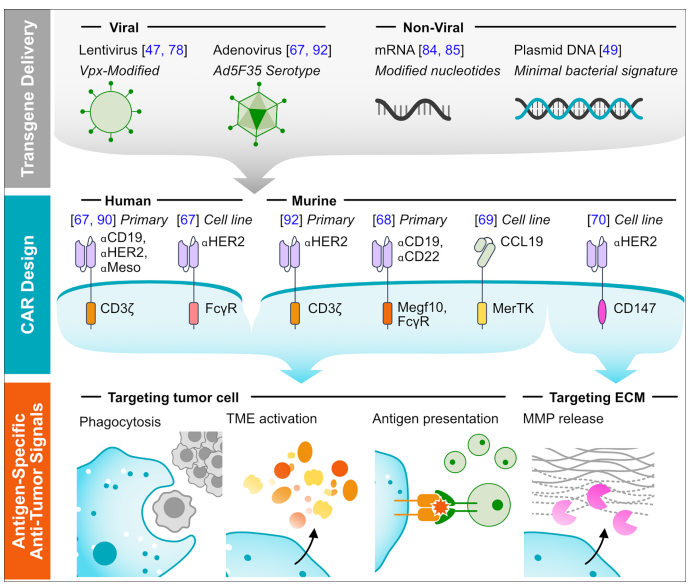

FIGURE 1 | Methods of targeting tumors using CAR-M. (Top) Representative viral and non-viral methods for delivering transgenes to macrophages are listed. (Middle) Representative CAR designs that have been functionally validated in macrophages, with annotated antigen-targeting and cytosolic domains. The system in which the receptors were validated is noted: either human or murine, testing with primary cells or exclusively with immortalized cell lines. (Bottom) Major mechanisms of tumor clearance by CAR-M.

in the RAW264.7 cell line, CAR-M bearing the MerTK activation domain exhibited the greatest tumor cell toxicity. Interestingly, while anti-CCR7 MerTK-based CAR-M performed well in this context, Morrissey et al's anti-CD19 CAR bearing the same cytosolic domain was unable to bind antigen-functionalized beads, despite expression at the cell surface (68). Such discrepancies hint that optimization and careful functional evaluation is necessary when generating new CAR-M architectures. In a final example, Zhang et al. designed a CAR bearing the activation domain from CD147 (CAR-147), a protein that regulates matrix metalloproteinase (MMP) expression and ECM remodeling (70). Instead of triggering phagocytosis, CAR-147 targeted the tumor ECM by upregulating expression of MMPs upon antigen recognition. While CD147 itself is not macrophage-specific, the utilization of this CAR design allowed for CAR-dependent secretion of MMPs within the tumor. These studies collectively illustrate that the modular CAR template can customize how macrophages respond to target antigens. Future efforts to engineer CAR-M will likely tap into the plethora of sophisticated CAR designs that have been developed for T cells, incorporating tandem activation domains (71), multi-antigen logic gates (72, 73), or drug-sensitive modules (74–76).

Delivering CARs and other transgenes to macrophages can present a challenge for researchers, but recent advances in gene delivery have enabled several viral and non-viral strategies for doing so. Myeloid cells are proficient at detecting and responding to foreign nucleic acids (77), making macrophages and monocytes resistant to genetic manipulation. Bobadilla et al. created novel HIV-1-derived lentiviral particles capable of infecting myeloid cells by leveraging the viral accessory protein Vpx (78). Upon infection, Vpx mediates degradation of SAMHD1, a myeloid-specific HIV-1 restriction factor that inhibits lentiviral transduction by limiting the deoxynucleotide pool and preventing efficient reverse transcription (79). The group demonstrated that modified lentiviral virions containing Vpx can efficiently deliver transgenes to myeloid cells. The Vpx platform can accommodate any pre-existing HIV-based lentiviral vector and thus provides an accessible strategy for modifying myeloid cells (47, 64, 78). Given that macrophages have limited proliferative capacity, we hypothesized that non-integrating, replication deficient adenoviral vectors may allow for efficient and long-term transduction. However, human myeloid cells do not express the Coxackie-adenovirus receptor, which serves as the primary docking site for traditional Ad5 vectors. Monocytes and macrophages highly express CD46, which mediates docking of group B adenoviruses such as Ad35 (80, 81). We thus evaluated the replication-incompetent chimeric adenoviral vector Ad5f35 and demonstrated that Ad5f35 exhibited robust transduction of primary human macrophages and monocytes – with CAR% and viability routinely >80% (67, 82). Ad5f35-transduced macrophages maintained CAR expression for at least 1 month *in vitro* and at least 62 days *in vivo*, as measured by co-expression of CAR-P2A-luciferase. Notably, Ad5f35 activated the macrophage inflammasome and provided a beneficial proinflammatory priming signal, which synergized with CAR activity and rendered the CAR-M locked into an M1 phenotype (83). Such results highlight the prospect of leveraging, rather than evading, the inflammatory response that can occur when delivering genetic material.

Several non-viral strategies have also been developed for engineering monocytes and macrophages. The bacterial origin of plasmid DNA can contribute to inflammation and gene silencing. Plasmids devoid of unmethylated cytosine-phosphoguanine (CpG) dinucleotides – a signature of bacterial DNA – were shown to evade detection by TLR9 and exhibit prolonged gene expression in RAW 264.7 macrophages and primary murine BMDMs (49). Other work has optimized the transient delivery of mRNA to monocytes and macrophages, carefully selecting mRNA modifications and transfection reagents to minimize transfection-induced macrophage toxicity or activation (84, 85). Lastly, transposon systems, which enable non-viral integration into the host genome, have been explored in porcine aortic macrophages (86).

Macrophages may be sourced through several production pipelines. While proof-of-concept studies can be performed in model cell lines such as THP1 and Raw 264.7 or with primary/immortalized murine BMDM, clinical translation necessitates a scalable source of primary human cells. For autologous cell therapies, 2-3x10^9 peripheral blood monocytes can be obtained by leukapheresis (87), and mobilization with filgrastrim or sargramostim further increases the number of available monocytes by approximately threefold (88). Our CAR-M therapy is manufactured over 1 week using filgrastrim-mobilized $CD14^+$ monocytes (67). Monocytes are cultured and differentiated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), which is associated with a pro-inflammatory differentiated phenotype (67, 89). Cells are then transduced with CAR-encoding Ad5f35, which further cements a pro-inflammatory phenotype. To further accelerate manufacturing time, a rapid, same day CAR monocyte process has been developed which yields CAR+ CD14+ monocytes with the capacity to differentiate into M1 CAR-M or CAR-expressing dendritic cells (CAR-DC) (82). Macrophages may be attractive as allogeneic cell therapies since there is no risk of graft *versus* host disease. Immune cells derived from induced pluripotent stem cells (iPSCs) hold potential as a renewable, allogeneic source for CAR-M therapies. Zhang et al. generated iPSC-derived CAR-Macrophages (CAR-iMac) by reprogramming PBMC's into iPSC's over the course of several weeks, transducing with CAR-encoding lentivirus, then differentiating into macrophages following a 4-week differentiation process (90). CAR-iMacs were capable of antigen-dependent macrophage functions, such as cytokine secretion and phagocytosis *in vitro*. However, CAR-iMacs differentiated with the current protocol had a lingering anti-inflammatory phenotype, and efficacy was limited when tested in murine models. Additionally in oncology applications, a significant consideration with iPSC-derived CAR-M is MHC-matching; antigen cross-presentation is likely an important component of CAR-M activity downstream of TAA engagement, thus careful study is required to determine whether CAR-M derived from MHC knockout iPSCs can potentiate a sufficient anti-tumor T cell response.

Furthermore, continued optimization of the iPSC-to-macrophage differentiation protocol, method of transduction, method of phenotype control, and GMP scale-up are necessary to translate these early findings into the clinic. Provided the process is appropriately scaled, there is theoretically no limit to the number of macrophages that can be expanded from iPSCs or differentiation intermediates, though current optimized protocols yield 2-6×10^7 macrophage progenitors per harvest (91). Benchmarking iPSC-derived macrophage phenotype against *bona fide* macrophages will be critical for advancing this approach to CAR-M production.

Mechanisms of Tumor Control by CAR-M

CAR-M therapies are able to clear tumor cells *in vitro* and in preclinical *in vivo* models. *In vitro*, human CAR-M exhibit antigen-specific phagocytosis, cytokine/chemokine secretion, and killing of target antigen expressing targets (67). In two immunodeficient NSGS xenograft models, a single dose of anti-HER2 CAR-M reduced tumor burden and prolonged overall survival against HER2+ SKOV3 tumors. Furthermore, IV-administered CAR-M localized to tumors in several xenograft models and persisted in tumor-free mice (primarily within the liver) for at least 62 days, detected by whole-body bioluminescent imaging of CAR-P2A-luciferase. RNA sequencing revealed that Ad5f35 transduction induced a proinflammatory profile resembling that of classically activated M1 macrophages, which resisted polarization by M2-inducing cytokines *in vitro*. Furthermore, supernatant from CAR-M was sufficient to induce a proinflammatory phenotype in cultured M2 macrophages. These phenotypic results held true in a humanized immune system (HIS) solid tumor xenograft model, where adoptively transferred CAR-M maintained a durable M1 phenotype and induced pro-inflammatory gene expression in host macrophages. *In vitro* analysis further showed that CAR-M could coordinate an antitumor T cell response by recruiting T cells and cross-presenting antigens from phagocytosed cells. Recently, our group established an immunocompetent, syngeneic CAR-M model and demonstrated that murine CAR-M increased intratumoral T cell infiltration, NK cell infiltration, dendritic cell infiltration/activation, and TIL activation (92). We found that CAR-M locally administered in $HER2^+$ tumors simultaneously controlled growth of contralateral $HER2^-$ negative tumors and prevented antigen-negative relapse upon HER2-negative tumor rechallenge, indicating epitope spreading and induction of long-term immune memory. Notably, this work also demonstrated for the first time that CAR-M synergize with PD1 blockade in PD1-monotherapy resistant solid tumor models (92).

Tumor killing by CAR-M was similarly achieved by Niu et al. using CCR7-targeting CAR-M in the RAW264.7 cell line (69). These CAR-M, which exhibited antigen-specific cytotoxicity *in vitro*, prolonged survival and prevented metastasis to distal tissues in a 4T1 breast cancer model. CAR-M recruited $CD3^+$ T cells and decreased PD-$L1^+$ cells in the tumor site, confirming that engineered macrophages themselves are not the sole driver of the antitumor response. Adoptive macrophage therapy also increased levels of pro-inflammatory cytokines IL1-β, IL-6, and TNF-α in the serum, indicative of a systemic immune response (69).

CAR-M's ability to facilitate an immune response was underscored by the CAR-147 technology, which targeted the tumor ECM rather than tumor cells directly (70). Zhang and colleagues hypothesized that degrading the dense tumor ECM would improve immune cell infiltration and thereby trigger antitumor activity. CAR-M engineered with a CD147 cytosolic domain upregulated MMP expression in an antigen-specific manner *in vitro*, but exhibited no changes in phagocytosis, killing, or cytokine release. In a $HER2^+$ 4T1 breast cancer model, CAR-M slowed tumor growth by reducing its collagen content, enhancing the presence of T cells, and increasing IL-12 and IFN-γ signaling. Taken together, these pioneering studies showcase the ability of CAR-M to infiltrate the tumor niche and initiate a broad anti-tumor response by the host immune system.

DISCUSSION

Toward CAR-M Combination Therapies

Co-administration of pharmacological immunotherapies or chemotherapy could further improve CAR-M efficacy. For example, antibody-based immunotherapies rely on macrophage phagocytosis to stimulate an immune response and could be evaluated for augmenting CAR-M efficacy (93, 94). The Fc region of antibodies binds and stimulates macrophage-expressed Fc receptors, leading to ADCP. Antibodies such as trastuzumab and rituximab thus direct macrophages to phagocytose opsonized target cells (95). Antibodies that block phagocytosis-inhibiting signals, such as CD47/SIRPα or the inhibitory Fc receptor FcγRIIB, have enhanced macrophage-mediated immunotherapies (96–98). T cell checkpoint inhibitors blocking PD1 signaling have also been shown to improve macrophage phagocytic capabilities *in vivo* (99). Given the impact of CAR-M on surrounding immune cells, we therefore hypothesized that CAR-M could synergize with PD1 checkpoint inhibitors. In a syngeneic CT26 model, which resists anti-PD1 monotherapy, we demonstrated that the combination of CAR-M with PD1 blockade indeed additively improved overall survival (92). Chemotherapy or radiation therapy could also synergize with CAR-M by inducing immunogenic cell death (100). The efficacy of combining radiation therapy and engineered macrophages was demonstrated by Bian et al. using SIRPα-KO macrophages (61). Furthermore, it is noteworthy that CAR expression is not mutually exclusive from other engineering manipulations described herein. Therefore, future iterations of CAR-M could likely synergize with gene editing or accessory transgene overexpression.

Clinical studies will be crucial to elucidating the toxicity profile of CAR-M in patients. The FDA-approved anti-CD19 CAR-T products tisagenlecleucel, brexucabtagene autoleucel, and axicabtagene ciloleucel carry black box warnings for CRS and neurotoxicity (101). CRS is driven by significant CAR-T expansion and secretion of pro-inflammatory cytokines for

sustained periods of time in the peripheral blood. Given that CAR-M have limited expansion potential and do not persist in peripheral blood, severe CRS is not expected, and indeed was not seen in older studies of M1 polarized non-engineered macrophages (41). Engineered macrophages have been shown to persist in pre-clinical glioblastoma models without associated toxicity, indicating that CAR-M may safely interact with the central nervous system (44, 47). A particular concern that may be more relevant for CAR macrophages than CAR T cells is that the TME could subvert tumor-localized CAR-M into a tumor-supporting phenotype (102). Although preclinical models suggest the opposite – that CAR-M reprogram the TME (67) – correlative studies in patients will be necessary to understand the bidirectional dynamics. At present, the first-in-human CAR-M Phase I clinical trial is underway using Carisma Therapeutic's lead product CT-0508 for treating HER2 overexpressing solid tumors (NCT04660929). Results from this Phase I trial and others will provide invaluable insights to guide the design of safe and effective CAR-M therapies.

Outlook: Beyond Oncology

Future therapies using engineered macrophages may extend beyond oncology indications. CAR-T have been shown to target fibrotic cardiac and liver tissues, and CAR-M may be even better suited for acellular pathogenic targets (103, 104). Novel therapies could also leverage macrophage tissue remodeling and anti-inflammatory capabilities, rather than their proinflammatory functions. For example, adoptive transfer of anti-inflammatory macrophages has been shown to reduce fibrotic tissue in liver injury models (105). From remodeling synapses to repairing cardiac tissue, macrophages are ubiquitous in maintaining tissue homeostasis, and their therapeutic application should be compatible with myriad tissue contexts (106, 107). In conclusion, macrophage phenotypic plasticity, when combined with synthetic biology, presents an exciting new platform for therapeutic applications to advance cellular engineering and deliver effective immunotherapies.

AUTHOR CONTRIBUTIONS

CS wrote the article and designed the figure. SG and MK contributed to writing and critically revised the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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iPSC-Derived Natural Killer Cell Therapies - Expansion and Targeting

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Treatment of cancer with allogeneic natural killer (NK) cell therapies has seen rapid development, especially use against hematologic malignancies. Clinical trials of NK cell-based adoptive transfer to treat relapsed or refractory malignancies have used peripheral blood, umbilical cord blood and pluripotent stem cell-derived NK cells, with each approach undergoing continued clinical development. Improving the potency of these therapies relies on genetic modifications to improve tumor targeting and to enhance expansion and persistence of the NK cells. Induced pluripotent stem cell (iPSC)-derived NK cells allow for routine targeted introduction of genetic modifications and expansion of the resulting NK cells derived from a clonal starting cell population. In this review, we discuss and summarize recent important advances in the development of new iPSC-derived NK cell therapies, with a focus on improved targeting of cancer. We then discuss improvements in methods to expand iPSC-derived NK cells and how persistence of iPSC-NK cells can be enhanced. Finally, we describe how these advances may combine in future NK cell-based therapy products for the treatment of both hematologic malignancies and solid tumors.

Keywords: NK cell, chimeric antigen receptor (CAR), immunotherapy, iPSC (induced pluripotent stem cells), cell engineering

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INTRODUCTION

Over the past decade, cellular therapies have advanced from pre-clinical studies through clinical trials and now to several U.S. Food and Drug Administration (FDA) approved therapies. Despite these successes, the FDA approved chimeric antigen receptor (CAR) T cell therapies for B-cell acute lymphoblastic leukemia (B-ALL), B-cell lymphomas and multiple myeloma are limited by their manufacturing processes and treatment-related toxicity (1).

Some of the major challenges with autologous CAR-T cell therapy are antigen escape, limited capability of CAR-T cells to migrate to and infiltrate the immunosuppressive tumor microenvironment (TME), and treatment-associated toxicities. The most significant adverse effects of CAR-T cells are cytokine-release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (2). CRS occurs in approximately 25% of patients treated with anti-CD19 CAR-T cells, severe ICANS in 12-42% and non-relapse related death in 1-2% of treated patients (3–6). An additional manufacturing issue with autologous CAR-T cell therapies is that patients who have previously received multiple rounds

of chemotherapy may not be able to mobilize sufficient T cells for CAR-T cell production with up to 10-30% of patients that fail CAR-T cell manufacturing (3, 5, 7). Additionally, in the time required for CAR-T cell manufacturing, patients can experience disease progression. For example, 38 out of 165 patients enrolled in one study of anti-CD19 CAR-T cells dropped out before receiving therapy (5). Therefore, 30% or more of patients who could potentially benefit from CAR-T cell therapy do not receive the treatment. Additionally, the cost of CAR-T cell manufacturing is typically $300,000-$500,000 for each patient, even before the costs of care.

Due to these limitations of autologous CAR-T cells, allogenic T cell approaches and alternative cell sources for cellular therapy have been investigated. Collecting allogenic, healthy and functional T cells from volunteer donors instead of the cancer patient undergoing chemotherapy has been one approach; however, allogenic T cells must be modified to prevent the development of graft versus host disease (GVHD) (8). Allogeneic T cells have been engineered to delete their human leukocyte antigen (HLA) class I and II molecules and disrupt T-cell receptor (TCR) expression to evade immune rejection and reduce GVHD in patients (7, 9–12).

Natural killer (NK) cells, key effector cells of the innate immune system, possess features that can overcome many of the challenges associated with autologous CAR-T cells. NK cells are an ideal cell population for anti-cancer cell therapy as the repertoire of receptors that regulate NK cell activity are distinct from the TCR system and allow for use of NK cells as an allogeneic therapy (13, 14). Therefore, NK cells do not require HLA matching and multiple clinical studies demonstrate a lack of GVHD despite these being allogeneic cells, making them a relatively safer therapeutic approach compared to allogeneic CAR-T cells that can still lead to GVHD if there are any cells with residual TCR (15–19). NK cells are known to play a key role in immunosurveillance that can limit or prevent tumorigenesis (20). This ability for NK cells to provide natural immunity to malignancies has been demonstrated in both mice and humans (21, 22). Agents that enhance endogenous NK cell activity can lead to improved anti-tumor responses (13). For example, the anti-NKG2A monoclonal antibody Monalizumab that blocks this inhibitory receptor expressed on NK cells and cytotoxic T cells has demonstrated potent anti-tumor activity in clinical trials (23, 24). NK cells are also recognized to play a key role in the anti-tumor activity of allogeneic hematopoietic cell transplantation (25). Because of these potential advantages, NK cells obtained from various sources have been tested as a specific cell population for adoptive transfer to treat cancer patients in clinical trials. These sources include the NK-92 cell line, peripheral blood cells, umbilical cord blood (CB), and induced pluripotent stem cells (iPSCs) (16, 26–28). iPSC-derived NK cells provide added benefits in terms of relative ease of genetically engineering, clonal selection post-genetic modification and no requirement for cells to be collected from a donor at any point in time. However, the scale-up and manufacturing of NK cells starting from iPSCs can be more challenging, though has been routinely accomplished (29).

NK CELLS AS CELLULAR THERAPY

Distinct NK cell sources each possess advantages and disadvantages for use in cellular therapy targeting cancer (30). Peripheral blood NK (PB-NK) cells must be collected from a donor by apheresis and expanded prior to use (16, 31, 32). CB-NK cells are required to be obtained from an umbilical cord blood unit and expanded (28, 33, 34). CB-NK cell populations can be expanded and exhibit similar cytotoxicity to PB-NK cells against tumor cells post expansion (34, 35). For both PB- and CB-NK cells there is variability in the NK cell yield from each blood unit which is influenced by donor variability and dependent on NK cell yield post-purification (15, 36). NK cell lines such as NK-92 provide homogeneous cell populations that expand indefinitely in culture and are more amenable to genetic alteration (26). However, these cell lines lack important receptors typically expressed on NK cells. For example, NK-92 cells do not express Killer Ig-like receptors (KIRs) or CD16, an Fc receptor that plays an important role in activating antibody-dependent cellular cytotoxicity (ADCC) (26). Additionally, NK tumor cell lines such as NK-92 cells are aneuploid and for safety reasons must be irradiated prior to patient administration. This irradiation limits their ability to expand and persist *in vivo*, decreasing anti-tumor efficacy (26). Pre-clinical studies and clinical trials of cellular therapies have demonstrated that improved CAR-T cell persistence corresponds with better treatment efficacy (37, 38). Similar studies for NK cells have also shown that persistence in pre-clinical *in vivo* models correlates with better tumor killing (39, 40). Therefore, this limited expansion and persistence after being administered to patients may account for the limited efficacy of NK-92 cells in several clinical trials (41, 42).

Multiple clinical trials using these different NK cell products demonstrate the efficacy of allogeneic NK cell adoptive transfer therapy. The ability of unmodified allogeneic NK cells to kill tumors that are resistant or refractory to standard therapies has been most clearly demonstrated in the treatment of acute myeloid leukemia (AML) (16, 17, 43–45). The first, seminal study using PB-NK cells was done by Miller et al. who treated patients with relapsed/refractory AML with allogeneic PB-NK cells from haploidentical donors. Complete hematologic remission was obtained in five of nineteen patients (16). In a larger study of 42 patients with AML treated with haploidentical NK cells and IL-15 by the same group, approximately 40% of patients achieved complete remission (45). A separate study of AML patients treated with haploidentical NK cells combined with an immunotoxin to deplete IL2 receptor-expressing T-regulatory cells led to 53% compete response rate (44).

Romee, Fehniger and colleagues demonstrated that stimulation with the cytokines interleukin-12 (IL-12), IL-15, and IL-18 produces so-called cytokine-induced memory-like (CIML) NK cells that exhibited a 56% overall response rate and 44% complete response rate in treatment of acute myeloid leukemia (46–48). Another phase 1 clinical trial by Green Cross LabCell Corporation used allogeneic NK cells (named MG4101) derived from peripheral blood in combination with rituximab for

patients with B cell lymphomas (49, 50). No patients experienced dose-limiting toxicities and five out of nine patients experienced a response.

Additional trials using NK cells engineered to improve targeting of tumors and NK cell expansion have been initiated (15, 51–53). For example, a recent trial utilizing adoptive transfer of *ex vivo* expanded, HLA-mismatched, CB-NK cells engineered to express both an anti-CD19 CAR and secreted IL-15 were used to treat 11 patients with CD19-positive relapsed or refractory B cell malignancies and demonstrated objective response in 73% of the patients (15). Importantly, none of the patients developed serious toxicities associated with CAR-T cells including cytokine release syndrome, neurotoxicity, and GVHD (15).

iPSC-NK CELLS – A STANDARDIZED, OFF-THE-SHELF ALTERNATIVE FOR CELLULAR THERAPY

NK cells generated from pluripotent stem cells have also emerged as a promising strategy to produce standardized, off-the-shelf NK cells with improved anti-tumor activity. This approach circumvents many of the challenges seen with other NK cell populations and T cells for adoptive cell therapy, such as the requirement for collection from a donor or cord blood unit. In contrast, pluripotent stem cells, either human embryonic stem cells (hESCs) or iPSCs, can grow indefinitely in an undifferentiated state *via* self-renewal (54–56). Therefore, the ability to routinely derive NK cells from hESCs and iPSCs allows for an unlimited number of uniform NK cells to be produced from the starting pluripotent stem cell population to provide a standardized, off-the-shelf approach.

The use of hESCs or iPSCs to derive engineered cell products also enables individual clone isolation and detection of off-target genomic alterations *via* whole-genome sequencing (39, 57). This approach also allows for the efficient addition of multiple genetic alterations to augment NK cell cytotoxicity. Genetic engineering approaches such as transposons and lentiviral delivery ensure efficient transgene insertion and stable expression in iPSCs (58, 59). TALENS and CRISPR/Cas9 can also be used for more precision in knocking in or deleting specific genes (60–64). Once engineered, the engineered and undifferentiated iPSCs can be frozen and stored to allow for consistent production of NK cells with an identical phenotype.

The first studies of human pluripotent stem cells demonstrated that hESCs can be differentiated into the three primary germ layers (54). Further studies led to the differentiation of $CD34^+$ hematopoietic progenitor cells and specific myeloid, erythroid, and lymphoid lineage populations (65–70). With the advent of iPSC technology, laboratories worldwide have developed protocols to differentiate target cells of many lineages with hopes of use for cellular therapy for complex diseases. Improvement in methods to derive NK cells from hESCs/iPSCs now enables the production of homogeneous, functional NK cells at a clinical scale (29). Initial methods to derive cells of hematopoietic origin involved coculturing of hESCs with irradiated stromal cell lines to generate $CD34^+CD45^+$ hematopoietic progenitors (29, 71). This was followed by use of a second stromal cell line combined with defined cytokines to produce mature NK cells (29, 71). Subsequent studies refined NK cell production from hESCs/iPSCs to eliminate the use of serum-containing media and stromal cells. A "spin embryoid body (EB)" protocol produces hematopoietic organoids that contain hematopoietic progenitor cells, as well as endothelial and mesenchymal cells. These hematopoietic progenitor cells then differentiate into NK cells under defined conditions (72, 73). The hESC/iPSC-derived NK cells can also be further expanded in the presence of IL-2 and K562 cells engineered to present 4-1BB ligand and IL-21 to the NK cells (29, 31). hESC/iPSC-derived NK cells recapitulate many key features of primary NK cells. They express important NK cell markers such as CD56, CD94, NKG2D, NKp44, NKp46, CD16, and KIRs, and exhibit potent cytotoxicity toward diverse solid tumors and hematological malignancies (69, 74, 75). Other methods to derive NK cells from human iPSCs have also been demonstrated, including developmental and functional differences between NK cells derived under Wnt-dependent versus Wnt-independent conditions (76).

Like CB- and PB-NK cells, hESC/iPSC-derived NK cells exhibit cytotoxicity against diverse target cells *via* lytic granule release of perforins and granzymes, production of proinflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα), and direct cell contact mediated apoptosis through TRAIL and Fas-FasL interaction (57, 77). However, NK cells derived from iPSCs are equally or more effective as primary NK cells and NK cell lines. *In vivo* ovarian cancer xenograft models demonstrated iPSC-NK cytotoxicity was comparable to PB-NK cells (27). A different group found that iPSC-NK cells have greater cytotoxicity against multiple ovarian, colon and breast cancer cell lines compared to donor PB-NK cells (78).

IMPROVEMENT OF iPSC-NK CELL EXPANSION AND FUNCTION THROUGH GENETIC ENGINEERING

Multiple recent studies have genetically engineered iPSCs to create iPSC-NK cells with enhanced expansion, *in vivo* persistence and tumor killing capability are being explored (52, 79, 80). Many of these technologies were first developed and tested in PB-NK cells and/or CB-NK cells and subsequently translated into iPSC-derived NK cells. The iPSCs provide a stable platform for routine genetic modifications that only need to be done on a one-time basis. Once a stably engineered iPSC clone is identified, this can be expanded and used to produce a standardized population of appropriately engineered iPSC-derived NK cells. Some examples of strategies to enhance NK cell functions are described in this section (**Figure 1**).

Improving NK Cell Anti-Tumorigenic Activity and Expansion *via* IL-15 Pathway

IL-15 plays an important role to stimulate NK cell expansion and cytotoxic functions (13, 81–84). IL-15 activation has also been shown to mitigate the immunosuppression mediated by

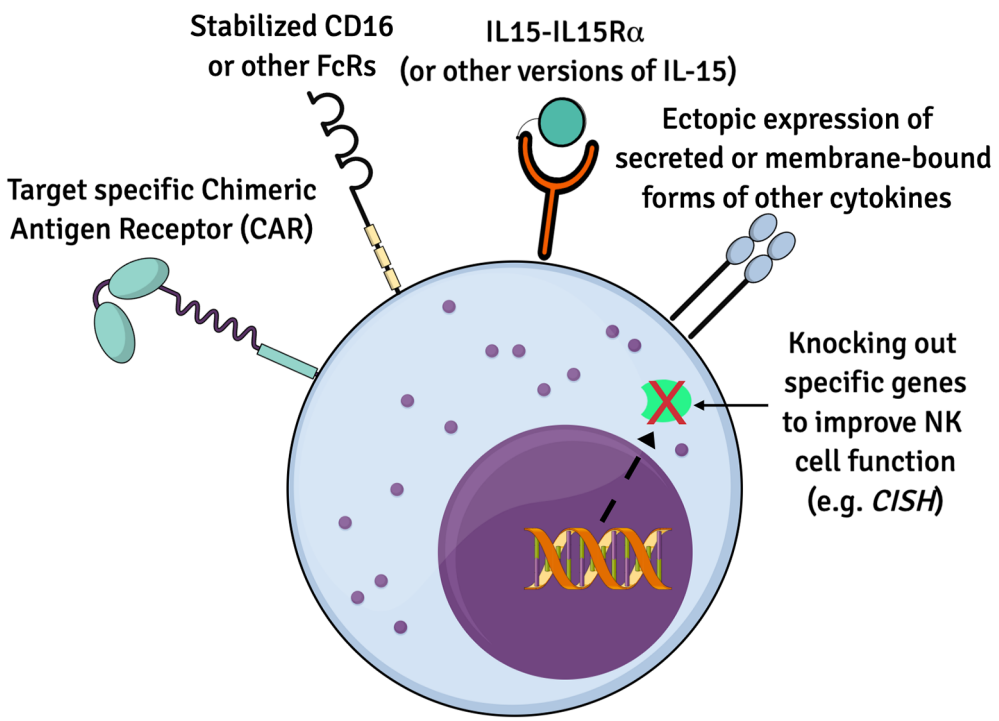

FIGURE 1 | Summary of genetic modifications to improve iPSC-NK cells. Numerous genetic alternations have been engineered to enhance the biology and function of iPSC-derived NK cells for therapeutics. Ectopic expression of IL15 and/or other cytokines, CARs to boost anti-tumor cytotoxicity, recombinant CD16 and knockout of specific genes such as *CISH* are some of the approaches.

transforming growth factor (TGF)-β1, released from the TME (85). These traits have made manipulation of IL-15 expression an appealing strategy to enhance the anti-tumor activity of a variety NK cells populations without the need to supplement the cell production cultures with high doses of cytokines. Various methods to activate the IL-15 signaling pathway have shown to improve NK cell biology and function. The Rezvani group demonstrated that expression of IL-15 combined with an anti-CD19 CAR improved CB-derived NK cell cytotoxicity towards CD19-expressing cell lines and primary leukemia cells *in vitro*, and markedly extended survival in a Raji lymphoma xenograft model (86). This approach was translated into a clinical trial of anti-CD19 CAR-expressing CB-derived NK cells that were well tolerated and demonstrated a 73% overall response rate for patients with relapsed/refractory B cell malignancies (15). Another study by Imamura et al. demonstrated expression of a membrane bound form of IL-15 (mbIL-15) in human PB-NK cells enhanced anti-tumor killing against hematologic malignancies and solid tumors by augmenting NK cell survival and expansion *in vitro and in vivo* without the need of additional exogenous cytokines (87). Another approach employed by two groups used an IL-15 receptor fusion construct comprising of an IL-15 superagonist and IL-15 receptor α (IL-15SA/IL-15RA) to increase anti-tumor activity of PB-NK and iPSC-NK cells, respectively, *in vitro* and *in vivo* (40, 88).

Regulators of IL-15 signaling also provide a target to improve NK cell function. Cytokine-inducible Src homology 2–containing (CIS) protein, encoded by the *CISH* gene, is a key negative regulator of IL-15 signaling. Initial studies demonstrated that deletion of *CISH* in mice leads to increased sensitivity to IL-15, enhanced metabolism and improved anti-tumor activity of NK cells (89, 90). The findings were adapted to human iPSC-NK differentiation platform by using CRISPR/Cas9 edited *CISH*-knockout ($CISH^{-/-}$) iPSCs and differentiating them into $CISH^{-/-}$ iPSC-NK cells which demonstrate improved metabolic profile, *in vivo* persistence and increased anti-tumor activity through increased IL-15-mediated JAK-STAT signaling activity (39, 91). Similar work also demonstrates that deletion of *CISH* in PB-NK cells or UCB-NK cells can also improve their anti-tumor activity (89, 91, 92).

Effects of Other Cytokines and Chemokines on NK Cell Expansion, Metabolic Fitness and *In Vivo* Persistence

NK cell activity is regulated by interactions with diverse immune cells including, but not limited to, T cells, dendritic cells, macrophages, and bone marrow stromal cells. These cells secrete diverse cytokines and chemokines that bind to specific receptors on NK cells. These cytokine receptors include IL-2R, IL-12R, IL-15R, IL-18R, IL-21R (93, 94).

Treatment of NK cells with cytokines allows NK cells to acquire an increased spectrum of effector functions (95). IL-18 is a key player of this priming process. Studies found that NK cells isolated from IL-18-KO mice secreted significantly less IFN-γ than wild-type NK cells in response to stimulation with IL-12 or IL-2 plus IL-12, demonstrating cooperation between the IL-2 and IL-18 signaling pathways (96). Another recent study demonstrated increased *ex vivo* expansion and cytotoxic activity of treated with a cytokine cocktail of IL-2, IL-15, IL-18 (97).

In pre-clinical and clinical studies, the Fehniger group has shown that treatment with a cytokine cocktail consisting of IL-12, IL-15 and IL-18 results in development of CIML NK cells with enhanced interferon-γ (IFN-γ) production and cytotoxicity against leukemia cell lines or primary human AML blasts (46–48). Their phase I clinical trial resulted in 4 out of 9 patients achieving complete remissions (48). CIML NK cells further demonstrated heightened cytotoxicity, enhanced IFN-γ production and persistence against ovarian cancer and other malignancies (98, 99).

IL-21 is another common γ-chain cytokine crucial for NK cell maturation and proliferation (100). In an interesting study, Li et al. demonstrated while increasing concentration of IL-21 (1-10 ng/ml) resulted in higher cytotoxicity through upregulation of IFN-γ and granzyme B, at high concentrations (50 ng/ml) IL-21 resulted in NK cell apoptosis (101). Notably, several groups now routinely utilize irradiated NK cell-sensitive tumor cells that express membrane-bound IL-21 (mbIL-21) and other stimulatory ligands (e.g., 4-1BBL or Ox40L) to stimulate prolonged and large-scale expansion of NK cells (29, 31, 102–104).

Efficient tumor infiltration and homing of NK cells is vital for effective anti-tumor activity. However, cells within the TME secrete chemokines such as C–X–C motif chemokine ligand 8 (CXCL8) or C–C motif chemokine ligand 2 (CCL2) that suppress the activity of intratumoral NK cells (105). High concentrations of adenosine in primary and metastatic TME, specifically myeloid cell adenosine A2A receptors (A2ARs) have a myelosuppressive effect that leads to suppression of NK cell anti-tumor activity (106). Additionally, IL18 binding protein (IL18BP) is a decoy receptor found in the TME that binds to IL-18 with high affinity (107). IL18BP reduces the efficacy of endogenous IL-18 or recombinant IL-18 (rIL-18) administered to try to mediate improved anti-tumor activity (108). Patients treated with rIL-18 have 10- to 100-fold higher concentrations of IL-18BP in their serum (107, 109, 110). In a fascinating recent study, IL-18 was engineered to override the IL18-BP inhibition *via* a 'decoy-resistant' IL-18 (DR-18) that was able to stimulate NK cells to effectively treat PD-1 resistant tumors despite the presence of IL18BP (111).

A detailed transcriptomic analysis demonstrated *ex vivo* expanded NK cells had drastic differences in expression pattern of chemotactic receptors and ligands, including a significant downregulation of CXCR4 and consequent upregulation of CCR5. The study further observed knocking out CCR5 resulted in reduced NK cell trafficking into liver and corresponding increase in NK cell presence in the blood circulation in immunodeficient mice post-infusion (105). PB-NK cells transfected with CCR7 had increased towards CCL19, a lymph node-associated chemokine (112). CXCR2-expressing primary NK cells also showed improved migration to renal cell carcinoma (113). Dual expression of an anti-EGFRvIII CAR and CXCR4 led to increased anti-tumor and better survival in xenograft mouse models (114).

Effect of Hypoxia on NK Cell Function

The hypoxic TME is a characteristic feature of solid tumors. Hypoxia-inducible factors (HIFs) are activated at low oxygen (115–118). Notably, deletion of HIF-1α in mouse NK cells inhibits tumor growth despite reducing cytolytic activity of NK cells. This was mainly shown to be mediated *via* increased bioavailability of the major angiogenic cytokine vascular endothelial growth factor (VEGF) (119). However, in a recent single cell transcriptomic analysis, conditional deletion of HIF-1α in mouse tumor-infiltrating NK cells lead to increased NK cell activation, upregulated NF-kB signaling and improved anti-tumor activity (120).

STRATEGIES TO IMPROVE TUMOR TARGETING OF NK CELLS

In addition to strategies to improve function of NK cells, diverse methods have now been used to improve NK cell targeting against more NK cell-resistant tumors. This section describes some of these strategies that include addition of CARs to NK cells, modification of Fc receptors on NK cells, use of immune checkpoint inhibitor antibodies and NK cell engager molecules.

Development of CAR-Expressing NK Cells

CARs are engineered cell surface receptor constructs that direct immune cell function *via* recognition of the target antigen on the tumor cell surface leading to activation of the immune effector cell *via* an intracellular signaling domain (121–123). CAR constructs typically contain three main components: an ectodomain for recognition of the target antigen (the binder), a transmembrane domain (TM) and an intracellular signaling endodomain(s) (124–126). The ectodomain is typically an immunoglobulin-like single-chain variable fragment (scFv) that imparts antigen specificity against the target tumor. For example, scFvs that target CD19 to treat B cell leukemia and lymphoma are now used for the FDA-approved CAR-T cells (3, 5). Binders that target mesothelin, epidermal growth factor receptor, prostate specific membrane antigen or other tumor antigens have been developed and are in clinical trials to treat diverse malignancies (51, 127–129).

NK cell CAR-based therapy has been shown to benefit from utilization of NK cell-specific CAR constructs compared to CARs that were developed for T cells. For example, our group tested four different transmembrane domains (CD16, NKp44, NKp46, and NKG2D) and four different costimulatory domains (2B4,

DAP10, DAP12, and CD137) in combinations with CD3ζ to optimize an NK cell-specific CAR construct. These studies demonstrated a CAR that contains the NKG2D transmembrane domain and 2B4 co-stimulatory domain mediated improved anti-tumor activity both *in vitro* and *in vivo* (128). Other groups have engineered NK cells to express CARs targeting CD19, CD33 or GPC3 using the 4-1-BB and CD3ζ components to kill otherwise resistant tumor cells (53, 130, 131). Additional studies have used NK cells that express CARs that incorporate either DNAX-activation protein 10 or 12 (DAP10 or DAP12) as the activating domain or as a costimulatory domain alongside CD3ζ (114, 128). A CAR consisting of NKG2D-DAP10-CD3ζ domains increased NK cell-mediated cytotoxicity and cytokine secretion against leukemia and solid tumor cell lines (132). A DAP12 signaling domain expressed in NK cells outperformed CD3ζ expression alone in first-generation prostate stem cell antigen targeting CAR-NK cells (133).

Increased CD16 and CD64 Expression Enhances ADCC Mediated by iPSC-NK Cells

NK cells express the activating immunoglobulin gamma Fc receptor CD16a which recognizes the Fc region of IgG antibodies bound to target targets. CD16a engagement provides a potent stimulus to activate NK cells (134). The clinical anti-tumor activity of monoclonal antibody therapy is in-part dependent on this NK cell ADCC activity (135). For example, there are allelic variants of CD16a with different Fc binding affinities, and the high affinity CD16 variant (F158V) has been shown to lead to improved antitumor responses in patients treated with monoclonal antibodies (136, 137). Additionally, as a negative feedback mechanism, CD16a is cleaved from the surface of activated NK cells by the metalloprotease ADAM17, resulting in decreased CD16a expression and decreased ADCC. With genetic modification, the ADAM17 cleavage site on CD16a can be mutated to block CD16a shedding and increase ADCC (82, 138). In iPSC-NK cells, a CD16 molecule with the high affinity F158V mutation that is resistant to ADAM17 cleavage (termed hnCD16) maintained CD16a surface expression and demonstrated increased cytotoxicity and cytokine production in combination with anticancer monoclonal antibodies (57). *In vivo* efficacy was confirmed in a xenograft mouse model of B cell lymphoma, where anti-CD20 rituximab monoclonal antibodies in combination with hnCD16-iPSC-NK cells improved survival over the combination of PB-NK cells with rituximab or WT iPSC-NK cells.

A second Fc receptor, CD64, binds to the same IgG1 and IgG3 isotypes as CD16A with more than 30-fold higher affinity. However, CD64 is typically only expressed on myeloid cells and not on NK cells (139). Expression of a recombinant receptor consisting of the extracellular region of CD64 and the transmembrane and intracellular regions of CD16a was tested in iPSC-NK cells to determine if this higher affinity Fc receptor could cytotoxicity against tumor cells in combination with monoclonal antibody treatment (140). iPSC-NK cells expressing the CD64/16A chimeric receptor killed $EGFR^+$/$HER2^+$ SKOV3 ovarian cancer cells when combined with the anti-HER2 therapeutic mAb trastuzumab, or the anti-EGFR1 monoclonal antibody cetuximab, while little anti-tumor activity killing was seen without addition of these antibodies (140). Additionally, the higher affinity of CD64 allowed for monoclonal antibodies to be pre-adsorbed to the NK cells expressing the recombinant CD64 and improved tumor targeting without additional antibody use (140).

NK Cells Enhance Anti-Tumor Activity in Combination With Immune Checkpoint Inhibitors

Immune checkpoint inhibitor therapies such as anti-programmed death 1 (PD-1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) monoclonal antibodies, that block inhibitory signals on immune effectors cells thereby activating the immune system, have revolutionized oncology (141, 142). The combination of cellular therapy with immune checkpoint inhibition can mediate improved anti-tumor activity. For example, the ability of adoptive transfer of NK cells to augment checkpoint inhibition therapies has been investigated in hematologic and solid tumors models (143–145). iPSC-NK cells combined with PD-1 checkpoint blockade produced more inflammatory cytokines and exerted increased cytotoxicity. In these studies, iPSC-NK cells were shown to cooperate with T cells to enhance inflammatory cytokine production and tumor killing (143). Other NK cell immune checkpoints, such as the inhibitory receptor NKG2A can be blocked to improve anti-tumor activity. The humanized anti-NKG2A antibody Monalizumab was shown to increase NK cell activation, increase tumor killing, decrease tumor volume and increase survival *in vivo* (146). This effect was augmented by simultaneous PD-1 inhibition and now is under study in phase II clinical trials (146).

Engager Molecules Direct iPSC-NK Cells to Target AML

Following the clinical success of bispecific engagers such as blinatumomab, a CD3-CD19 bispecific antibody that engages CD3+ T cells and traffic them to $CD19^+$ B cell acute lymphoblastic leukemia, several groups have developed multi-valent targeting molecules that specifically engage NK cells in close proximity to the target tumor to improve tumor killing (147). These bispecific killer engagers (BiKEs) or trispecific killer engagers (TriKEs) have been designed to stimulate NK cell activating cell surface cell receptors. For example, engagers targeting NK cells to $CD30^+$ lymphomas, CD33+ myelodysplastic syndrome, $CD133^+$ colon cancer, $CLEC12A^+$ and $CD33^+$ AML are all in clinical development. A bispecific CD30xCD16 engager was able to direct PB-NK and CB-NK cells to increase cytotoxicity against $CD30^+$ lymphomas in a pre-clinical study both *in vitro* and *in vivo* (148). A CD16xCD33 bispecific engager and TriKE targeting CD16, CD33 and stimulating IL15 improved NK cell killing of $CD33^+$ myelodysplastic syndrome cells (149, 150). NK cells were directed to more effectively kill $CD133^+$ or $EPCAM^+$ colon

cancer cells by CD16xCD133 or CD16xEpcam TriKEs that included an IL-15 crosslinker (151, 152). In AML preclinical models BiKEs and TriKEs targeting CD33 and CLEC2A on AML increased NK cell mediated killing of $CD33^+$ or $CLEC2A^+$ AML cells, respectively (153, 154).

NKG2C is another NK cell surface receptor that delivers a strong activating signal to NK cells. To determine if NKG2C signaling could enhance NK cell-mediated antitumor responses an anti-NKG2C/IL-15 engager was developed. The engager has multiple functions, it is designed to bind CD16 to target the NK cells to CD33 that is expressed highly on AML cells, as well as to activate IL-15 signaling and NKG2C. The engager was demonstrated to direct $NKG2C^+$ iPSC NK cells to target $CD33^+$ AML cells and induce degranulation, IFN-γ production and cytotoxicity against the $CD33^+$ cells and primary AML blasts (155).

These strategies to enhance NK cell function can also be combined. Again, iPSCs become very useful for these combined approaches, as it is possible to do all the engineering steps in the undifferentiated iPSCs. Once stable iPSCs are obtained, they can be characterized for any off-target effects of the genetic modification to help ensure safety and uniformity of the differentiated product. The stably engineered iPSCs can then be differentiated into NK cells and expanded for clinical use. This approach was recently described for a product with expression the non-cleavable, high-affinity version of CD16 to allow improved ADCC combined with an IL15-receptor fusion protein to enhance expansion of the cells (40). Additionally, as these NK cells are intended to be combined with an anti-CD38 antibody (Daratumumab) to target multiple myeloma, CD38 was deleted from the iPSCs to produce CD38-knockout (KO) iPSC-NK cells that also contain the engineered CD16 and IL15 molecules. Since CD38 also mediated NAD metabolism, these CD38-KO iPSC-NK cells have features similar to so-called adaptive NK cells that arise after cytomegalovirus infection (40). Interestingly, while these triple-engineered iPSC-NK cells demonstrate potent anti-tumor activity *in vitro*, they were no better than iPSC-NK cells with just the engineered CD16 and IL15 receptor (and not the CD38-KO) in killing tumor cells *in vivo* using myeloma and AML xenograft models (40). Clinical trials utilizing these engineered iPSC-NK cells are underway.

CONCLUSION

CAR-T cells have produced impressive clinical results in patients with relapsed or refractory B-cell malignancies and multiple myeloma, with ongoing studies in progress against many other tumor types (1–3, 5). However, the current CAR-T therapeutic strategy has several safety and logistical limitations that reinforce the need to identify alternative immune cell populations for use for cellular therapy. NK cells, and particularly iPSC-NK cells, are a promising alternative to T cells for cellular therapy based their proven safety record, ability to be used as an allogeneic treatment, and ability to be produced in large numbers and be stored to make an off-the-shelf therapy. Questions about NK cell persistence, the durability of the response, homing to the target tumor and the ability to overcome immune checkpoints remain to be answered. Advances in iPSC-derived NK cell expansion and targeting *via* genetic engineering and gene-editing techniques promise to solve many of these issues and move iPSC-derived NK closer to being an approved clinical option for the treatment of hematologic and solid malignancies.

AUTHOR CONTRIBUTIONS

BG and PH contributed equally to this work. BG and PH wrote the manuscript. DK reviewed and edited the manuscript. All authors read and approved the submitted manuscript.

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Engineered NK Cells Against Cancer and Their Potential Applications Beyond

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Cell therapy is an innovative therapeutic concept where viable cells are implanted, infused, or grafted into a patient to treat impaired or malignant tissues. The term was first introduced circa the 19th century and has since resulted in multiple breakthroughs in different fields of medicine, such as neurology, cardiology, and oncology. Lately, cell and gene therapy are merging to provide cell products with additional or enhanced properties. In this context, adoptive transfer of genetically modified cytotoxic lymphocytes has emerged as a novel treatment option for cancer patients. To this day, five cell therapy products have been FDA approved, four of which for CD19-positive malignancies and one for B-cell maturation antigen (BCMA)-positive malignancies. These are personalized immunotherapies where patient T cells are engineered to express chimeric antigen receptors (CARs) with the aim to redirect the cells against tumor-specific antigens. CAR-T cell therapies show impressive objective response rates in clinical trials that, in certain instances, may reach up to 80%. However, the life-threatening side effects associated with T cell toxicity and the manufacturing difficulties of developing personalized therapies hamper their widespread use. Recent literature suggests that Natural Killer (NK) cells, may provide a safer alternative and an 'off-the-shelf' treatment option thanks to their potent antitumor properties and relatively short lifespan. Here, we will discuss the potential of NK cells in CAR-based therapies focusing on the applications of CAR-NK cells in cancer therapy and beyond.

Keywords: natural killer cells, chimeric antigen receptors, cancer, autoimmunity, clinical trials, CAR-NK, Preclinical studies

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NK CELL BIOLOGY

Natural killer (NK) cells are characterized as cytotoxic lymphocytes of the innate immune system. They account for 5-15% of the circulating mononuclear lymphocytes and are phenotypically defined as $CD3^{-}CD56^{+}NKp46^{+}$. NK cells develop in the bone marrow (BM) niche from hematopoietic stem cell (HSC) progenitors and undergo maturation in the BM or other secondary lymphoid organs, such as uterus, liver and tonsils (1). NK cells play important roles in host defense due to their ability to recognize and eradicate viral infected and malignant cells without the need for prior sensitization. They are equipped with a repertoire of receptors responsible for

delivering activating or inhibitory signals, the relative balance of which dictates the cytotoxic activity and the canonical functions of the cell, such as proliferation and cytokine release (1, 2). Typical activating receptors are the Natural Cytotoxicity Receptors (NCRs) NKp44 (CD336), NKp46 (CD335), and NKp30 (CD337), as well as the Killer Immunoglobulin-like Receptors (KIRs) KIR2DS1, KIR2DS2, KIR2DS4, KIR2DS5, and KIR3DS1. Other important activating receptors are CD224 (2B4), CD226 (DNAM-1) and NKG2D. Several ligands to these activating receptors are upregulated upon cellular stress, infection, or malignant transformation. In cancer, commonly upregulated ligands include MICA and MICB (MHC-class I polypeptide-related sequence A and B), the UL16-binding proteins (ULBPs) and the adhesion molecules PVR (poliovirus receptor, also known as CD155) and Nectin-2. MICA/B and ULBPs are mediating activating signals *via* binding to NKG2D, whereas PVR and Nectin-2 ligate DNAM-1 (3). NCRs recognize a diverse set of ligands, such as heparan sulfate proteoglycans, cell surface proteins and proteins that reach the surface after their intracellular cleavage. These ligands are not exclusively activating but can also have an inhibitory effect depending on the splice variant of the receptor. The research on the identification of NCR ligands is ongoing. Some of the better studied ones include B7-H6 and HLA-B associated transcript 3 (BAT3) that bind to NKp30, and the proliferating cell nuclear antigen (PCNA) that binds to NKp44. The mechanism of upregulation of NK cell activating ligands is not fully elucidated, although increasing evidence suggests transcriptional and post-translational modifications taking place as a result of cell response to stressful stimuli and DNA damage (4, 5).

Besides the activating KIRs, many of the KIR group receptors are known to propagate inhibitory feedback upon interaction with their ligands. Such ligands are self-MHC (major histocompatibility complex) class I molecules that are expressed in all nucleated cell types and play a critical role in mitigating autoimmune reactions. The downregulation of surface MHC class I can occur under cellular stress conditions leading to increased targeting by NK cells. This is also known as 'missing-self recognition' and is a unique feature of NK cells. Inhibitory signals are also mediated by the receptors sialic acid-binding Ig-like lectin-7 (siglec-7) and 9 (siglec-9) that bind to sialic acid-containing carbohydrates (e.g. mucins) aberrantly expressed on tumor cells (6). Other inhibitory receptors are the complex NKG2A/CD94 and the receptors CD161 and KLRG1, that bind to HLA-E, lectin-like transcript 1, and cadherins, respectively.

In addition to the expression of activating and inhibitory receptors, mature NK cell subsets express the FcγRIIIa receptor CD16 that allows the recognition and elimination of antibody-coated cells through antibody-dependent cellular cytotoxicity (ADCC) (7). The mechanism of ADCC is being increasingly explored in cancer therapy. Such therapeutic approaches involve the use of monoclonal antibodies (mAbs) to specifically bind cell surface moieties. Subsequently, CD16- Fc region interaction triggers the antitumor effector immune response resulting in target cell killing. Today, a large number of mAbs have received regulatory approval for cancer treatment, including Rituximab (anti-CD20), Daratumumab (anti-CD38) and Elotuzumab (anti-SLAMF7) (8).

NK CELL-MEDIATED CELLULAR CYTOTOXICITY

Upon target recognition and immunological synapse formation, NK cells induce target cell lysis *via* the secretion of lytic granules containing perforin, granzymes (mainly granzyme B) and granulysin (9). The process, also known as degranulation, involves the delivery of granzymes into the cytosol of the target cells, through pores formed by perforin. The granzymes are then cleaving several substrates including caspase-3, Bid and DNA-PKc, and initiate target cell death. A second NK cell killing mechanism is mediated by the engagement of the death receptors Fas and TNF-related apoptosis-inducing ligand (TRAIL)-R1/2 expressed on the surface of target cells, to their respective ligands FasL and TRAIL on NK cells (10). This interaction triggers cell death *via* the activation of caspase-8. The two pathways follow different kinetics, as granzyme B mediates killing in shorter time compared to death receptors (11).

Similar to $CD8^+$ cytotoxic T cells, NK cells are 'serial killers' (12). Observations from time lapse video microscopy revealed that a single NK cell can eliminate up to six target cells. Moreover, it has been shown that NK cells switch from the 'faster' granzyme B to the 'slower' death receptor–mediated killing during serial target elimination (10). However, the exertion of cytotoxicity, especially when sequential, can lead to depletion of the cytotoxic granule payload, and consequently to NK cell anergy. Strategies to prevent NK cell exhaustion and restore cell fitness have been explored (12). Exposure to cytokines including IL-2, IL-15 and IFNα has been shown to prevent NK cell exhaustion.

Alongside the direct effector cell functions, activated NK cells release major inflammatory cytokines, such as IFNγ, TNFα, GM-CSF, and chemokines, like CCL1-5 and CXCL8 (13, 14). which play important immunomodulatory roles in cellular activation, differentiation, and migration.

ADOPTIVE NK CELL THERAPY

Unlike T cells, the large-scale *ex vivo* expansion of autologous and donor-derived peripheral blood (PB) NK cells has been a challenge for many years. Today, NK cell expansion is performed either by a cytokine-based system or by a feeder cell-based one. The first method includes the use of IL-2, IL-12, IL-15, and IL-21, alone or in a combination of them, to provide the necessary activating and proliferating stimulus (15–17). Alternatively, the stimuli are provided by feeder cells. As feeder cells can be used autologous cells, recombinant human fibronectin fragment-stimulated T cells (18), Epstein-Barr virus-transformed lymphoblastoid cell lines (19), or genetically modified cells of the chronic myelogenous leukemia cell line K562 (20). The latter

are engineered to express membrane bound IL-15 (mbIL15), mbIL21, MICA, and/or 4-1BB ligands (21, 22). Of note, feeder cells need to undergo an irradiation step prior to the initiation of the expansion to prevent cell division and obtain high purity of the final NK cell product.

Although an in-depth comparison between the different expansion strategies has yet to be done, it has been shown that the proliferative capacity, the cytotoxic potency (23), the metabolic function (24) and the receptor expression profile of the generated NK cells are heavily influenced by the expansion protocol (21). For instance, NK cell fold expansion can be negatively affected by telomere shortening; a process occurring due to the repeating replication cycles leading to NK cell senescence (25). The degree of telomere shortening is evidenced to be affected by the protocol used. Denman and colleagues showed that NK cell expansion with mbIL21-expressing feeder cells sustained or even increased telomere length while inducing a mean of 47,967-fold expansion (22). In comparison, the NK cell expansion with mbIL15-expressing feeder cells was limited to 825-fold and telomere shortening was observed (22, 26). Regarding the phenotype of the *ex vivo* expanded cells, studies have focused on the effect of the expansion in the upregulation of immune checkpoints and immunosuppressive molecules, as this can indicate limited efficacy. Markers associated with T cell exhaustion, such as PD-1 and Tim3, have been indeed found upregulated in healthy donor NK cells expanded with a clinically validated protocol of mbIL15-mb4-1BBL-K562 feeder cells + soluble IL-2 (27). Nevertheless, *in vitro* responsiveness assays verified the high cytotoxicity of the cells, suggesting that despite the upregulation of these markers, the cells were not functionally exhausted. Similar conclusions were drawn from cytokine-based NK cell expansions (28). Today, an increasing number of clinical trials is choosing feeder cell expansion systems. However, feeder-free expansion is still a feasible option as it is easier to adapt to GMP regulations and does not involve the hazard of infusing viable feeder cells to the patients (29).

In 2011, Parkhust and colleagues reported the infusion of *ex vivo* cytokine stimulated PB-derived autologous NK cells (30). Although the infusion was well-tolerated, none of the eight recruited patients responded to the treatment. This was hypothesized to be due to the inhibitory interactions between the NK cells and self MHC-class I molecules that are upregulated within the tumor microenvironment (TME). Moreover, the patients were heavily pre-treated, which by itself could have a negative impact on the function of NK cells. Nonetheless, the study provided valuable insight on the persistence of the cells *in vivo* reporting the presence of NK cells in peripheral blood of the patients between a week and several months post infusion. In an attempt to improve NK cell targeting of tumors, Lundqvist and colleagues investigated pre-treatment with the proteasome inhibitor Bortezomib (31, 32). Infusions of *ex vivo* expanded NK were well-tolerated with the exception of thyroiditis and constitutional symptoms related to IL-2 therapy. The study showed preliminary clinical evidence of antitumor immunity with best clinical response observed in 7/14 patients having stable disease (33).

In comparison to autologous NK cells, NK cells from haploidentical donors can elicit greater cytotoxicity due to the alloreactivity caused by the KIR-HLA mismatch (34, 35). This observation was made in an acute myeloid leukemia (AML) mouse model transplanted with HSC, and its translation to the clinic provided grounds to investigate haploidentical or HLA-mismatched NK cells in adoptive cell transfers (36). In 2005, Miller and colleagues conducted a phase I clinical trial in patients with poor-prognosis AML (37). Results showed that infusions of alloreactive NK cells derived from haploidentical donors are safe, have better *in vivo* persistence and resulted in remission of 5 out of 19 patients. Overall, the fact that allogeneic NK cells have low risk of causing graft-versus-host disease increased the applicability of NK cell therapy and encouraged discussions on off-the-shelf cell products (38).

ALTERNATIVE NK CELL SOURCES

To date, the majority of clinical studies on NK cell adoptive cell therapy (ACT) has utilized NK cells derived from peripheral blood. Through the years other NK cells sources have been explored (See Table 1). An example is the umbilical cord blood (UCB). UCB is a NK cell-rich source readily available as cryopreserved biobank material (39). The high proliferative capacity of the cells is particularly attractive, since it allows the generation of large amounts of clinical-grade NK cells (40). Furthermore, UCB-NK cells are suitable candidates for genetic manipulation strategies (41), as well as for combinational treatments with monoclonal antibodies (40), which allows their applicability in different immunotherapeutic strategies. A limitation of UCB-derived NK cells is the inevitable heterogeneity between the final NK cell products due to the use of different UCB donors among batches (42). In addition, comparative studies between PB NK and UCB-NK showed that the latter have some immature characteristics and phenotypic differences. More specifically, UCB-NK cells have increased expression of NKG2A and decreased expression of CD16, KIRs, target adhesion molecules (CD2, CD11a, CD18 and CD62L), perforin and granzyme B (43, 44). Strategies to overcome these issues include the culture of UCB-NK cells with the EBV-transformed HLA-I^+ B lymphoblastoid cell line PLH, which provides the necessary inhibitory and activating signals to drive their maturation (45). Moreover, UCB-NK cells are often combined with cytokine support (e.g., IL-2 or IL-15) that enhances their *in vivo* activity and persistence. Nevertheless, it is worthy of mention that a direct comparison between genetically modified PB and UCB-NK led by Herrera and colleagues found both cell sources to induce similar levels of targeted *in vitro* cytotoxicity (46).

Stem cell-derived NK cells have been proposed as a viable alternative due to their suitability in standardized off-the-shelf settings. Different sources of stem cells have been explored so far, such as human embryonic stem cells (hESCs) (47), $CD34^+$ HSCs (48) and induced pluripotent stem cells (iPSCs) (49). iPSCs have the advantage of being easier to generate and satisfy the clinical

interest of providing greater donor diversity regarding KIR haplotypes (47). It has also been shown that irrespective of their KIR expression profiles, iPSC-NKs have similar killing capacity between them (50). iPSCs were firstly reported in 2006, when human somatic cells were reprogrammed by the simultaneous introduction of four factors: *OCT3/4, SOX2, c-Myc and Klf4* (51, 52). In 2007, the combination of the factors *OCT4, SOX2, NANOG* and *LIN28* was also proved effective (53). After successful reprogramming, iPSCs can undergo an essentially indefinite expansion *in vitro* and, subsequently, produce unlimited amounts of NK cells (54). Their production method is well described (42, 55, 56). Briefly, TrypLE-adapted iPSCs are cultured with human stem cell (SCF) and vascular endothelial growth (VEGF) factors for one week to induce their hematopoietic differentiation. Cells are then further differentiated into NK with the addition of IL-3, IL-15, IL-7, SCF and ftl3 ligand and expanded using feeder cell systems. Similar to UCB-NK cells, iPSC-NK are well susceptible to genetic manipulation and exhibit potent cytotoxicity (57). However, in comparison, the iPSC-derived NK cell population is reproducibly homogenous and consistent (58). Phenotypically, iPSC-NK cells have many similarities to the PB NK cells, with the exception of higher NKG2A and lower KIR expression, which indicates a degree of immaturity (49). The popularity of iPSC-NK cells has been increasing over the past years, with multiple preclinical studies and one clinical trial underway (See **Table 2**).

A newer addition to the NK cell sources has been the memory-like (ML) NK cells. ML-NK cells are generated after viral infection (59), exposure to haptens (60) or cytokines, such as IL-12, IL-15 and IL-18 (61). These cells exhibit characteristics of adaptive immunity and have been reported to have higher anti-cancer reactivity in a clinical setting compared to conventional NK cells (62). The cells are well-susceptible to genetic manipulation and have been recently investigated in the context of chimeric antigen receptor (CAR)-NK therapy where introduction of an antiCD19-CAR further improved their targeting and cytotoxic activity (63).

In addition to primary NK cells, NK cell lines have also been used in ACT. Cell lines have the advantage of being easy to culture, expand and cryopreserve. Out of the available ones, NK-92, an IL-2 dependent non-Hodgkin lymphoma NK cell line, has been tested the most in proof-of-concept, preclinical and clinical settings. NK-92 cells have characteristics of activated NK cells, while lacking KIR expression (except KIR2DL4), which explains the potent cytotoxic responses they exhibit upon target cell recognition (64). The cell line NK-92, as well as its genetically modified IL-2- independent counterpart NK-92MI, have been considered for the use in 'off-the-shelf' NK cell-based immunotherapies. KHYG-1 and YT are two other NK cell lines with great potential (65). Nevertheless, the use of transformed cell lines in patients is in general met with safety concerns regarding the tumorigenic nature of the cell lines and the hazard of causing secondary NK lymphoma to the patients (66). These limitations can be overcome by implementing a high-dose (5-10 Gy) γ-irradiation step prior to the cell infusion (67, 68). While irradiation halts cell division, it can also negatively impact their long-term *in vivo* persistence which is necessary for better tumor control (69). Indeed, a study of irradiated and non-irradiated CAR-NK-92MI cells, demonstrated that irradiation with 5Gy reduced *in vitro* and *in vivo* proliferation of the cells and shortened their life span (69). Still, the cytotoxicity against target cell lines is not significantly compromised as shown by two different studies (69, 70). Another concern of γ-irradiation is the cellular damage that it causes, ranging from DNA breakage to radical formation and impairment of the cell membrane integrity (71, 72). An alternative method to γ-irradiation was proposed by Walcher and colleagues. Specifically, they demonstrated that low energy electron irradiation has considerable advantages, as it requires shorter treatment times, has more reproducible dose rates, is easier to implement in a laboratory or GMP setting and -importantly- maintains the high cytotoxic effector function of the NK-92 cells (73).

The unique advantages and weaknesses of each NK cell source are listed in **Table 1**.

CRYOPRESERVATION OF NK CELLS

The great potential of NK cells as off-the-shelf cellular treatments is often overshadowed by their sensitivity to cryopreservation. For instance, it has been shown that although cryopreserved NK cells can eliminate target cells in standard *in vitro* cytotoxicity assays, their efficacy against three-dimensional tumor models is reduced due to a 6-fold decrease in motility (74, 75). Moreover, in a mouse model where infusion of fresh or cryopreserved expanded NK cells was compared, the disadvantage of cryopreserved cells in homing, persistence and expansion was evident (76). Maintaining NK cell viability and cytotoxic function post-thaw is essential in a clinical setting, where high consistency and quality must be guaranteed. A number of studies are investigating the optimal cryopreservation

TABLE 1 | Advantages and limitations of NK cell sources.

NK Cell Source	Advantages	Limitations
Peripheral blood	Easy collection; Safe; High cytotoxic potency	Time consuming and costly expansion process; Low numbers in patients; Variability between the final products
Umbilical cord blood	Readily available; Safe; High starting percentage of NK cells; Strong proliferation potential	Small volume of starting material; Diverse products depending on the UCB unit; Need of cytokine support for adequate cytotoxic function
Induced pluripotent stem cells	Easy generation of high NK cell numbers; Homogenous product; High cytotoxic potency	Additional step of generating NK cells from iPSCs; High production cost
NK cell lines	Accessible; Easy to culture and amplify; Fast proof-of-concept studies	Safety concerns; Potential decreased cytotoxicity due to the necessary irradiation step

conditions, assessing various freezing/thawing media, cooling rates, storing conditions, culture protocols and resting times. Although a gold standard has not been established yet, it is worth mentioning that substituting dimethyl sulfoxide (DMSO) with other small molecule sugar or protein-based cryoprotectants (e.g sucrose, proline, mannitol) in the freezing media formulation is a generally accepted alternative, associated with improved viability of immune cells post-thaw (77). Given the fact that cryostoring is a necessary step between the development of a cellular product and its infusion, intensifying the efforts to optimize the procedures will positively impact the universal application of immunotherapy.

GENETIC MANIPULATION OF NK CELLS

In the clinical setting, the potency of ACT is dependent on the persistence of the infused NK cells. Accumulation of inhibitory factors, insufficient tumor targeting and failure to persist and expand *in vivo* are only a few of the challenges that need to be overcome. For most of these issues, genetic manipulation of the cell product provides a viable solution. NK cells, however, are typically less susceptible to such manipulation compared to other immune cell types (78). Therefore, although the genetic reprogramming of NK cells shares similar principles and methodologies to that of T cells, additional steps are often necessary.

Transfection is a method in which plasmid DNA, mRNA or proteins are introduced to a cell with the aim to initiate their expression. Depending on whether a short or a long-term expression is desired, different methods are applied. One of the most common transient transfection techniques is electroporation. The method uses electric pulses to permeabilize the cell membrane and create pores from where the genetic material is inserted. Electroporation is highly efficient in T cells, however, similar yields have yet to be observed in NK cells (79). Moreover, electroporation results in short-term expression of the transgene which can limit its applicability in the context of immunotherapy (80). Still, the cost-effectiveness of the technique and the ease of its application in large-scale clinical settings are attractive. Efforts to improve NK cell transfection efficiency focus on optimizing fundamental parameters, such as the number of cells, the voltage, and the concentration of material to be electroporated (81). Moreover, ways to minimize the considerable cell death that follows electroporation are being explored (82). Examples of the application of the method in NK cell therapy are mostly concerning CRISPR-mediated genome engineering (knock outs or edits) (83). If long-term expression is desired, the transposon-based technology may be an attractive method. Two such systems have been reported, namely the Sleeping Beauty (84) and the PiggyBac (85). These systems combine the efficiency of electroporation with the precise insertion of the genetic material into the host genome thanks to its integrating element. Nonetheless, to this day, the use of transposon-based systems is more commonly used in T cell rather than in NK cell studies.

Viral transduction is one of the most common methods for immune cell engineering. It results in stable transgene expression while maintaining higher cell viability compared to electroporation. Moreover, the method is well validated in preclinical and clinical studies, where it has repeatedly proven safe and efficient. The vast majority of the clinical studies are using either lentiviral (LV) or retroviral (RV) vectors, although some studies with adenoviral vectors have also been completed with mixed results (86). The first report of viral transduction of NK cells was made by Nagashima et al., in 1998, where he described the transduction of NK-92 cells using RVs (87). This study showed a transduction efficacy of about 2-3%, which although impressive for the time, it is considered low with today's standards. Of note, recent publications of retroviral transductions of expanded NK cells resulted in about 70% transduction efficiency (88–90). RVs, and more specifically, self-inactivating γ-RVs were also the first viral vectors to enter clinical trials. Their application was further increased by the development of clinical grade RV-producing packaging cell lines. These cell lines, such as the murine cell line PG-13, are able to continuously generate large quantities of clinical-grade virus supernatant following their stable transduction by the vector of interest (91). A disadvantage of the RVs is the fact that they require active cell division in order to successfully integrate their vector to the host genome (92). Moreover, the integration itself can be at random sites, which rises concerns on tumorigenicity and the overall safety of the approach. Careful optimization of the transduction process is, therefore, necessary in order to limit the viral copy number per cell to the absolute minimum. Apart from the γ-RVs that have been monopolizing the transductions, α-RVs are gaining more attention, especially after studies showing superior transduction efficacies in primary NK cells of α-RVs compared to γ-RVs and LVs (93).

Unlike the RVs, LVs are able transduce cells irrespective of their cell cycle phase, leading to theoretically higher transduction efficacies. The vector of choice for T cell-based immunotherapies is the vesicular stomatitis virus (VSV-G) pseudetotyped vector, because of its broad tropism. However, these vectors have proved less effective in NK cells. Alternatively, LVs pseudotyped with a modified baboon envelope glycoprotein (BaEV-LVs) hold greater potential since their entry receptors sodium-dependent neutral amino acid transporter-1 and -2 (ASCT-1 and ASCT-2) are abundant in NK cells (94). Indeed, two independent investigations showed a 20-fold higher transduction efficacy with BaEV-LVs compared to VSV-G-LVs (94, 95). As far as safety is concerned, LVs, and more specifically the 3rd generation vectors, are considered safer viral vector options since the packaging genes *gag/pol* and *rev* are found in separate plasmids, thus making the generation of wild-type recombinant virus harder (96).

Due to their innate antiviral defense mechanisms, viral transduction of NK cells has proved to be challenging. A way to enhance the transduction efficacy is by reducing the NK cell virus repulsion, which in turn increases the internalization of viral particles into the cells independently of the viral receptors (97). Examples of such reagents are the cationic polymers polybrene and protamine sulfate. Although both reagents are routinely used in viral transduction protocols, they are also

associated with decreased post-transduction cell viability. An alternative approach is to use recombinant human fibronectin fragment such as RetroNectin. RetroNectin is a 63kDa with adhesion sites for cells (integrin receptors VLA-4 and VLA-5) and viral particles (heparin domain) (98). Upon adhesion of both parties to the filament, transduction is facilitated thanks to the close proximity between them (99, 100). Transduction using RetroNectin is efficient and suitable for difficult to transduce or frail cells (98). Understanding the intracellular patterns of foreign genomic material recognition has contributed to finding additional strategies to increase transduction efficacy. More specifically, the inhibition of the TBK1/IKKε complex acting downstream of RIG-1, MDA-5 and TLR3, has significantly improved the viral transduction (101). Such reagent is the VyOz, which is reported to increase the efficacy of NK cell transduction by 4-fold (102). Of note, all the afore-mentioned reagents are available in GMP grade, further enabling the translation of the preclinical studies to the clinical setting.

Virus-like particles have also recently emerged as gene editing tools. One of these approaches uses engineered murine leukemia virus-like particles loaded with Cas9-sgRNA complexes (103). The system, commonly known as Nanoblade, is suitable for both *in vitro* and *in vivo* manipulations, providing high gene editing efficacy and precision in a cost-effective way. Although they have yet to be applied to NK cells, nanoblades have shown promising results in genome editing of human T, B and $CD34^+$ cells (104).

CHIMERIC ANTIGEN RECEPTORS (CARs)

Irrespective of the source of NK cells, the cytotoxic potential and the targeting capacity of the cells can be further increased with the expression of CARs. CARs are synthetic receptors comprising of three main regions; the antigen-binding single chain variable fragment (scFv), a short transmembrane region (TM) and one or more signal transduction domains (105).

Single Chain Variant Fragment (scFv)

The extracellular part, or ectodomain, derives from a tumor-specific antibody and consists of a heavy and a light chain that connect *via* a linker. Selecting the right targeted epitope, scFv and linker for each application is crucial. Indeed, a study comparing distinct scFvs targeting the same antigen demonstrated that the scFv domain can influence the expression of the CAR, as well as its functionality (106). A step further is the affinity optimization of the scFv. This is particularly relevant in the occasions where the targeted antigen is expressed on normal tissues (although in lower levels), and thus there are increased chances of on-target off-tumor toxicities. A strategy to reduce the recognition of $antigen^{low}$ normal cells was described by Drent et al. (107). The researchers used 'light-chain exchange technology' to construct 124 new antibodies with 10 to >1000-fold less affinity to CD38, a target for multiple myeloma (MM). The selected scFvs were then assessed in both CAR-T (107) and CAR-NK (65) models, where effector cells expressing the affinity optimized CARs effectively discriminated between MM and normal cells. The selection of the scFv domain can be aided by modern computational methods of protein design (108).

Hinge Region

The ectodomain is connected to the TM *via* the spacer or Hinge region. Similar to the linker, the type and length of the Hinge region can influence the binding capacity of the CAR, as it provides stability and flexibility to the receptor (109). The right selection of Hinge region can additionally protect the patient from off-target activation of the engineered cells and/or unintentional innate immune response; a phenomenon firstly observed in CARs with an immunoglobulin G (IgG) 1 Fc spacer domains interacting with IgG FcγRI receptors (110). It has also been reported that incorporation of a Hinge region to the CAR design enhances the expansion of CAR-transduced T cells (111). The most commonly used Hinge regions in CAR-NK cell therapy derive from the CD8a, CD28 and the IgG-based sequences (105). To our knowledge, there is no study comparing the Hinge regions in CAR-NK cells. Nonetheless, a study comparing the human CD28 and CD8a regions in anti-CD19 CAR-T cells showed that CD8a induced lower production of IFNγ and conferred better resistance to activation-induced cell death, while maintaining equal cytotoxicity to the CD28 counterpart (112). Apart from the functional purpose, Hinge regions act as a target for CAR-detection antibodies facilitating the confirmation of CAR expression (111).

Transmembrane Region

After the Hinge region follows the TM. The TM region is responsible for anchoring the receptor on the cell membrane, as well as for transducing the signal from the extracellular to the intracellular domains of the CAR. Typical TMs used in T and NK cell studies are derived from CD3, CD8α and CD28 (112). In the context of NK cell therapy, TM domains of activating receptors, such as CD16, NKp44, NKp46, NKG2D, 2B4 and DNAM-1, have also been tested in an *in vitro* iPSC-NK based study (113). The comparison revealed that the combination of NKG2D-derived TM with 2B4 co-stimulatory domain and CD3ζ signaling domain confers strong antigen-specific cytotoxicity.

Intracellular Domains

Regarding the intracellular region, the design of the CARs has advanced throughout the years, from having only a signal transduction domain (1^{st} generation CAR) (114), to having one or two co-stimulatory domains additionally (2^{nd} and 3^{rth} generation respectively) (115, 116). The intracellular domains for the first CAR designs were inspired by the activating signaling pathways of T cells. To this day, the most broadly used co-stimulatory domains are CD28 and 4-1BB (CD137), while the most common signal transduction module is CD3ζ (117). Both CD28 and 4-1BB are shown to recapitulate natural co-stimulation and provide increased potency to the transduced effector cells. It is also known that CD28 confers a different set of advantages over 4-1BB (118). Studies comparing the two in a 2^{nd} generation CAR setting showed that 4-1BB promotes survival and proliferation, whereas CD28 attributes a stronger cytotoxic

potential. Depending on the clinical application, the use of either or both domains is preferred.

Transitioning into NK cells, the expression of already validated T cell-based CAR designs was initially assessed. The first CAR-NK cell was reported by Tran et al. (119). In this *in vitro* study NK cells successfully expressed a functional CD4-CD3ζ CAR, which redirected the cells against HIV-infected $CD4^+$ T cells and NK cell-resistant tumor cells. Soon after, applying the CAR know-how from T to NK cells without adjustments was deemed sub-optimal. This is because besides some common signaling moieties that the two cell types share, such as CD3ζ and 4-1BB, most of the frequently used co-stimulatory domains are absent in NK cells. Therefore, questions were raised on whether the true potential of CAR-NK therapy was harnessed with the early CAR designs. Novel constructs for NK cell therapy are substituting the signaling domains of the CARs with those of activating NK cell moieties. The domains that NK cells typically use for downstream signaling are the CD3ζ, DAP10, DAP12 and FcRγ chains (120, 121). In comparison to CD3ζ that has three ITAM domains, the rest of the molecules have a single ITAM. It is also worth mentioning that DAP10 is the adapter protein of NKG2D, whereas DAP12 mediates signaling of activating KIRs, NKG2C and NKp44. In experiments of primary NK cells, DAP10 was found to be functional only when NKG2D was used as the ectodomain, whereas when NKG2D was utilized as the TM region, DAP10 decreased the functionality of the CAR (122, 123). On the contrary, DAP12, as a signaling domain, was met with greater success outperforming CD3ζ-based CARs in two independent *in vitro* and *in vivo* studies of primary NK cells (124, 125). Another co-stimulatory domain under investigation is the NK cell specific receptor 2B4. A couple of studies comparing NK-92 and PB-NK cells expressing 2^{nd} generation antiCD5 or antiCD123 CAR constructs with either 2B4 or 4-1BB co-stimulatory domains, showed that 2B4-CD3ζ CARs provided superior antitumor efficacy compared to 4-1BB-based CAR constructs (121, 126). A similar effect was observed in a study comparing NK-like CARs (NKG2D TM and 2B4 + CD3ζ intracellular domains) and T-like CARs (CD28 TM and 4-1BB + CD3ζ intracellular domains), which showed that iPSC-derived NK cells expressing NK-like CARs induced tumor regression and prolonged mice survival (113). NK cell-like stimulatory domains are being increasingly used in the design of CAR constructs for NK cell immunotherapies, especially for the treatment of solid tumors (113, 125, 127, 128). Regarding 3^{rd} generation CARs, the investigation on finding the optimal combination of signaling domains is currently of high interest. Overall, the findings suggest that NK cell-like co-stimulatory domains unleash superior antitumor responses by CAR-NK cell products.

ADVANCEMENTS IN CAR AND TRANSGENE DESIGN

Alternative Antigen Recognition Domains

The first CAR designs were comprising of scFv domains deriving from mouse antibodies. It has been shown, however, that murine scFvs can be immunogenic and that the triggering of the host immune response often results in early elimination of the CAR products (129). In an effort to prevent that, fully human CARs are being developed, showing comparable targeting capacity to the originally reported scFvs (130). Besides scFvs, which are typically used as the antigen recognition domain, alternatives for improving the CAR features or facilitating their design process have emerged throughout the years. For instance, when target cells express a cell-specific surface antigen whose ligand is known, CAR constructs can incorporate the ligand itself as the extracellular antigen-recognition domain, instead of an scFv. Of note, Zhuang and colleagues demonstrated that CD28H-based CAR-NK cells could be used to recognize and kill target cells expressing B7H7, its natural ligand (131).

A more recent advancement is the use of nanobody-based constructs. Nanobodies, also known as V_HH antibodies, derive from the variable domain of heavy chain-only antibodies naturally existing in Camelidae and shark species (132, 133). Nanobodies have several advantages over traditional antibodies, such as increased capacity of reaching inaccessible epitopes thanks to their long CDR3 sequence, ability to maintain their physiochemical properties in extreme conditions, easier humanization process and less probable folding and assembly issues (134–136). A study of generating CD7-nanobody based CAR-NK-92MI cells demonstrated potent antitumor effect against T-cell leukemia cell lines and primary cells (137). Similarly, nanobody-based CAR-NK-92 cells targeting CD38 in multiple myeloma showed high specificity and cytotoxic activity in primary human bone marrow samples (138). A comparison between scFv- and nanobody- based CARs was done in clinical studies of CAR-T cells, where comparable efficacy and safety were reported (136). A further step is the design of affinity optimized nanobodies, using a recently developed algorithm that helps predict the residues whose modulation would confer specific binding characteristics (139).

Ectopic Cytokine Production

As previously mentioned, the long-term persistence of NK cells after adoptive cell transfer can be a concern without cytokine support (21). Due to the association of cytokine administration with serious adverse effects, recent efforts are incorporating an ectopic cytokine support system into the CAR plasmid, developing thus a so-called 'armored' CAR. In a study of Liu et al, CB-NK cells were transduced with a viral vector encoding for an anti-CD19 CAR and the IL-15 gene (41). The results were promising as the generated NK cells exhibited increased cytotoxicity against CD19-positive targets *in vitro* and led to prolonged survival *in vivo* (41). In a phase I/II clinical trial, the same approach showed response to 8/11 patients, without the cause of serious adverse effects (140). In a different study, Wang et al. coupled IL-15 transgene expression to an inducible MyD88/CD40 system and achieved increase of the *in vivo* CAR-NK cell persistence for a minimal of 40-50 days (141).

Safety Switches

Ensuring the safety of the adoptive cell transfer is pivotal, especially after the reports of neurotoxicity, on target-off tumor effects and cytokine storm in CAR-T cell clinical studies (142). Although NK cells have in general a safer profile than T cells, additional safety

measures can be lifesaving during an emergency situation. A method to rapidly terminate a cell-based treatment is by incorporating a suicide gene into the therapeutic transgene. In NK cells, the strategy has been tested using the inducible safety switch caspase-9 (iCasp9) suicide gene that expresses a modified caspase-9 fused to the human FK605 binding protein (41). The system is pharmacologically activated, meaning that upon administration of AP1903 (a chemical inducer of dimerization) a caspase-mediated apoptosis is induced (143). Similar drug-induced CAR-NK cell elimination was achieved by an orthogonal rapamycin-regulated caspase-9 switch (141). Notably, cell-cycle dependent suicide genes, such as HSV-TK, are not recommended for use in CAR therapy, as they require active cell division in order to function and can, additionally, induce immunogenicity (144). An alternative strategy for increasing safety and minimizing on-target off-tumor effects was applied in an anti-CD147 CAR-NK study for hepatocellular carcinoma (70). Tseng and colleagues designed a GPC3-synNotch-inducible anti-CD147 CAR, in which the CAR-mediated antitumor responses were unleashed only when GPC3 and CD147 were co-expressed on the surface of the target cell.

Dual-Specificity CARs

Antigen escape, the partial or total loss of the targeted antigen expression from the surface of malignant cells, is a known mechanism that impedes the efficacy of CAR therapy in cancer. A study addressed this issue by designing a CAR construct with dual specificity for the tumor associated antigens (TAA) EGFR and its mutant form EGFRvIII for the treatment of glioblastoma (145). Dual-specific NK cells eliminated cells positive for both or either of the antigens, in contrast to the CAR-NK cells targeting a single epitope, which resulted in the significant extension of survival of glioblastoma bearing mice. A different approach was proposed by Li and colleagues, who engineered a plasmid encoding for NKG2D and an antiPD-1-CAR, with the intracellular domain of DAP10, aiming to induce synergistic activation of NK-92 cells by parallel recognition of PD-1 and NKG2D ligands (146). Functional assays showed increased cytotoxic activity of the engineered cells and underlined the potential of the method. Notably, dual targeting can be also achieved with the use of tandem CAR constructs. Tandem CARs are constructed by consecutively linking two different antigen binding domains (either scFv or nanobody-based) to a single intracellular domain. Although this approach has been assessed in CAR-T studies, tandem CARs have not yet been evaluated in the NK cell setting (147).

Adapter CARs

Grote S. et al. further increased the versatility of CAR-targeting by proposing modular CARs (148). Modular or 'adapter' CARs (AdCARs) recognize biotin-labeled antibodies specifically targeted against TAAs. This would mean that the CAR-mediated cytotoxicity is fully dependent on the selection of the biotin-labeled antibodies and can be easily modified if needed. The novel AdCAR-NK-92 cell product demonstrated superior cytotoxic responses against $CD19^+$ and/or $CD20^+$ primary cell targets and gave ground for discussions on off-the-shelf universal CAR-NK cell therapy.

APPROACHES TO MODULATE CAR-NK CELL FUNCTIONALITY

Expression of Chemokine Receptors and Cytotoxic Ligands

Cell therapy is traditionally administrated *via* intravenous infusion. Therefore, trafficking of CAR-NK cells to the targeted tissue is critical for the exhibition of a therapeutic effect. To aid this process, the incorporation of a chemokine receptor transgene into the CAR design was assessed. In a model of AML, Jamali and colleagues, generated transgenically augmented anti-CD19 CAR-NK cells (TRACKs) expressing the chemokine receptor CXCR4, which is implicated in the retention of NK cells in the bone marrow niche (149). Improved migration and superior lysis of target cells was demonstrated *in vitro*. The strategy was also applied in a solid tumor setting, where expression of CXCR4 by EGFRvIII CAR-NK cells induced specific chemotaxis towards the CXCL12/SDF-1α positive glioblastoma cell line U87-MG (150).

In the occasion that one of the tumor-associated antigens overexpressed in the tumor is a death receptor, transduction of CAR-NK cells with its respective apoptosis-inducing ligand could effectively redirect CAR-mediated antitumor responses towards the cancer cells. The approach was assessed by Lee Ye and collaborators in a pancreatic ductal adenocarcinoma model, where TRAIL transgene was cloned in the FRα-specific CAR vector (151). The generated CAR-NK-92 cells demonstrated enhanced and targeted cytotoxicity.

CRISPR/Cas9-Mediated Gene Editing

CRISPR technology is a powerful gene editing tool that has been increasingly used in cellular immunotherapy. CRISPR/Cas9 is successfully used to insert the CAR transgene into specific loci of the effector cell genome with high precision (152). Apart from that, the technology is extensively studied in the context of increasing the functionality and persistence of CAR-modified cells. Such strategy involves the knocking in of genes associated with effector cell activation, and/or the knocking out (KO) of inhibitory genes. In a study on EGFRvIII CAR-T cells, the authors used the CRISPR/Cas9 system to specifically disrupt the PD-1 gene without causing further alterations to the CAR-T phenotype (153). This resulted in the *in vitro* inhibition of the glioblastoma cell growth. Similarly, CRISPR-mediated KO of the endogenous TGF-β receptor II (TGFBR2) in CAR-T cells unleased immunosuppressive breaks and reduced the CAR-T exhaustion (154). In NK cells, blockade of NKG2A expression resulted in highly functional NK cells that overcame NKG2A-mediated inhibition (155). Although the last study used specifically designed protein expression blockers, rather than the CRISPR-technology, it is believed that both methods could be applied for the generation of $NKG2A^{null}$ NK cells.

An alternative way of using the CRIPSR/Cas9 system is enabling the application of an approach in a particular setting. For example, CD38 is a validated target for MM therapy, as it is overexpressed on the malignant cells. However, CD38 is also highly expressed by effector immune cell types, such as the NK cells (156).

Because of that, antiCD38 CAR-NK therapy is characterized by effector cell fratricide shortly after the CAR is expressed. In a study of antiCD38 CAR-NK cells, CRISPR/Cas9 KO of CD38 on the NK cells provided a practical solution to avoid fratricide while maintaining their functionality and cytotoxic potential (65).

Enhancing NK Cell Metabolism

The metabolic state of the CAR-NK cells in the hostile environment of the TME affects their functional fate to a great extent. For example, limiting glycolysis or oxidative phosphorylation (OXPHOS) is known to impair the production of cytokines, such as IFNγ and Granzyme B in NK cells (157, 158). Efforts are made to elevate NK cell metabolism in the TME using gene editing techniques. Mammalian target or rapamycin complex 1 (mTORC1) is a key regulator of NK cell development and effector responses, activated upon cytokine stimulation (e.g IL-15). A method to maintain mTORC1 within the TME is *via* deletion of the cytokine-inducible SH2-containing protein (CIS) (159). This results in increased JAK/STAT and mTORC1 signaling after IL-15 stimulation, and consequently improves metabolic fitness, cytotoxicity and *in vivo* persistence (160, 161). However, caution should be taken during long exposure of the edited NK cell to IL-15, as it may lead to opposite results, such as NK cell exhaustion and reduced cytotoxicity (24). Another pathway that could be targeted is the cMyc signaling. Adequate levels of cMyc protein are of vital importance for sustaining elevated rates of glycolysis and OXPHOS in NK cells (162). Nevertheless, within TME it is observed a rapid loss of cMyc expression. A strategy to sustain the levels of cMyc is by targeting its degradation pathway mediated by the kinase glycogen synthase kinase-3 (GSK3). Indeed, GSK3 inhibitors are found to restore NK cell cytotoxicity and enhance IFNγ and TNFα production in *in vivo* models of AML and ovarian cancer (163, 164). Alternatively, expression of cMyc protein is rescued by increasing the availability of glutamine. Suppression of glutamine metabolism in tumors can be achieved by treatment with the compound JHU083 (165). Last but not least, the potential benefit of the hypoxia-inducible factor 1α (HIF1α) deletion is being discussed, although the complexity of its regulation requires further investigation (166).

Increasing CAR-NK Cell Homing and Tumor Infiltration

With the advances of CAR technology and the discovery of alternative NK cell sources, many of the initial obstacles that CAR-NK therapy faces were addressed. However, there are still opportunities for further improvements. As we have previously discussed, NK cell homing is crucial for the success of immunotherapy. Reaching the appropriate effector to target cell ratio within the malignant site has proven a challenge, especially with regards to solid tumors (167). Independent studies have provided evidence that 1) pre-conditioning of patient with lymphodepletion (168), 2) complement cytokine support (76), 3) pharmacological intervention (169, 170), 4) reduction of immunosuppression in TME (171–173) and 5) expression of chemokine receptors by effector cells (174), among others, have a positive effect. Combining different strategies into a multi-dimensional novel approach appears to be the best chance of improving the baseline trafficking and infiltration.

CAR-NK CELLS IN COMBINATIONAL APPROACHES

Modification of Targeted Antigen Expression

Pharmaceutical intervention can counteract antigen escape by increasing or maintaining the expression of the targeted antigen. The drugs used for this application are mediating epigenetic modulations, post-translational modulations or inhibit antigen cleavage from the cell surface (175). An example is the use of all-trans retinoic acid (ATRA), an anti-leukemic agent known to enhance the expression of CD38 and folate receptor β (FRβ), to increase the potency of anti-CD38 (176) or anti-FRβ (177) CAR-T therapy respectively. Similarly, treatment with histone deacetylase inhibitors, such as valproic acid, causes upregulation of the NKG2DL MICA/B and ULBP2 from the tumor cells (178). This effect was successfully harnessed in a study of NK cell-mediated lysis of hepatocellular carcinoma cells (179). It could additionally be exploited, however, by the NKG2D-based CAR modified T (180) and NK cells (124, 181). Other examples of drug CAR therapy combinations reported in literature are: 1) adenosine 2a receptor antagonists and mesothelin CARs (182), 2) DNA methyltransferase inhibitors (e.g decitabine) and mucin 1 CARs (183), 3) enhancer of Zeste homolog 2 (e.g GSK126 and tazemetostat) and GD2 CARs (184), protein kinase C modulators (e.g bryostatin-1) and CD22 CARs (185), and 4) γ-secretase inhibitors and BCMA CARs (186).

Anti-Angiogenic Agents

The efficacy of CAR-NK therapy against solid tumors is limited. To tackle problems associated with insufficient migration of the modified cells to the solid tumor, several studies have investigated the synergistic effect of anti-angiogenic agents with CAR-NK cell infusion. Zhang and colleagues investigated the combination of regorafenib and EpCAM-specific CAR-NK-92 cells in a mouse model of human colorectal cancer xenografts and found the combination to have superior antitumor response compared to each monotherapy (169). Another study by Wu et al. combined apatinib with anti-HER-2 CAR-NK-92 cells and assessed the efficacy against gastric cancer xenografts (170). The strategy achieved improved CAR-NK cell infiltration into the larger tumor xenografts and resulted in better tumor growth suppression (169, 170).

Immune Checkpoint Inhibition

Checkpoint inhibition and adoptive cell transfer have revolutionized modern cancer treatment. The combination of these individually successful immunotherapeutic approaches has been assessed. Specifically, blockade of the immune checkpoint CD73 enhanced the cytotoxicity of NKG2D-targeting CAR-NK-92 cells in $CD73^+$ human lung cancer xenograft models (128). Furthermore, the administration of an anti-PD-1 monoclonal antibody together with anti-HER2 CAR-NK-92 cells for the

treatment of glioblastoma has been reported in an abstract by Strassheimer et al. (187).

Therapeutic Antibodies

The combination of adoptive NK cell transfer with therapeutic monoclonal antibodies (mAbs) is a promising therapeutic approach, due to the innate ability of NK cells to induce ADCC *via* its FcγRIII receptor CD16 (188). The efficacy of this combination is dependent on the CD16 polymorphisms, as well as the affinity of the mAb to the CD16 receptor (189). Engineered Fc receptors, with optimized affinity and enhanced durability within the *in vivo* environment have emerged the last years in an effort to increase the efficacy of cell and antibody therapy combination. Indeed, iPSC-derived NK cells expressing a high affinity non-cleavable CD16-construct (hnCD16-iNK cells) showed enhanced ADCC-mediated effector functions against target cells coated with an anti-CD20 mAb (190). To our knowledge, CAR-NK cells have not been combined with mAbs to this day in a peer-reviewed journal. However, a relevant approach was proposed by Goodridge and colleagues in an abstract form, where iPSC-NK cells co-transduced with hnCD16 and antiCD19-CAR constructs showed promising results in combination with Rituximab (191).

A similar approach was described in a study reporting the combination of anti-TF CAR-NK cell therapy with chimeric antibody-like homodimer immunoconjugates that also target TF, called ICON and L-ICON, in triple-negative breast cancer (192). The combination was assessed *in vitro* showing enhanced cytotoxicity deriving both from the CAR and the ADCC response, compared to the individual treatments.

Radiotherapy

Radiotherapy is a commonly used regime for the treatment of cancer, particularly in solid tumor malignancies. Radiation introduces cell damage to the tumor and the adjacent cells which generates neoantigens or induces stress ligand upregulation (193). The accumulation of activating ligands triggers the immune system and facilitates cancer cell recognition. For this reason, radiotherapy and immunotherapy have been assessed in a combinational approach with promising results (194). In 2020, Kim et al. reported their findings from the synergy of radiotherapy and ACT of *ex vivo* activated NK cells in a human triple-negative breast cancer xenograft model (195). The combination treatment showed enhanced NK cell tumor infiltration, reduced tumor burden, prolonged NK cell retention to the tumor site and suppression of metastasis. CAR-based therapy and radiotherapy have been assessed together only in the context of CAR-T therapy for glioblastoma treatment. In this study, NKG2D-specific CAR-T cells were combined with radiation therapy due to the upregulation of NKG2DL occurring post-radiation (196). The study showed increased CAR-T cell activation and improved outcomes in terms of survival and tumor control. Taken together, the combination of CAR-NK cells and radiotherapy is worth exploring.

Oncolytic Virotherapy

Oncolytic virotherapy is a fast-developing field within the cancer immunotherapy, attracting particular interest the last two decades. The field is based on the notion of utilizing viruses to selectively replicate within malignant cells, leading to target cell lysis while normal cells remain unaffected (197). Indeed, oncolytic viruses (OVs) have shown impressive cancer cell elimination in murine models, without causing severe side effects in multiple studies (198–200). There are currently three OVs that have received governmental regulatory approval: 1) the herpes simplex virus T-VEC (Imlygic), approved in USA and EU, 2) the adenovirus H101 (Oncocrine), approved in China, and 3) the Rigvir enterovirus, approved in Latvia, Armenia, Georgia and Uzbekistan (201). OVs and CAR therapy have been successfully combined, although mainly in the context of CAR-T cells. The combinational approaches have the form of either sequential treatment courses of OV and CAR therapy, or CAR cells are used as the vessels to deliver OVs to the tumor site (202). There are many advantages of combining two immunotherapies with different mechanism of action. These include better tumor infiltration of the CAR cells following the initial direct tumor cell lysis by the OVs, upregulation of stress markers from the OV-infected cells leading to enhanced effector cell persistence, proliferation and functionality and additional antitumor activity in the case the CAR cells become anergic in the TME (203). CAR-NK cells and OVs have also been studied together. More specifically, in a mouse model of breast cancer brain metastasis, sequential intratumoral administration of OVs (herpes simplex virus) and anti-EGFR CAR-NK-92 cells resulted in better tumor control and prolonged survival, compared to the effects of the individual treatments (204). Moreover, in the *in vitro* experiments of the same study, CAR-NK cells displayed higher cytolytic activity and cytokine release after co-culture with breast cancer cell lines. Additional evidence on the potential of such combinational approach was given by a recently published study on glioblastoma, where OVs expressing the IL-15/IL-15Rα complex (OV-IL15C) were combined with off-the-shelf EGFR CAR-NK cells demonstrating strong antitumor response (205).

Recombinant Viruses

The combination of recombinant viruses with CAR-NK cells has been reported in a preliminary abstract format. Specifically, HER2-specific AAV-mediated gene transfer of a PD-1 inhibitor together with local administration of anti-HER2 CAR-NK-92 cells has been suggested for the treatment glioblastoma (206).

CAR-NK Cells as Drug Carriers

An interesting approach on limiting the insufficient delivery of nanoparticle-based drug formulations was suggested by Siegler et al, which involved the use of CAR-NK cells as carriers (207). The researchers used Abraxane, an FDA-approved nanoparticle-based formulation of the chemotherapeutic agent paclitaxel, to load multilamellar liposomal vesicles, which they then cross-linked to the CAR-NK cell surface. The combinatorial approach was assessed *in vitro* and *in vivo* against $HER2^+$ and $CD19^+$ cancers where enhanced targeted cytotoxicity was observed.

CAR-NK Cell-Derived Extracellular Vesicles (EVs)

Although less of a combinational approach and more of an unexplored field, CAR-NK cell-derived EVs are worth

mentioning. EVs are membranous vesicles secreted by multiple cell types. They enclose proteins, lipids, nucleic acids and other cytoplasmic components of the parent cell and, upon uptake, they mediate biological effects, phenotypic changes and cell-to-cell communications (208). EVs can be categorized into microvesicles, exosomes and apoptotic bodies, depending on their generation mechanism and size. Of particular interest are the exosomes. These are typically 30-150nm in diameter and originate from the endosomal cell compartment. Studies on the content of the NK-cell derived exosomes (NK-exos) revealed that they are loaded with perforin, granzymes, DNAM-1, IFNγ, and other functional molecules, which provides reason for their exploration in cancer therapy (209, 210). Indeed, exosomes derived from the NK cell line NK-92 were found to exert potent antitumor activity in *in vitro* and *in vivo* studies of aggressive melanoma (211). Importantly, NK-exos can be modified, either directly [e.g *via* electroporation (212)] or by the genetic manipulation of the parent cells (213). The latter approach has been extended into the CAR-T field, offering a new and potentially 'off-the-shelf' treatment option. More specifically, primary T cells were lentivirally transduced to express a 2nd generation CAR construct (214). The deriving exosomes were analyzed for their content, where it was found that apart from the cytotoxic molecules, the CAR protein was also present. In contrast, the PD-1 receptor was not detected. The cytolytic potential of the exosomes was assessed against relevant cancer cell lines, showing targeted cancer cell death in a concentration-dependent manner. Taking everything into consideration, we believe that the CAR-NK derived exosomes is also an immunotherapeutic platform worthy of exploring.

CAR-NK CELLS IN PRECLINICAL CANCER RESEARCH

Hematological Malignancies

CAR-NK-based therapies have been extensively studied for the treatment of hematological malignancies, showing a clear *in vitro* and *in vivo* advantage of CAR expressing NK cells over control NK (41, 46, 63, 149, 160, 190, 215–217). CD19 targeting has been in the epicenter of this research, following the FDA approval of the antiCD19 CAR-T products Yescarta and Kymriah. Overall, multiple studies demonstrated that CAR-NK cells were efficient in eradiating CD19$^+$ targets (41, 46, 63, 149, 160, 215–217). Regarding the nature of the assessed CAR constructs, it was shown that 2nd generation anti-CD19 CAR-NK cells containing CD3ζ and CD28 costimulatory domains outperformed 1st generation CARs, whereas 2nd and 3rd generation CAR constructs did not seem to confer substantial differences in NK cell functionality (215, 217). Investigations on the optimal NK cell source were also conducted, where it was suggested that primary NK sources might be a better option than antiCD19 CAR-NK-92 cells (218, 219). In a comparison of CAR PB-NK versus CAR UCB-NK expressing the same antiCD19 CAR construct, it was shown that the former was better in eliminating CD19+ target cells in an effector to target ratio of 1:1 (46). However, the latter could be obtained at higher numbers with less inter-donor variability and stimulation with IL-2 and IL-15 improved more their functionality compared to CAR PB-NK cells. Naturally, the question of whether anti-CD19 CAR-T or CAR-NK cells had better efficacy was addressed. A study comparing CD19-targeting CAR T cell and CAR PB-NK cell anti-leukemia responses *in vivo* demonstrated prolonged survival and reduced adverse effects in mice treated with the CAR-NK cell product, highlighting the potential of CAR-NK therapy in CD19$^+$ malignancies (216).

Other studies have evaluated the preclinical efficacy of CAR-NK cell products for the treatment of hematological malignancies targeting CD5, CD20, CD38, FLT3 or B7H7. All these CAR-NK cell products showed enhanced antitumor responses against target cells expressing the respective antigen (65, 125, 131, 138, 190, 220).

Solid Tumors

CAR-NK therapy has been evaluated as a therapeutic option of various solid tumors, targeting different antigens for each application. More specifically, CAR-NK cell therapy has been assessed in ovarian cancer (NKG2D ligands (NKG2DL), PSMA, FRα, CD24, HER2 or mesothelin) (113, 124, 221–224), glioblastoma (NKG2DL, EGFRvIII and ErbB2) (128, 225, 226), colorectal cancer (NKG2DL and EpCAM)124,187, prostate cancer (PSMA and NKG2DL) (128, 227), hepatocellular carcinomas (c-MET, GPC3 or CD147) (70, 127, 228), pancreatic cancer (mesothelin and FRα) (151, 229, 230), high-risk myosarcoma (ErbB2) (231), gastric cancer (HER2) (170), breast cancer (ErbB2, EGFR and TF) (192, 232, 233), head and neck cancer (PD-L1) (234, 235), neuroblastomas and melanoma (GD2) (236) and lung cancer (NKG2DL and EGFR) (128, 190). Overall, these preclinical studies showed superior antitumor responses *in vitro* and/or *in vivo* compared to non-transduced or control NK cells. However, solid tumors pose additional challenges for CAR-NK cell efficacy, namely intra-tumor infiltration, tumor trafficking, immunosuppressive microenvironment, among others (237). Strategies to overcome these issues and enhance CAR-NK cell functionality against solid tumors have been previously discussed.

CAR-NK CELLS BEYOND CANCER THERAPY

Infectious Diseases

A number of studies have explored the potential of CAR-NK therapy for the treatment of infectious diseases, such as AIDS (acquired immunodeficiency syndrome) and COVID-19 (coronavirus disease). Regarding AIDS, a universal CAR-NK cell product was designed to recognize 2,4-dinitrophenyl (DNP)-tagged antibodies that target the gp160 glycoprotein expressed on the HIV-infected cells (238). The study demonstrated effective degranulation against gp160+ cells, as well as killing of HIV-infected primary CD4+ T cells. Although a comparison between DNP CAR-NK cells and anti-gp-160 CAR-NK cells showed the first to be less cytotoxicity, the versatility of the approach and the ability to target multiple variants/isoforms of the HIV gp160

glycoprotein depending on the DNP-tagged antibodies used is an advantage. Therefore, considering the high mutational rate of HIV, the universal DNP CAR-NK cell product poses a very attractive and potentially effective strategy to treat AIDS.

COVID-19 is an infectious disease caused by the recently emerged SARS-CoV-2 virus (239). The virus can cause severe acute respiratory syndrome, for which no effective treatment exists at the moment. Independent studies have explored the potential of 'off-the-shelf' CAR-NK cell therapy in this setting, although only one of them is published in a peer-reviewed paper. In that study, Ma and colleagues generated a CAR-NK-92 cell product using the scFv domain of the neutralizing antibody S309, which recognizes a highly conserved region of the virus' spike glycoprotein (240). S309 CAR-NK-92 cells showed increased degranulation and cytotoxicity *in vitro*, while targeting four different variants of the spike protein. Other studies generated similar spike protein-targeting constructs displaying promising results (241).

Autoimmune Diseases

The incapability of follicular helper CD4+ T cells (T_{FH}) to prevent aberrant immune responses is associated with the development of several autoimmune diseases (242–244). Current therapeutic strategies are insufficient in providing a permanent solution and are additionally causing serious side effects. CAR-NK therapy holds potential, as it can confer targeted elimination of the pathological immune cells in the autoimmune milieu. For their approach, Reighard and colleagues targeted PD-1, a marker moderately expressed on physiological cells, but overexpressed on T_{FH} cells (245). They generated antiPD-1 CAR-NK-92 cells and reported cytotoxicity against PD-1^{high} but not PD-1^{low} cells *in vitro* studies. The results were further validated in an NSG lupus-like mouse model. Autoantibodies have been described in many other autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis, where the potential of CAR-NK cell therapy could be investigated.

CLINICAL STUDIES ON CAR-NK CELLS

To our knowledge, only a few studies evaluating the clinical efficacy of CAR-NK cell for the treatment of hematological malignancies and solid tumors have been published to date. The clinical studies evaluated NK-92, PB-NK and UCB-based CAR-NK cell products.

NK-92-Based CAR-NK Therapy

A phase I clinical study in patients with AML evaluated the safety of (60) Co-irradiated antiCD33 CAR-NK-92 cells. The cells were transduced with a lentiviral vector encoding for a 3rd generation CAR with CD28, 4-1BB and CD3ζ co-stimulatory domains (246). All three of the enrolled patients were recruited after experiencing relapse from at least one chemotherapeutic regimen. They displayed up to 37,5% blasts in the BM, of which 20.4 to 99.9% were $CD33^+$. Overall, the treatment was found well-tolerated by all patients and the maximum tolerable dose was not reached even with the dose of 50 billion cells. The first two patients were diagnosed with grade I cytokine release syndrome (CRS). To evaluate the response to the treatment, bone marrow aspirates were collected 1-4 months post-infusion. Only one of the patients achieved objective response (OR), but shortly relapsed and all three patients eventually reached a concentration of blasts of at least 75%. Analysis of CD33 positivity in two of the patients revealed 49% and 94,6% CD33+ blasts. The limited efficacy was hypothesized to be primarily due to the decreased cytotoxic potency that the CAR-NK-92 cells had after the irradiation step, as well as due to the insufficient phenotypic evaluation of the $CD33^{+/high}$ AML populations. As a solution, the researchers proposed treatment with non-irradiated CAR-NK-92 cells engineered with a suicide gene. This could allow a better control of the lifespan and proliferation of the NK-92 lymphoma cell line in the patients whilst maintaining high viability and cytotoxicity. Further improvements would be the optimization of the CAR construct towards recognizing different CD33 isoforms present in AML patients, and/or the targeting of alternative antigens. Finally, the authors acknowledge the need to elucidate the factors that determine CAR-NK-92 responsiveness in AML in order to better predict the response in patients.

Another study published the results of the clinical evaluation of anti-Robo1 CAR-NK-92 to a patient with pancreatic ductal adenocarcinoma and liver metastasis (NCT03941457) (247). Here, cells were generated with a lentiviral vector carrying a 2nd generation CAR with 4-1BB and CD3ζ co-stimulatory domains. The patient was refractory to chemotherapy and had $Robo1^+$ non-operative tumor. The patient was infused with 10 billion anti-Robo1 CAR-NK-92 cells on days 1 and 3, and liver metastasis was treated with percutaneous administration of this CAR product on days 2 and 4. Overall, no substantial treatment-related adverse events were reported. The patient progressed two months after the final infusion, with an overall survival of 8 months.

Primary Cell-Based CAR-NK Therapy

CAR-transduced UCB- and PB-NK cells have also been investigated in clinical trials. The promising preclinical results obtained with CAR UCB-NK cells co-transduced with IL-15 and iCasp9 genes encouraged the investigation of the clinical efficacy of this therapy (41). Therefore, a phase I/II study was published 2 years later using UCB-NK cells retrovirally transduced with a 2nd generation antiCD19 CAR construct endowed with CD28 and CD3ζ intracellular signaling domains (NCT03056339) (140). In addition, these UCB-NK cells were also transduced with IL-15 gene and iCasp9. Briefly, a single dose of HLA-mismatched antiCD19 CAR UCB-NK cells was administered to 11 relapsed/refractory patients with $CD19^+$ lymphomas after undergoing lymphodepleting chemotherapy. These patients already received a median of 4 lines of therapy before. The administered doses ranged from 1 to 100 x 10 (5) cells per kg. The maximum tolerated dose was not reached, and no CRS, neurotoxicity or GvHD was detected. Despite the HLA-mismatch, CAR-NK cells were found at least 12 months after infusion, probably due to the inclusion of IL-15 gene in the engineered cells. With a median follow-up of 13.8 months, the ORR is 73% (8 patients), with 7 patients showing CR and 1 PR.

Last, colorectal cancer patients were treated with anti-NKG2DL CAR-expressing autologous or allogeneic PB-NK cells in an haploidentical setting (125). The product was generated by

mRNA electroporation of the CAR gene which contained a single DAP12 co-stimulatory domain. Intraperitoneal infusion reduced $EpCAM^+$ cancer cells in two patients, while a third patient showed tumor size reduction four days after the first injection with allogeneic CAR-NK cells. In addition, the third patient showed almost no uptake of fludeoxyglucose by PET/CT imaging after completion of the treatment, an indication of tumor regression. Tumor sites injected with CAR-NK cells demonstrated necrotic lesions, which were not apparent in non-injected tumor sites. Moreover, CAR-NK cell injected tumor regions showed loss of expression of the NKG2D ligands MICA/B, Villin and CDX2 (markers of adenocarcinoma of intestinal origin), supporting the argument of local antitumor effect in the patients.

Ongoing Clinical Trials

The current ongoing clinical studies evaluating the safety and efficacy of CAR-NK cell therapy in various indications are summarized in **Table 2**. We found that about half of the listed trials are based on the NK-92 cell line. These much-anticipated results are believed to shed light on the potential of NK cell lines as the source of off-the-shelf CAR-NK cell products. The rest of the trials with disclosed information concern mostly PB- and UCB-based CAR-NK cells, while there is also one iPSC-based CAR-NK cell trial (NCT04245722). Being the first registered trial investigating the clinical efficacy and tolerability of this approach, the insights gained from this study could boost further investigations of iPSC-derived CAR-NK cells in the clinical setting.

Even though the applicability of CAR-NK cell therapy in various indications has been proved preclinically, it is clear that the vast majority of the registered clinical trials is focusing on cancer. CD19 remains the most commonly targeted antigen (34% of the trials), while ROBO1 and NKG2DL are being increasingly investigated, counting for about 10% of the listed trials each. It is also worth mentioning that although cell therapy is traditionally used to treat hematological malignancies, there are currently 10 registered trials focusing on solid tumors. Moreover, for the first time, CAR-NK cells are under clinical investigation for the treatment of the pandemic-causing infectious disease COVID-19 (NCT04324996).

As previously mentioned, NK cell-based co-stimulatory domains may induce more potent CAR-NK cell-mediated antitumor responses compared to T cell-based CARs (113, 122–126). The potential of NK cell-based CARs is being increasingly investigated in the clinical setting, counting for 37% of the trials with relative disclosed information. The results of these studies are believed to influence the next generation of CAR-NK therapies.

TABLE 2 | Ongoing clinical trials using genetically modified NK cells.

Target	Disease	NK cell source	Intracellular domains	Clinical stage	NCT number
CD7	Lymphoma and leukemia	NK-92	CD28 + 4-1BB + CD3ζ	I/II	NCT02742727
CD19	Acute lymphocytic leukemia, Chronic lymphocytic leukemia, Follicular Lymphoma, Mantle Cell Lymphoma, B-cell Prolymphocytic Leukemia, Diffuse Large Cell Lymphoma	NK-92	CD28 + 4-1BB + CD3ζ	I/II	NCT02892695
CD19	B cell lymphoma or leukemia	UCB-NK	CD28 + CD3ζ	I/II	NCT03056339
CD19	B cell lymphoma	NK-92	2B4	I	NCT03690310
CD19	Non-Hodgkin lymphoma	Not known	Not known	I	NCT04639739
CD19	Non-Hodgkin lymphoma	Not known	Not known	I	NCT04887012
CD19	Non-Hodgkin lymphoma, Chronic lymphocytic leukemia and B cell acute lymphocytic leukemiaB-ALL	Allogeneic NK	Not known	I	NCT05020678
CD19	B cell lymphoma	UCB-NK	Not known	I	NCT04796675
CD19	B cell lymphoma, Chronic lymphocytic leukemia	iPSC	NKG2D + 2B4 + CD3ζ	I	NCT04245722
CD19	B cell lymphoma, Myelodysplastic syndrome	UCB-NK	Not known	I/II	NCT05092451
CD19/CD22	B cell lymphoma	Not known	2B4	I	NCT03824964
CD22	B cell lymphoma	Not known	2B4	I	NCT03692767
CD33	Acute myeloid leukemia	Not known	N Not known	I	NCT05008575
CD33	Acute myeloid leukemia	NK-92	CD28 + 4-1BB + CD3ζ	I/II	NCT02944162
BCMA	Multiple Myeloma	NK-92	4-1BB + CD3ζ	I/II	NCT03940833
BCMA	Multiple Myeloma	UCB-NK	Not known	I	NCT05008536
HER2	Glioblastoma	NK-92	CD28 + CD3ζ	I	NCT03383978
Mesothelin	Epithelial Ovarian Cancer	PB-NK	2B4	I	NCT03692637
MUC1	Hepatocellular carcinoma, Non-small cell lung cancer, Pancreatic carcinoma, Breast cancer, Glioma of brain, Colorectal carcinoma, Gastric carcinoma.	NK-92	CD28 + 4-1BB + CD3ζ	I/II	NCT02839954
NKG2DL	Solid tumors	PB-NK	Not known	I	NCT03415100
NKG2DL	Acute myeloid leukemia, Myelodysplastic syndrome	Allogeneic NK	Not known	I	NCT04623944
NKG2DL and/or SARS-CoV-2 S protein	COVID-19	UCB-NK	Not known	I/II	NCT04324996
PD-L1	Head and neck squamous cell carcinoma, gastric cancer	NK-92	Not known	II	NCT04847466
PSMA	Castration-resistant prostate cancer	NK-92	2B4	I	NCT03692663
ROBO1	$ROBO1^+$ solid tumors	NK-92	4-1BB + CD3ζ	I/II	NCT03940820
ROBO1	Pancreatic Cancer	NK-92	4-1BB + CD3ζ	I/II	NCT03941457
ROBO1	Pancreatic cancer	NK-92	4-1BB + CD3ζ	I/II	NCT03931720
–	Non-small cell lung cancer	NK-92	Not known	I	NCT03656705

Lastly, it is worth mentioning that non-CAR genetically engineered NK cell products are also under clinical investigation (e.g., NCT03656705 or NCT04023071). The reports of these results are highly anticipated.

NON-CAR GENETIC MODIFICATION OF NK CELLS

In **Figure 1** we have provided a schematic representation of the different aspects involved in the development of a CAR-NK cell therapy, as well as recent advances of the field. The success story of CAR therapy, as well as the advances in receptor engineering, inspired the development of other constructs for T and NK cell therapy of cancer. Some of the ones that have been applied to NK cells are listed below.

Dimeric Antigen Receptors (DARs)

Dimeric antigen receptors, or DARs, are a novel category of artificial receptors that share many of the transmembrane and the signal transduction compartments of the CARs (248). While the antigen-targeting domain of the CARs is a scFv domain, however, DARs utilize the whole Fab part of the antibody. This is presumed to increase the stability of the synapse, as well as the targeting specificity. To this day, evidence on the preclinical efficacy of the DARs has only been provided by T cell-based approaches for the treatment of relapsed/refractory multiple myeloma. Nevertheless, the preclinical evaluation of anti-CD38 iPSC-derived DAR-NK cells for the same indication has also been announced.

Chimeric Switch Receptors (CSRs)

As TME is a major factor dictating the success of an immunotherapy, strategies have been developed to switch the negative effects of immune suppression into positive, using chimeric switch receptors (CSRs). CSRs are cleverly designed to bind to inhibitory ligands on the malignant cells and transmit activating signal instead, thanks to their intracellular signaling domain. The ectodomains of the checkpoint inhibitors PD1 (249), TIGIT (250) and CTLA-4 (251) are particularly attractive and have shown promising results in T cell studies with regards to resistance to immunosuppression and restored effector function (252). Application of the approach to NK cells is gaining popularity the last years. Notably, a study on NK-92

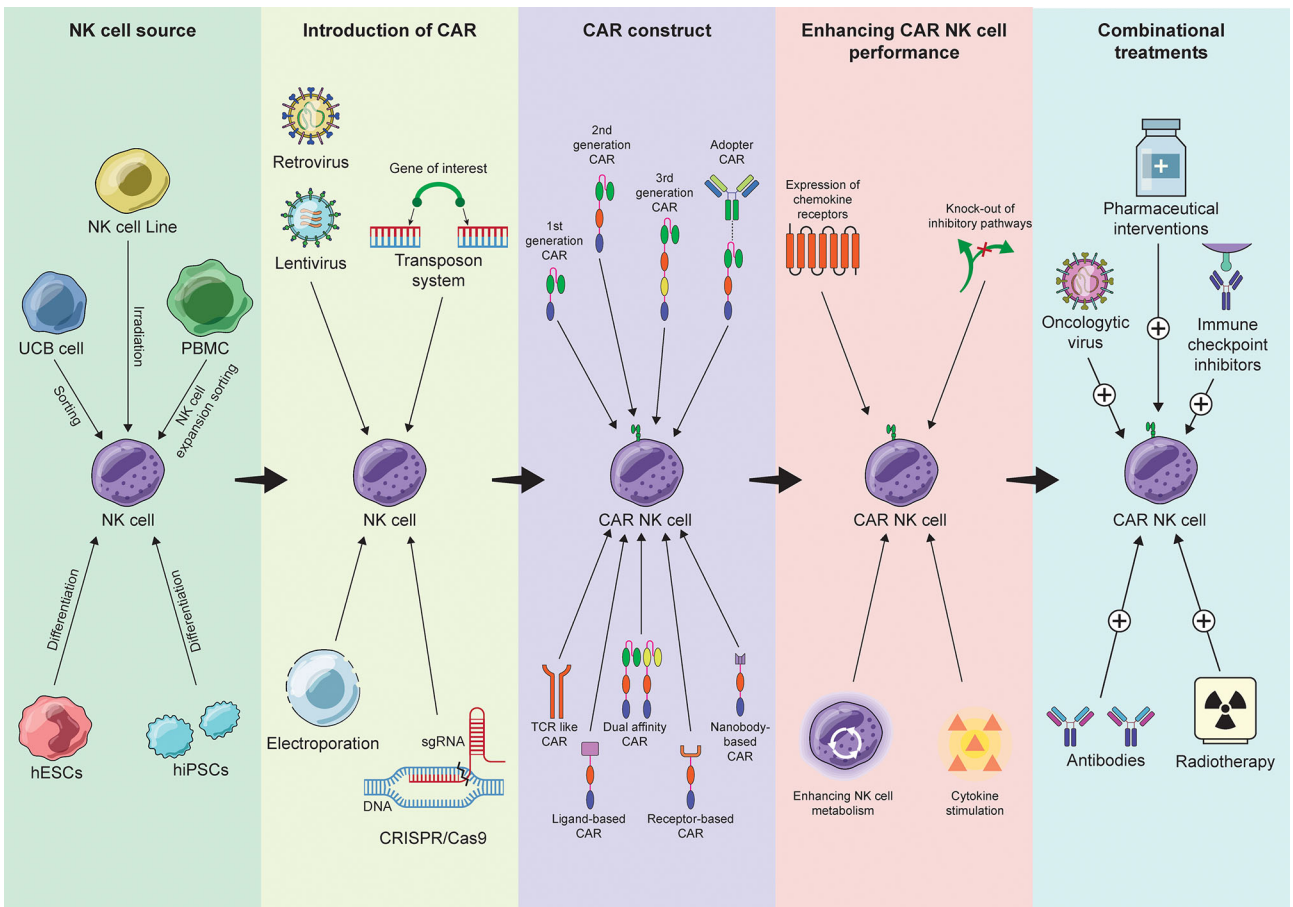

FIGURE 1 | Summary of the recent advances in the process of CAR-NK cell therapy development from NK cell source selection to combinational approaches.

expressing a novel PD1-NKG2D-41BB receptor demonstrated rapid elimination of PD1+ lung cancer target cells in an *in vitro* setting (253). A similar re-targeting approach is the chimeric chemokine receptors (CCRs). Although the potential of CCR-NK cells has yet to be explored, co-expression of chemokine receptors, such as CCR2b, has shown increased migration of CAR-T cells to the site of the malignancy (254).

T Cell Receptor (TCR)-Expressing NK Cells

The genetic modification of NK cells to express tumor specific T cell receptors (TCRs) has recently been attempted *in vitro*. In contrast to CARs that bind to cell surface antigens, TCRs can recognize antigenic peptides from degraded protein presented on the MHC, and therefore, are theoretically less restricted by the localization of the targeted molecule. An obstacle to the TCR expressing NK cells, however, is the lack of the accessory TCR signaling components that are present in T cells. Taking this into consideration, the engineering of TCR-NK cells becomes more challenging as the presence of the CD3 complex on the cell surface is necessary for the TCR to be functional. Mensali et al, in 2019, provided evidence that TCRs can be successfully expressed on NK-92 cells and that are able to mediate pMHC-specific cytotoxicity (255).

CONCLUDING REMARKS

CAR-NK therapy has given new hope to the patients battling 'incurable' diseases and a new platform for researchers to explore the potential of CAR-based cell therapy. Although the challenges regarding the *ex vivo* expansion of the cells, *in vivo* persistence and insufficient cell trafficking remain, recent advances in cell and molecular biology provide viable solutions. Furthermore, CAR-NK cells are proven versatile and customizable, which expands their applicability to diseases beyond cancer. Looking into the future, next generation CAR-NK therapy is incorporating more and more state-of-the-art technology, adapting from the discoveries of CAR-T research, but also harnessing the unique features of NK cells. Taken together, CAR-NK therapy is believed to play an even greater role in the clinics in the forthcoming years, by providing efficient and safe off-the-shelf products.

AUTHOR CONTRIBUTIONS

MK, MV-M, AL, and EA contributed equally for this review. All authors contributed to the article and approved the submitted version.

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REVIEW
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CyTOF® for the Masses

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Mass cytometry has revolutionized immunophenotyping, particularly in exploratory settings where simultaneous breadth and depth of characterization of immune populations is needed with limited samples such as in preclinical and clinical tumor immunotherapy. Mass cytometry is also a powerful tool for single-cell immunological assays, especially for complex and simultaneous characterization of diverse intratumoral immune subsets or immunotherapeutic cell populations. Through the elimination of spectral overlap seen in optical flow cytometry by replacement of fluorescent labels with metal isotopes, mass cytometry allows, on average, robust analysis of 60 individual parameters simultaneously. This is, however, associated with significantly increased complexity in the design, execution, and interpretation of mass cytometry experiments. To address the key pitfalls associated with the fragmentation, complexity, and analysis of data in mass cytometry for immunologists who are novices to these techniques, we have developed a comprehensive resource guide. Included in this review are experiment and panel design, antibody conjugations, sample staining, sample acquisition, and data pre-processing and analysis. Where feasible multiple resources for the same process are compared, allowing researchers experienced in flow cytometry but with minimal mass cytometry expertise to develop a data-driven and streamlined project workflow. It is our hope that this manuscript will prove a useful resource for both beginning and advanced users of mass cytometry.

Keywords: mass cytometry, workflow, experimental design, protocol, data analysis, panel design, barcoding, CyTOF

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INTRODUCTION

Mass cytometry, also termed cytometry by Time-Of-Flight (CyTOF®), is a powerful tool for high-dimensional and high-throughput single-cell assays. First introduced in 2009 by Bandura et al. (1), mass cytometry has become an important tool in the analysis of immune cell function/activation due to its high-parameter capabilities. Since 2015, the application of mass cytometry for immunophenotyping in hematopoietic stem cell transplantation (HSCT) (2–6), tumor microenvironment (TME) (7–13) and cancer immunotherapy (6, 9, 14–17) has significantly expanded.

Until recently, fluorescent-based (conventional) flow cytometry was the method of choice for phenotypic and functional analysis of single cells. Standard flow cytometry technologies using 4- or 5-laser data acquisition instruments allow analysis of up to 30 parameters simultaneously. The newer fluorescent-based flow cytometry machines (spectral flow cytometers) measure the total fluorescence in 1 sample and then use an unmixing technology to mathematically separate the specific fluorophore signals (18). These data acquisition machines can process up to 50 parameters simultaneously; however, practical application typically allows a maximum of 40 parameters (19). Due to the broader emission spectra of fluorescent probes following laser excitation, overlapping emission spectra remains a significant issue in flow cytometry. Mass cytometry replaces fluorescent labels with non-biologically available metal isotopes with concise mass spectrometry parameters, thereby overcoming the pitfalls associated with overlapping emission spectra and increasing the number of simultaneously analyzable parameters further (20, 21). In mass cytometry, cells are incubated with a mixture of probes/antibodies tagged with a unique non-radioactive heavy metal isotope. Single-cell suspensions are nebulized such that each droplet contains a single cell. Individual cells subsequently pass through argon (Ar) plasma, which atomizes and ionizes the sample. This converts each cell into a cloud containing ions of the elements present in or on that cell. A high-pass optic (quadrupole) removes the low-mass (mainly biologic) ions from each cloud (ions with mass below 75 Da), resulting in a cloud containing only those ions corresponding to the isotope-conjugated probes. In the Time of Flight (TOF) chamber, the ions are separated by mass-to-charge ratio. Upon encountering the detector, these ion counts are amplified and converted into electrical signals. Theoretically, 120 parameters can be studied simultaneously. However, realistically the availability of isotopes with sufficient purity as well as antibody conjugation chemistries limit applications to ~60 parameters per mass cytometry panel. A single-cell technology generating even more dimensions is single-cell RNA sequencing (scRNAseq), which gives a quantitative measure of gene expression levels per cell. scRNAseq is a powerful genomic tool for dissecting cell populations. However, scRNAseq can only be run on a small number of single cells (limited mainly by increased costs), whereas mass cytometry experiments can acquire data on several times that number (in the range of 10^6-10^7 cells), facilitating the characterization of rare cell populations. Additionally, mass cytometry can add critical functional information through protein analyses. When the complementary techniques of scRNAseq and mass cytometry are combined, one can rigorously phenotypically and functionally characterize diverse cell populations within a single sample. Mass cytometry can also be used to confirm data derived from scRNAseq. Considering the complexity of the TME, such a multimodal approach yields powerful data applicable to both tumor-intrinsic and tumor-extrinsic effects of immunotherapies in the TME, as well as the correlation of peripheral immune signatures with treatment response or failure or identification of new targets (22).

The purpose of this article is to detail considerations critical to designing and performing a mass cytometry experiment for immunologists and cancer biologists with limited expertise (**Figure 1**). Our target audience includes not only bench scientists and clinicians with knowledge of basic flow cytometry, but also computational scientists and immunotherapy-focused individuals working with mass cytometry datasets. For more detailed reference literature on conventional flow cytometry, we refer the reader to selected reviews, guidelines, and protocols (18, 23–27).

EXPERIMENTAL DESIGN

Study Endpoints and Sample Sources

Paramount to carefully planning mass cytometry experiment design is consideration of the study goal (e.g. identifying multiple new populations in a sample, characterizing an unknown cell population, proportional comparison of multiple well-characterized cell populations, novel biomarker discovery, and analysis of protein expression, cell cycle or phosphorylation state, and pharmacokinetics/pharmacodynamics). The large number of parameters simultaneously analyzable by mass cytometry facilitates assessing all of these endpoints leveraging a single antibody panel.

In addition, it is important to plan ahead for the sample sources to be used. Each sample source and tissue type has corresponding optimal pre-analysis sample processing and storage considerations; the researcher is referred to existing interactive resources noted elsewhere in this review to raise project-specific questions for clarification.

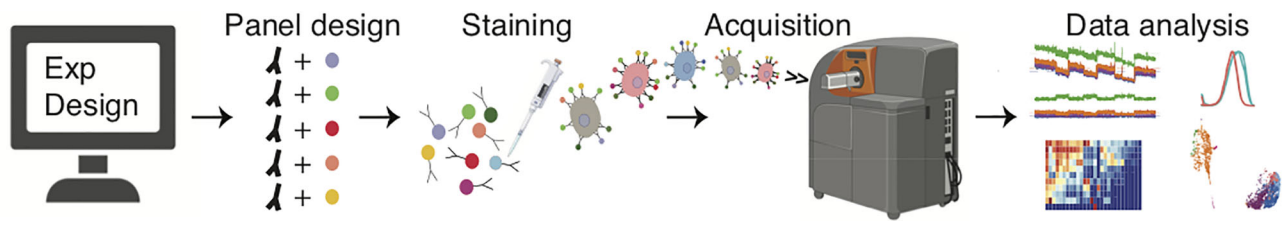

FIGURE 1 | Typical workflow used in mass cytometry experiments. An experiment starts with careful design of an antibody/probe panel. This is followed by sample processing, staining and acquisition, and finally data analysis.

Many experimental factors can affect mass cytometry data, including cell isolation, staining protocol, fixation, and donor-specific biological variation. All of these are described in more detail by *Olsen* et al. (28) Accounting for these factors, isolation and staining protocols may need several optimization rounds and unique quality controls. An important quality control (QC) approach for reproducibility and staining consistency is an internal control per sample tube, which can be achieved by sample barcoding (see *Barcoding*). Another QC element when studying cytokines or transcription factor activation is stimulated versus unstimulated conditions. The sample distribution itself can contribute important QC components, since all cell types are not positive for all markers (i.e. internal positive and negative controls).

Isotope- Antibody Pairing

Proper pairing of antibodies with metal isotopes (see *Panel Design*) is critical. When opting for standard pre-conjugated antibodies, the main limitation is the extent of the vendor portfolio. Alternatively, lanthanides can be conjugated *de novo* to purified antibodies using a Maxpar® X8 antibody labelling kit (Fluidigm, San Francisco, CA) (29–31). With lanthanides, a panel can contain up to 37 cellular markers/antibodies (**Figure 2A**). One limitation of this approach is that proteins such as bovine serum albumin (BSA) (often used in the buffers of purified antibodies) can bind lanthanides, resulting in failed antibody conjugation due to adsorption of the lanthanide.

In addition to lanthanides, the distinct isotopes of cadmium (31), palladium (32, 33), indium (32), platinum (34), and bismuth (32, 35) can be utilized, to a total of 60 distinct isotopes (**Figure 2A**). The Maxpar® MCP9 antibody labelling kit (Fluidigm, San Francisco, CA) is specifically designed to conjugate cadmium (Cd) isotopes (31). Most commercial Qdots used for conventional flow cytometry contain isotopes of cadmium ($^{106\text{-}116}$Cd) with either selenium ($^{72\text{-}82}$Se) or tellurium ($^{120\text{-}130}$Te), which are readily available as antibody

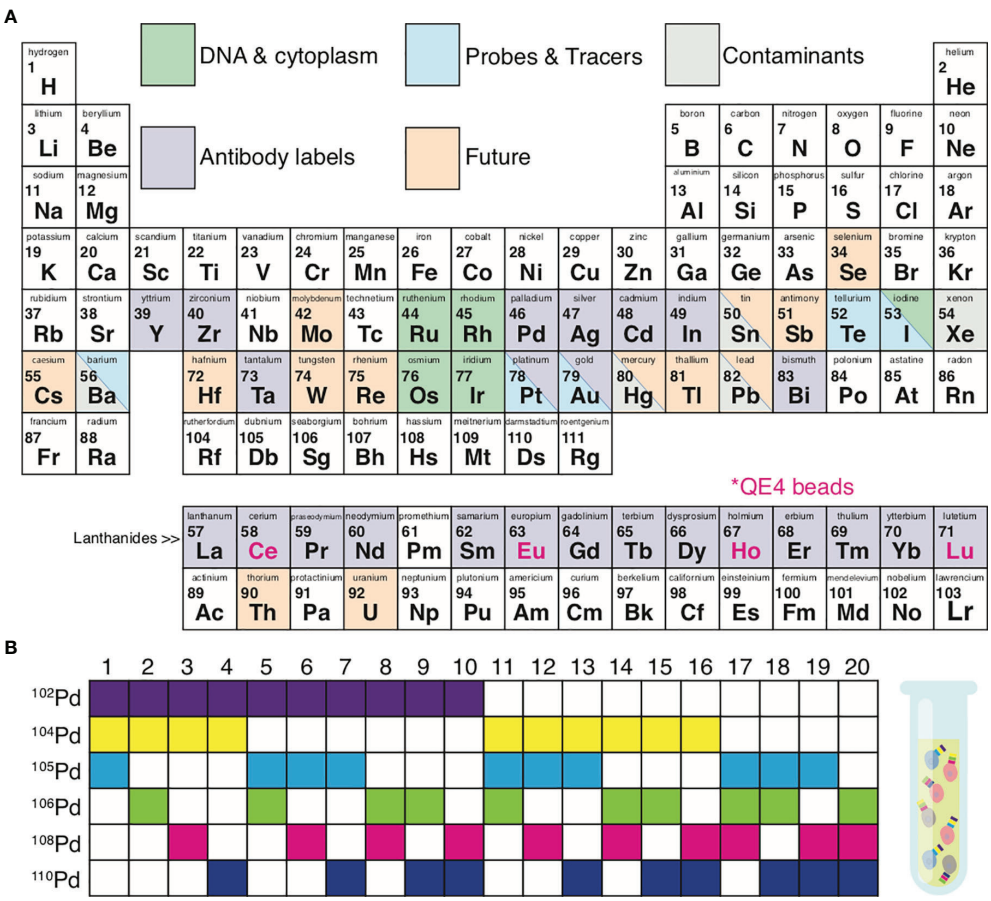

FIGURE 2 | Metal isotopes utilized in mass cytometry. **(A)** Periodic table summarizing the elements currently available for mass cytometry experiments. *Grey*, possible contaminating elements; *green*, elements used to indicate DNA content or cell size; *purple*, elements available for antibody conjugations; *blue*, elements available as probes and tracers; *orange*, elements not yet explored for mass cytometry but of potential future interest. *Pink font*; isotopes included in QE4 calibration beads. **(B)** Certain isotopes work well for the use in sample 1 barcoding. Shown is an example of palladium barcoding using 6 isotopes in unique combinations of 3. This strategy generates 20 separate barcodes, allowing 20 individual samples to be combined into 1 single tube.

conjugates (36, 37). More recent options for antibody labels include streptavidin-coated gold or silver nanoparticles (38, 39) and tantalum oxide nanoparticles (40).

Barcoding

Sample throughput can be enhanced, costs reduced, and data quality improved by utilizing sample barcoding (33, 41). Each experimental sample (e.g. across individuals or treatment groups) can be tagged with a unique isotope of a particular element (e.g., after which all samples are combined into 1 tube. **Figure 2B** demonstrates a 6-choose-3 barcoding example. Six Palladium (Pd) isotopes can be used to generate 20 different barcodes, where each barcode is created from a combination of any 3 Pd isotopes. Barcoding minimizes the possibility for inter-sample staining variability, reduces cell-cell doublets, and minimizes the propensity for inter-sample cross-contamination seen in serial runs across individual samples (33, 42–44). Sample barcoding minimizes inter-sample staining variability by avoiding sample-to-sample pipetting errors and inconsistent incubation times. In a 6-choose-3 barcoding scheme, a cell-cell doublet will yield an illegal barcode (I.e. a combination of 2 existing barcodes from the scheme) with a positivity for at least 4 out of 6 isotopes, which cannot belong to a single cell event (33). Doublets between cells within a sample cannot be detected and removed by barcoding alone. Moreover, the use of all possible Pd combinations (any combination of 6 isotopes generating 64 barcodes) can result in miscoding if one or more reagents fail, making it impossible to exclude cell-cell doublets. Sample barcoding can also be utilized to add an internal control into each tube prior to staining. This internal control generates the same results/clustering/cell proportions from one tube to the next and is therefore strongly recommended for optimal data quality and reproducibility. Internal control cells can be cryopreserved and rethawed pooled wild-type mouse splenocytes or PBMC from the same healthy donor or Veri-cells® (Biolegend, San Diego, CA) (45, 46).

There are 3 main options in sample barcoding:

1. The Cell-ID® 20-Plex palladium (Pd) barcoding kit (Fluidigm, San Francisco, CA) uses 6 distinct Pd isotopes to combine up to 20 samples per tube (41, 47, 48). This is an intracellular method; one limitation is that samples need to be fixed prior to panel staining, so epitopes may be crosslinked in such a way that the corresponding antibody no longer recognizes the intended epitope. Therefore, protocol troubleshooting and optimization is needed prior to applying this approach.
2. Antibody-based live cell barcoding is more flexible as it does not require fixation prior to panel staining. Unique Pd, cadmium (Cd), and/or platinum (Pt) isotopes are conjugated to antibodies directed against ubiquitous epitopes such as CD45 (hematopoietic lineages), β2 microglobulin (class I MHC and CD1 isomers), or CD298 (integral membrane cationic ATPase-associated proteins), and samples are stained with different combinations of these antibodies (42, 44, 49, 50). Pd, Cd, or Pt are ideal for live cell barcoding primarily because these isotopes are outside the CyTOF® optimum mass range of 153 to 176 and therefore tend to be less "bright" (51). Pd and Cd isotopes are well below and Pt is well above the 139-176 mass range of lanthanides and therefore do not influence lanthanide-based antibody detection. Pd live cell barcoding is more labor intensive than Cd, since there is no available kit for Pd conjugations. Recently, *Muftuoglu* et al. (52) showed that Cd-CD45 barcodes elicit higher signal intensities than Pd-CD45 barcodes, most likely attributable to superior signal resolution because MCP9 polymers used to conjugate Cd chelate a higher number of isotopes as compared to mDOTA (used to conjugate Pd). (This group also showed that it is possible to conjugate Pd isotopes to CD45 antibodies using MCP9, and that this results in an equal signal intensity as for the Cd-CD45 conjugates.)
3. Monoisotopic cisplatin-based live cell barcoding is the simplest and fastest method available. Cisplatin is used to directly label cells, without the need for antibody conjugations (53, 54). Cisplatin, a chemotherapeutic agent, contains platinum and is available from Fluidigm as any of the following isotopes: ^{194}Pt, ^{195}Pt, ^{196}Pt, and ^{198}Pt.

Thiol-reactive tellurium (TeMal) (55) or osmium and ruthenium tetroxide (56) can be added to any of the 3 barcoding strategies in order to further increase multiplexing capabilities.

Cell Numbers and Viability

Mass cytometry sample staining and acquisition induces a high rate of cell loss. Therefore, starting with 800,000 - 1 million cells per sample is advisable. Typically, only 50-70% of the sample can be recovered in the data; the remainder is loss due to aggregation on the walls of the spray chamber and injector (28, 57). Of note, these numbers are based on the CyTOF Helios® instrument (Fluidigm, San Francisco, CA). With the CyTOF2® instrument (Fluidigm, San Francisco, CA), cell recovery is even lower (30-40%) (20). Of note, this cell loss inside the machine is stochastic in nature and therefore does not appear to introduce sampling bias (51).

Optimal starting cell numbers are highly dependent on the study and the planned sample staining protocol. Studies involving rare cell populations or transcription factors require a larger starting sample size for adequate rigor as compared to studies investigating prevalent subsets. During sample processing and staining, an additional cell loss of 20-30% must be considered.

It is important to minimize inter-sample variability in analyzed cell number and viability in order to insure reproducible staining approaches across experiments. When processing samples, tissue digestions, freeze/thaw cycles, and incomplete fixation prior to permeabilization can introduce sampling bias by differentially affecting specific cell populations. Dead cells may compromise flow and mass cytometric data by non-specifically trapping antibodies (58). In addition, dead cells tend to release DNA, which adheres to cells, causes cell aggregation, and increases cell doublets. Tissue digestions can also cause an overall low cell viability; achieving a high cell viability (> 80%) is important in ensuring high-quality

data (59, 60). To address low cell viability, dead cell removal kits are available [e.g. Miltenyi Biotec Inc (Auburn, CA) and STEMCELL Technologies Inc (Cambridge, MA)] for application prior to sample staining to assist in data QC.

A convenient solution for low cell yields prior to sample staining is live cell barcoding. Not only can multiple low-yield samples be combined in a single tube, but a spiked-in internal control can also be added, increasing the total analysis cell number and distributing cell loss in downstream steps across both study samples and controls and thereby preserving a greater fraction of the study sample (7, 45). For example, *Winkels* et al. (61) combined barcoded mouse splenocytes with mouse aorta samples, preserving a greater fraction of the murine aorta samples.

Where low cell yields and/or poor viability persist, the cellular composition of these tissues can instead be studied *in situ* using a histologic approach. There are 2 platforms available for this purpose. In imaging mass cytometry [Hyperion® (Fluidigm)], a laser ablates histological sections stained with metal-labelled antibodies (62, 63). A more novel metal-based histology platform with increased speed, sensitivity, and image resolution is multiplexed ion beam imaging [MIBI (Ionpath)], which collects data through secondary ions released from the histological slide by primary ion beams (64–66).

PANEL DESIGN & ANTIBODY CONJUGATION

As in conventional flow cytometry, panel design is key to mass cytometry experiment success (36, 57, 67). The initial marker selection relies heavily on the scientist's combined biological knowledge and familiarity with statistical testing methods, varying depending on the sample type, cell type, and overall experimental objectives. Relevant biological knowledge includes that from literature and from data generated from prior RNA sequencing or conventional flow cytometry. Marker screen kits are available (68, 69). Beyond isotope-conjugated antibodies, other probes which can be included in mass cytometry panels include tetramers (70), carbohydrate-binding molecules (71), tellurium-based oxygen sensors (72), inorganic nanoparticles (73), RNA probes (74, 75), and modified nucleotides (75, 76) (**Figure 2A**). With the exception of oxygen (72), small molecules or proteins were not detectable by mass cytometry until the Nitz group developed a Tellurium-containing analog of phenylalanine, making it possible to monitor protein synthesis (77). Poreba et al. have since developed multiple protease-selective lanthanide-labelled probes for mass cytometry (78).

Since cells are atomized and ionized inside a mass cytometer, the resulting data lacks the Side Scatter (SSC) and Forward Scatter (FSC) parameters used for cell doublet and debris discrimination in conventional flow cytometry. Therefore, mass cytometry relies on the use of a DNA intercalator (see *Sample Staining*) (7). As an alternative to the FSC parameter, Osmium Tetroxide (OsO_4) has been suggested as a useful tool to reconstruct cell size in mass cytometry data (79). OsO4 is a nonpolar compound that penetrates charged membranes and can be detected directly by the mass cytometer. *Good* et al. have also adapted carboxy-fluorescein succinimidyl ester (CFSE)-based protocols for tracking cell proliferation in mass cytometry using a metal-conjugated CFSE cross-reactive anti-fluorescein isothiocyanate (anti-FITC) antibody (80).

The next step in panel design is pairing an antibody or probe with a metal isotope in a manner that insures optimal signal intensity with minimal to no signal overlap. Critical considerations include: 1) isotope sensitivity range of the detection instrument, 2) intensity of surface marker expression, 3) degree of variation and patterns of expression across samples, and 4) spillover/background.

1. CyTOF® is most sensitive in the range from atomic mass 153 to 176; therefore isotopes/mass tags within this mass range are preferable for antibodies against weakly expressed markers (51).
2. Surface marker intensity considerations follow a process akin to fluorophore-based panel design for flow cytometry; antigens/probes are first classified as either high expression (primary), medium/variable expression (secondary), or low/unknown expression (tertiary) (81). Antibodies with low binding affinity or directed against tertiary antigens should be paired with isotopes in the detection instrument's high-sensitivity detection range. Antibodies against primary or secondary antigens need pairing with isotopes on either end of this optimal mass range.
3. The same antigen may have vastly different expression patterns depending on cell type, organ, or disease state (36). For this reason, it is important to either have or obtain knowledge of the specific antigens in the study. Prior knowledge from conventional flow cytometry and literature will help assign antigens to the above-mentioned categories. For example, CD4 is a primary antigen and exhibits a clear bimodal expression, with clear negative and positive populations (82). Alternatively, chemokine receptors such as CCR7 are often classified as secondary antigens and have a broad, often non-modal spectrum of expression (83).
4. Relative to the cellular autofluorescence or channel cross-talk seen in conventional flow cytometry, sources of background are greatly reduced in mass cytometry (20, 36). Mass cytometry background is predominantly caused by signal spillover related to instrument detection sensitivity. In a TOF analyzer ions are separated based on velocity, which in turn is determined by their mass (M) and kinetic energy. Ions of the same kind have small differences in initial position and velocity from each other resulting in slightly different detector arrival times, which is reflected in the width of the resulting mass peak. An over-abundance of the same ions causes position and velocity spreads, resulting in broader mass peaks spilling over into the adjacent mass peak (M+/-1). If an antibody against a high-expressing antigen is conjugated to a metal isotope within the high-sensitivity range of the instrument, spillover will occur due to abundance sensitivity. The second cause of spillover is oxidization of certain metal isotopes following air exposure, resulting in a background

signal at 16 mass units (^{16}O) higher than the mass of the primary isotope (M+16). Oxide formation can occur in lanthanum (La), praseodymium (Pr), neodymium (Nd), and samanium (Sm)-labelled antibodies/probes. Only 7 metal isotopes form significant oxides: ^{139}La, $^{142\text{-}144}Nd$, ^{148}Nd, and ^{150}Nd. Spillover matrices are available to assist in this process (84). Before sample acquisition, CyTOF® machine parameters are optimized to limit the ^{139}La oxidation to less than 3% of ^{155}Gd (= M+16). Finally, the largest contribution to signal overspill is isotope impurity, i.e. a contamination of a metal with one of its other isotopes. 100% purity is not pragmatically feasible for all metal isotopes.

The overall principles of panel design (36) are as follows:

1. Tertiary antigens should be paired with isotopes within the CyTOF® high-sensitivity range (85–108) and primary antigens should be paired with isotopes outside this range;
2. Do not place a tertiary antigen-isotope pair in the oxide-mass (M+16) of a primary antigen-isotope pair;
3. Choose isotope tags for tertiary antigens in channels which receive no or little spillover from adjacent channels;
4. For less pure isotopes, select antigens that identify specific cell subsets (for example $CD4^+$ and $CD8^+$ subsets, which are mutually exclusive outside the gut and thymus);
5. Channels with high spillover can be reserved for markers to be excluded from downstream analysis (for example CD41 to gate out platelets and platelet-cell aggregates).

There are many reference resources that support mass cytometry panel design, including Fluidigm's online tools, institute mass cytometry core facilities, expert collaborators, and key publications (67). Additionally, available MaxPar panel kits (Fluidigm, San Francisco, CA) (109, 110) include a user-friendly kit-specific data analysis platform (GemStone Software, Topsham, ME).

Although the list of pre-conjugated antibodies for purchase is steadily growing, customized antibody panels often require in-house conjugations. Following panel design, unlabeled antibodies must be conjugated to selected isotopes. Fortunately, conjugation kits and published protocols are available (29, 31, 33–35). The isotope planned for conjugation is first linked to a polymer *via* a chelator. Common chelators used for mass cytometry isotope conjugations include diethylene triamine pentaacetic acid (DTPA), ethylenediamine tertraacetic acid (EDTA), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). The antibody is separately modified in its hinge region by a reduction of disulfide bonds to thiols using tris-2-carboxyethyl phosphine (TCEP). Finally, the polymer and associated chelate are coupled to a thiol group of the reduced antibody. These methods can be applied to conjugate metal isotopes to IgG antibodies. Buffers containing protein or glycerol as antibody stabilizers should be avoided. For purified antibodies only available with BSA, BSA removal kits are available that can be used before proceeding to the antibody conjugations (111).

Methods for confirming successful conjugation vary depending on the protocol. This is elegantly outlined by *Han* et al. (29) The shelf life of antibodies conjugated "in-house" can vary significantly. If stored properly they generally maintain functionality at least 6 months from conjugation (29, 112). Antibodies unused/stored for longer periods will require testing on the mass cytometer to confirm that the isotope remains conjugated. How these tests are best performed is explained in the section "*Sample Acquisition & Data Output*". Once confirmed, the antibody can be used for new cells of interest following standard titration. Results of subsequent titrations should be compared to the initial titration results to identify and troubleshoot new issues.

The next crucial step is to titrate all antibodies and perform a test run involving the entire experimental protocol. This achieves the best possible signal-to-noise ratio by reducing non-specific antibody binding and spillover (113, 114). In addition, using antibodies at non-saturated concentrations prevents ion detector saturation (33). Similar to conventional flow cytometry, a serial dilution strategy of at least 5 dilutions is advisable. Antibodies to primary antigens (e.g. common lineage markers such as CD3, CD19, and CD11b) should be titrated individually and separately. Subsequently, antibodies directed against secondary and tertiary antigens can be titrated within the combined antibody panel, in the presence of the antibodies already optimally titrated against primary antigens. This approach has 3 advantages: 1) to titrate antibodies for staining the population of interest, 2) to enrich the signal by gating cells known to express particular secondary and/or tertiary antigens, and 3) to provide internal positive and negative controls within the titration samples. If titrating for signaling molecules such as cytokines or transcription factors, both a baseline sample and an activated sample (stimulation or treatment) are needed (115). It is advisable to select a cell number per titration point that is comparable to the actual experiment. The antibody titers are determined by calculating the staining index, a method very similar to that used in conventional flow cytometry (116, 117). The main difference between mass cytometry titrations and those used in flow cytometry is that the standard deviation of the negative population is essentially non-existent in mass cytometry titrations and is therefore not included in the staining index formula (118).

Finally, the performance of the panel should be tested on a few control samples prior to proceeding to valuable experimental samples. If the specificity of a signal is unclear, "metal-minus-one" (MMO) controls can be used for resolution (117). If spillover persists with MMO, Chevrier et al. developed a set of computational tools to compensate for spillover in mass cytometry data (see *Compensation*) (84). In some situations, tertiary antigens are so weakly expressed that they are not cleanly discernible. Switching to a 2-step staining can augment the signal from the weakly staining primary antibody; a primary antibody conjugated to biotin, FITC, phycoerythrin (PE), or allophycocyanin (APC) is followed by a metal isotope-labelled secondary antibody or streptavidin. Intelligent mass cytometry panel design is an iterative process often requiring multiple revisions for optimization.

SAMPLE STAINING

Before sample staining, ensure that all buffers are clean by running these solutions at a 1:10,000 dilution (in ddH_2O or Fluidigm's cell acquisition solution [CAS]) in the machine's "solution" mode. This process is further elaborated in "*Sample Acquisition & Data Output*". Contaminants including barium (Ba) from dish soap used in labware cleaning; or lead (Pb), mercury (Hg) and tin (Sn) from water pipes/distilled water are a common challenge in mass cytometry (**Figure 2A**) (36). An abundance of Ba contacting the detector also damages the detector over time and decreases detector lifespan (51). When working with patient samples, certain therapeutic reagents (cisplatin in cancer chemotherapy, gold in autoimmune therapies), or contrast reagents (Iodine, Ba) can circulate in the patient and contaminate the tissue under study, thus confounding the data (**Figure 2A**) (54, 119). Alternative intercalators are available, including rhodium-103 (^{103}Rh) (119).

Samples often require pre-processing prior to staining. For PBMC, the anticoagulant used in the blood collection can affect specific cell types and thereby adversely impact the data (120, 121). It is important to optimize tissue digestion protocols to minimize cell debris (reduces staining quality) and maximize viability. Multiple published protocols exist for a variety of human and mouse tissues, including tumors (43, 57, 61, 122–130). In general, when attempting to enrich cells, protocols should leave the cells of interest unmanipulated (e.g., negative selection procedures for magnetic bead separation or fluorescence-activated cell sorting/FACS). Heavy metals in magnetic beads can interfere with the mass cytometer, so careful washing post-enrichment is required (131). Immune cells from digested tissues can be enriched without antibodies or magnetic beads *via* density gradient isolations using agents such as Percoll® (Cytivia, Marlborough, MA), Ficoll® (Cytivia, Marlborough, MA), and Lympholyte® (Cedarlane, Burlington, LC). Density gradients, can cause differential loss reduced numbers of certain cell types (for example, granulocytes after Ficoll®gradient isolations) (132).

When working with cryopreserved samples, the effect of freezing and thawing on target epitopes needs to be tested by conventional flow cytometry prior to initiating a mass cytometry experiment. Freezing can greatly alter surface expression of certain surface antigens (CD62L or PD-1) and cytokines, due to down-regulation under cellular stress (133–135). This can be mitigated by maintaining the samples overnight in cell culture media to allow them to equilibrate following thaw (136). For immune cells, a common media is RPMI with 10% heat-inactivated fetal bovine serum (ΔFBS). To measure cytokine expression potential, cells can be stimulated with phorbol myristate acetate (PMA) (an activator of NF-κb) and ionomycin. To block secretion and thus loss of intracellular signal, at the end of stimulation (2 hours minimum, maximum overnight), treatment with brefeldin A (an inhibitor of protein transport between the endoplasmic reticulum ad the Golgi apparatus) or monensin (an inhibitor of trans-Golgi transport) is required (137–139). Cryopreservation can also differentially affect the relative frequencies of viable subpopulations upon rethaw, and this needs to be optimized to minimize selection bias (140).

When a studying phospho-proteins, because phosphorylation and dephosphorylation is a very rapid and dynamic process, capture of the phospho-protein may require exploratory conventional flow cytometry studies to codify optimal conditions and timing. Samples are often fixed prior to staining, a procedure which must be optimized to minimize selection bias (141–143). It is critical that all antibodies in the panel be confirmed to bind target after fixation, due to epitope denaturation by fixation (144). Occasionally, a new antibody clone must be titrated to replace the clone no longer functional post-fixation. Another approach is to forego sample fixation entirely, and block dephosphorylation by pervanadate incubation for 5 minutes prior to staining (145, 146).

Because mass cytometry target detection relies on antibody Fab' - target interaction, constant fragment (Fc) receptors need to be blocked prior to antibody staining in order to reduce false positive signal from antibody binding *via* their Fc region (as in conventional flow cytometry) (147). If, however, CD16 (148–153) and CD32 (154, 155) are critical targets, other options include 1) non-specific protein saturation with extra serum or BSA or 2) staining with anti-CD16 and anti-CD32 antibodies in a separate first step, prior to the staining with the remainder of the antibody panel. (The latter approach also leverages competitive inhibition by using the detecting antibodies for simultaneous Fab'-mediated CD16 and CD32 detection and blockade).

Unlike conventional flow cytometry, in which antibody staining is performed at 4°C, mass cytometry staining can be performed at room temperature as internalization of antigens does not alter detection. There are however specific situations in which staining at 4°C is advisable, such as for myeloid populations that adhere to plastic if metabolically active above 4°C or to better preserve cell activation and viability prior to specific functional assays.

After Fc blockade and live sample barcoding, every staining begins with a live/dead cell discernment step using cisplatin. Cisplatin quickly and freely diffuses into dead cells with compromised membranes and forms covalent sulfhydryl bonds with intracellular protein nucleophiles. Cisplatin is commonly applied for live-dead discrimination in mass cytometry because it a) binds covalently to cellular proteins within cells and b) stains cell membranes of viability-compromised cells to a much greater extent than live cells (54). The first property allows cisplatin to remain bound through multiple downstream staining steps used in mass cytometry protocols, and the second property is leveraged for live-dead cell discrimination. Of note, cisplatin needs to be titrated on the sample as diffusion efficiencies vary by tissue (156). This is followed by surface staining (36, 157), after which many parts can be added into staining protocols, such as intracellular (36, 158, 159), intranuclear (160, 161), phospho-staining (141–143), or tetramers (160).

A cryopreservation method has been developed for long-term (9 months) storage of antibody mixes. Use of such a method is

highly advisable in large mass cytometry studies with multiple staining cycles to enhance staining reproducibility and data consistency across time (162). The general order of staining steps used by our group is summarized in **Figure 3**. For phospho-protein studies, a dephosphorylation block with pervanadate is inserted at the end of step III (**Figure 3**). The cells are fixed with cold methanol after step IX (intracellular/intranuclear staining) before proceeding to the phospho-staining (step XI) and the subsequent DNA intercalator (step XII) (163). Tetramer staining (e.g., MHC or CD1 tetramers to identify cells with unique TCR specificities) can be incorporated into surface staining (160). Multiple wash steps are incorporated after each staining step to thoroughly remove contamination from unbound reagents and minimize background. As already mentioned, in mass cytometry cells are separated from debris by addition of a DNA intercalator staining incorporated at the end of all staining steps (step XII, **Figure 3**). Natural abundance Iridium (^{191}Ir and ^{193}Ir) will bind to nucleic acid after cell membranes are permeabilized and the detection of both Ir isotopes allows single cells to be distinguished from debris and doublets (see *Manual Gating*) (7, 164).

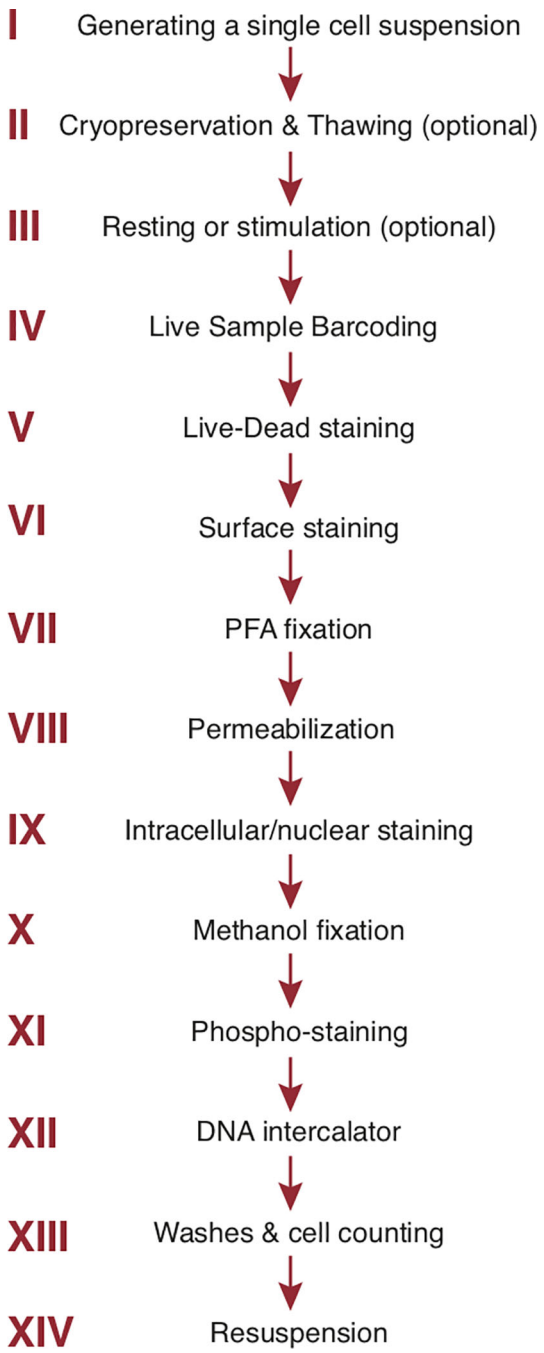

FIGURE 3 | Step-by-step sample preparation. Representative sample preparation protocol. The order of individual steps may vary depending upon the experimental design (see text). Sample washes with cell staining buffer separate each step. The final 3 steps are specific to mass cytometry. *PFA*, paraformaldehyde.

Samples are finally washed once with cell staining buffer and counted. Cell numbers need to be kept consistent throughout the experiment and samples need to be resuspended at the optimal cell concentration for sample acquisition, improving data quality and minimizing doublets. The optimal cell concentration depends upon the specific mass cytometry instrument model and its injector type. Each injector has an optimal and maximum event rate, and event rates higher than the maximum indicated for that injector will drastically increase doublets in the resulting data. After any paraformaldehyde (PFA) fixation step, sample staining can be conveniently paused and resumed later by simply storing the sample overnight in 1.6-2% PFA at 4°C. Unlike the case with denaturation of certain conventional fluorophores, metal isotopes are not affected by prolonged fixation steps. If performing phospho-staining, a pause stop can be incorporated with 100% methanol at –80°C. When opting for completing the entire staining protocol in one workday and acquiring the samples on CyTOF® on a subsequent day, samples can be stored in DNA intercalator (e.g. ^{191}Ir/^{193}Ir cocktail) overnight at 4°C. When storage needs to occur longer than overnight at either of these 2 steps, samples can be washed once with 1 mL cell staining buffer and pellets stored up to one week (165). If stained samples need to be stored longer, *Sumatoh* et al. have developed a method to preserve them in 10% dimethylsulfoxide (DMSO) + 90% FBS at –80°C (165). This long-term storage method was tested on human PBMCs only and therefore may not be applicable for other types of tissues. Our group has confirmed that this protocol also works well for long-term storage of stained mouse splenocytes (*data not shown*).

SAMPLE ACQUISITION & DATA OUTPUT

Sample acquisition on a CyTOF® mass cytometer is performed in one of 2 modes: 1) solution mode or 2) event mode for beads and single-cell analyses. Solution mode is used for testing buffers for contaminants, verifying antibody conjugations, or re-testing older conjugated antibodies (> 6 months). A buffer or conjugated antibody is diluted 10,000 times into either ddH_2O or CAS and acquired in solution mode. The data output of this type of

acquisition includes a mass spectrum showing each element detected as a peak, accompanied by average ion counts. This method does not give the exact number of metal ions conjugated to each antibody. To generate such data, conjugated antibodies must be diluted in Tuning Solution SKU 201072® (Fluidigm, San Francisco, CA), which contains 6 metal isotopes at known concentrations and is used for daily calibration of the instrument. Fluidigm provides users with the necessary protocols and worksheets upon request.

Immediately prior to data acquisition, at least 2 washes with ddH_2O are required to remove residual buffer salts; these otherwise accumulate at the detector and can cause detrimental instrument drift. Tuning, the process by which the CyTOF® instrument is calibrated before any sample acquisition, maximizes the signal intensity of the metal isotopes within the optimum range from atomic mass 153-176 while minimizing isotope oxide formation (M+16) by the inductively coupled plasma (ICP). The acquisition instrument requires re-tuning every 6 hours during prolonged data acquisition. For more detail on machine tuning and sample acquisition, we refer the reader to helpful Fluidigm machine manuals and a video by Leipold and Maecker (51, 166, 167).

The fluid tubing of a mass cytometer is of much smaller diameter than that of conventional flow cytometers (51). This makes the CyTOF® relatively more prone to obstruction by accumulated debris. For this reason, samples need to be passed over a double 30 mm cell strainer (Partec North America Inc., Swedesboro, NJ) immediately prior to sample acquisition. Even following filtration, there remains a periodic need to unclog the lines during sample runs. Our lab routinely uses BD FACSAria® 50 mm sample inline filters (BD Biosciences, San Jose, CA), which perfectly fit the sample probe line of the Helios®.

When using sample barcoding, updating the CyTOF® software with separate labels for all barcodes is strongly recommended throughout the sample run [e.g., for CD45 live barcoding, rather than a uniform label of "CD45", it is preferable to use "CD45_102" (isotope mass), "CD45_healthy" (experimental group), or "CD45_tumor" (tissue type)]. The importance of this labeling schema is clarified in "*Debarcoding*".

Importantly, when samples sit in ddH_2O or CAS, staining intensity and quality declines over time (168, 169) Therefore, samples should be resuspended in these media only immediately prior to data acquisition. Whether ddH_2O or CAS is the preferred solution for acquisition depends on both the CyTOF® model and injector type associated with that model. For the ball joint injector on CyTOF2® and the narrower HT injector on early Helios® machines [the narrow bore injector (NB)], cell pellets must be resuspended in ddH_2O. The Helios® injector has a narrower inner diameter, resulting in smaller ion clouds (roughly one half the size of those attained with wide-bore injectors), reducing doublets and doubling the machine's event acquisition rate (cells/second) (51). However, unintended consequences to the data such as lower median signal intensities and higher coefficients of variation (CVs) have been described using these injectors (170). The Helios® system introduced the wide-bore (WB) injector and CAS to address this pitfall. The WB injector has an inner diameter intermediate between the (narrower) HT injector and the (wider) CyTOF® injector. The CAS has a higher ionic content than water and in combination with the newer WB injector resolves the data quality issues seen with narrow-bore injectors in Helios® datasets (170). One disadvantage of the newer WB injector, however, is that it drastically decreases the maximum event rate from 500 event/sec to 250 events/sec, requiring significantly longer sample acquisition/run times.

During mass cytometry runs, signal drift occurs over time due to gradual accretion of cellular material inside the instrument and associated progressive acquisition delays (51). Signal drift can cause variations between and even within individual data files. Since the sample acquisition speed on a CyTOF® is quite low compared to conventional flow cytometers, experiments are often run across multiple days. The instrument is tuned each day and may be cleaned periodically within a prolonged experiment run, causing additional variance between days. Additionally, in consortia or clinical trials, data is collected at multiple sites and on multiple instruments. To correct for these variances and minimize measurement variations, samples are resuspended in EQ Four Element Calibration Beads® (EQ 4 beads) (Fluidigm, San Francisco). EQ4 beads are polystyrene bead standards containing known relative quantities of metal isotopes from 4 metals (^{140}Ce, ^{151}Eu, ^{153}Eu, ^{165}Ho and ^{175}Lu), diluted 10-fold in either ddH_2O or CAS and used for normalizing data within and across experiments (see *Normalization & Concatenation*) (171, 172). This method for minimizing experimental variation is efficient enough that data acquired from different machines can be combined into cumulative datasets. This is important, since different machines have been shown to have discrepancies in their atomic mass sensitivity ranges (173, 174). Recently, Liu et al. further improved the isotopic range of EQ 4 beads by adding 3 elements (^{89}Y, ^{115}In, and ^{209}Bi), resulting in a total of 7 isotopes. The authors demonstrated that the EQ4 beads did not consistently normalize isotope signals outside the mass range of 140-175, whereas the 7-element calibration bead system resolved this issue (175). As of the time of this manuscript publication, 7-element beads are not available for purchase and interested parties must synthesize these themselves.

The output data from the machine is in the form of a Flow Cytometry Standard (.fcs) file (FCS file). The FCS file structure is a standardized array with columns representing channels and rows representing events. This is used for downstream data analysis through programming languages (e.g., R) or FCS file-processing platforms [e.g., FCS Express® (DeNovo Software, Pasadena, CA), Cytobank® (Cytobank Inc, Santa Clara, CA), or FlowJo® (FlowJo LLC, Ashland, OR)]. Generally required items are: 1) the generated FCS file; 2) a panel file listing the metals and corresponding markers; and 3) a metadata file listing the names of all FCS files used, categorizing FCS files into experimental groups (e.g., control, knockout, etc.). The panel and metadata files can be in Comma Separated Values file (CSV)

or Microsoft Excel (XSLX) format. Refer to the analysis program/package analysis instructions for guidance in selecting file format.

NORMALIZATION & CONCATENATION

To correct machine signal variation and thereby minimize measurement variation, "global normalization" (normalization across all FCS files) must be performed. Though this normalization is performed computationally after sample acquisition, it requires the use of bead-based standards added to the sample before sample acquisition and therefore merits advance consideration.

The two algorithms for normalization are **1)** MATLAB®-based bead normalization shown by *Finck* et al.(171) and **2)** Fluidigm's bead identification and normalization (172). The primary difference between these is that MATLAB® normalization compares each file to the other files in the same data set, whereas Fluidigm's algorithm compares the acquisition files to a set of external values. One pitfall of the Fluidigm algorithm is that the data may be slightly reshaped to fit the external values used as compared to data normalized by the MATLAB® method (176).

In some circumstances, a single sample must be acquired across multiple FCS files and subsequently recombined into a single data file prior to analysis. Common scenarios necessitating such an approach include: 1) a clog occurs in the sample line, sample capillary or the nebulizer requiring the acquisition to be halted and restarted 2) a particularly large sample requiring collection from multiple tubes (168, 169). The recombining of these files into a single FCS file before analysis is termed concatenation (85). Concatenation should be performed in order of file acquisition, to avoid introducing errors from incorrect sequencing (86).

Because events are normalized independently, the order of normalization and concatenation should not affect data quality. However, many algorithms remove artifacts and metadata during the normalization process, which can cause errors during concatenation. For this reason, unless the sample uses a large number of bead standards (> 200 hundred beads), it is advisable to first concatenate and subsequently normalize.

Several different algorithms exist to aid normalization and concatenation. For the MATLAB® algorithm, R packages such as premessa or CATALYST can be used (87, 88). CATALYST fuses all FCS files into a single cell experiment object in R. Therefore if using CATALYST, concatenation is not required. Fluidigm's algorithmic bead normalization is performed through the instrument's software. (As of this publication, the software version is CyTOF® 6.7). Note that each batch of EQ 4 beads has its own corresponding passport of external values, which must be updated in the CyTOF® software whenever a new batch of EQ 4 beads is used.

Though doublets have historically been considered noise or unwanted confounders, *Burel* et al. found that the molecular signature of T-cells in complex with other immunological cells such as monocytes or B-cells showed phenotypic differences when compared to unattached single T-cells. Moreover, they observed an increase of T-B-cell complexes in the circulation of type-1 diabetes patients (89). The implication of these findings is that removal of doublets may in certain studies skew the data in ways that miss important biological outcomes. However, since most doublets are indeed unwanted noise, it is generally advised to study doublets separately from the singlet data analysis to determine their value to any individual experiment, to avoid compromising experimental rigor. A good QC step to visualize the effect of normalization is to plot the bead intensity over time before and after normalization (**Figure 4**).

DEBARCODING

In barcoding, each sample is labeled with a unique identifier and samples are mixed in a single tube before data acquisition. The output is a single FCS file consisting of the multiplexed data from all of the barcoded samples in the tube. Therefore, an extra step must be taken following data acquisition to split the output data from each barcode into its own FCS file. This process is termed "debarcoding".

In debarcoding, events are stored in new FCS files corresponding to the individual barcodes, identified by the debarcoding algorithm. The number of output FCS files is equal to number of barcoded samples used. Samples are re-assigned a unique identifier in the debarcoding process. During debarcoding, the single-cell deconvolution algorithm must define which channels are positive and negative for each cell. For this, a threshold value is selected, and cells above this threshold value will be assigned to their corresponding barcode sample (90). The threshold can be selected manually or with the help of an algorithm (e.g. CATALYST provides estimation of threshold values). Rarely, high stringency in such parameters can reduce overall event number in the dataset.

The vast majority of unassigned events are cell-cell doublets or debris (33). Occasionally, cells can be sorted into incorrect barcode channels (false assignment). This false assignment rate is generally under 0.5% under uniform sample staining/experimental conditions. A valuable internal control is to leave 1 barcode from the scheme unused, allowing an estimation of the false assignment rate for debarcoding.

Both premessa and CATALYST packages in R can be used to debarcode (88, 91). Debarcoding can also be done using the MATLAB Compiler Runtime (33). Graphical User Interfaces (GUIs) are available for these processes. GUIs are pre-programmed interfaces that run through the code, allowing analysis without directly interacting with the code. Most of these algorithms require a debarcoding scheme alongside the FCS file, typically in the form of a table that correlates the sample identity to the element mass of the barcode isotope. This instructs the algorithm which barcode corresponds to a sample's FCS file. Formatting varies by the debarcoding resource.

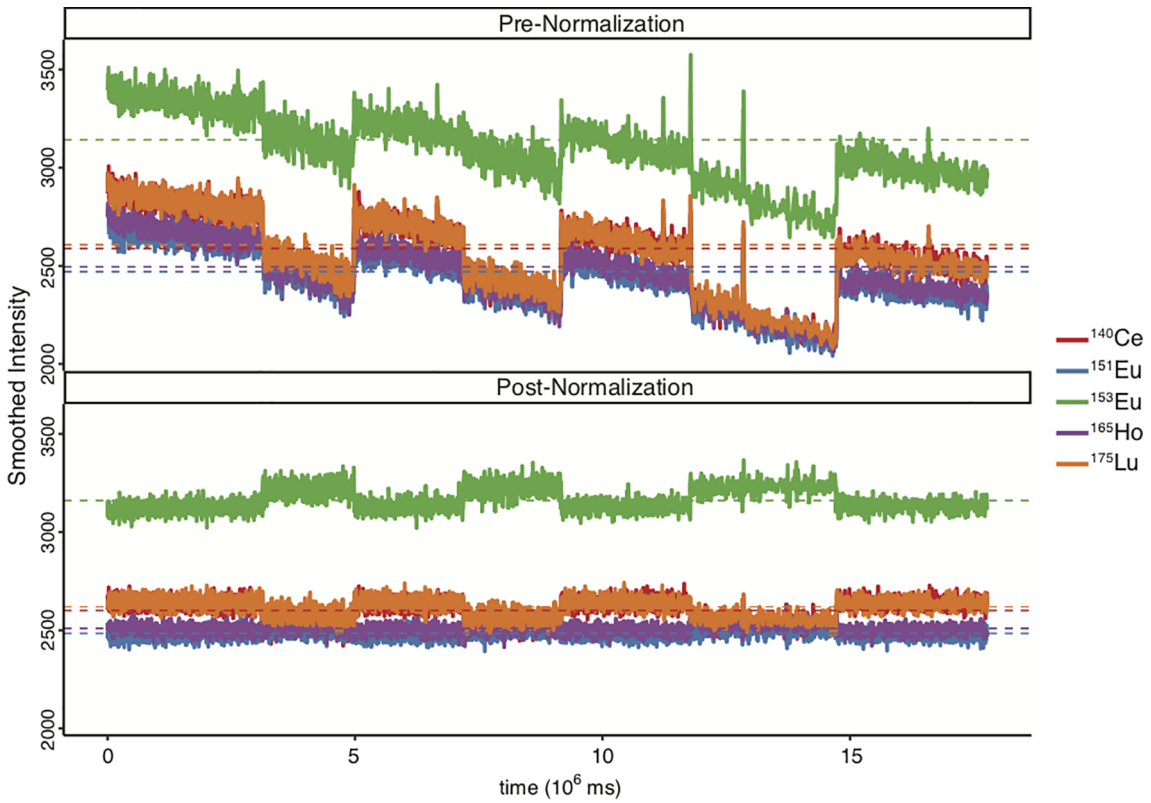

FIGURE 4 | Comparison of bead intensity over time before and after data normalization. *Ce*, Cesium; *Eu*, Europium; *Ho*, Holmium; *Lu*, Lutetium. EQ4 beads were used during sample acquisition. Figure was generated in R *via* the CATALYST package using their standard settings.

COMPENSATION

After normalization, concatenation and debarcoding, compensation is generally the final data pre-processing step in mass cytometry workflow. Compensation is the process whereby detection signal spillover is resolved. In conventional flow cytometry, this is a routine practice due to overlapping excitation and emission spectra of fluorophores and spillover which correlates linearly with fluorophore signal intensity (92). Compensation subtracts the percentage of a fluorophore's spillover from the measured signal in that particular channel.

Mass cytometry can have spillover, but antibody titrations and intelligent panel design can optimize the signal-to-noise ratio such that compensation is no longer necessary (67). *Chevrier* et al. performed a detailed analysis and found that mass cytometry spillover has a similar linear relationship with the primary signal (84). This relationship has been applied to generate a spillover matrix allowing compensation to be performed with the CATALYST package either in R or *via* a web application created by the same group (87, 93). Another helpful algorithm is CytoSpill, a statistical program that aims to minimize spillover effects. Unlike the CATALYST algorithm or conventional flow cytometry analysis programs, this algorithm does not require the use of single-cell controls, making the process significantly cheaper and easier (94, 95).

MANUAL GATING

During the experiment, samples have been put through various stresses which results in the events in the FCS file being a combination of a) live cells, b) dead cells, c) cell-cell doublets, d) beads e) cell-bead doublets and f) bead-bead doublets. The goal of this step is to purge the data as much as possible of b-f, such that the output consists of live single cells. Manual gating can be performed using many established platforms from conventional flow cytometry, such as FlowJo® or FCS Express®. Though usually done *via* manual gating, some algorithmic methods such as that in the CATALYST pipeline can efficiently remove d-f.

In **Figure 5A**, a standard mass cytometry gating strategy is shown. Here, the "event length" parameter is used in conjunction with a DNA intercalator to select single cells, followed by live/dead gating based upon cisplatin intercalation. Though single-cell gating using this method has proven difficult, the Helios® mass cytometer is now equipped with additional Gaussian parameters (e.g. center, offset, width, residual), which can be used to streamline data (96). These parameters are generated in the TOF chamber. The ions enter the TOF chamber *via* a narrow slit (**Figure 5B**). Every 13 μs (exact interval varying by machine), a high-voltage pulse provides equal energy to all ions that have accumulated in the chamber during the interval, accelerating

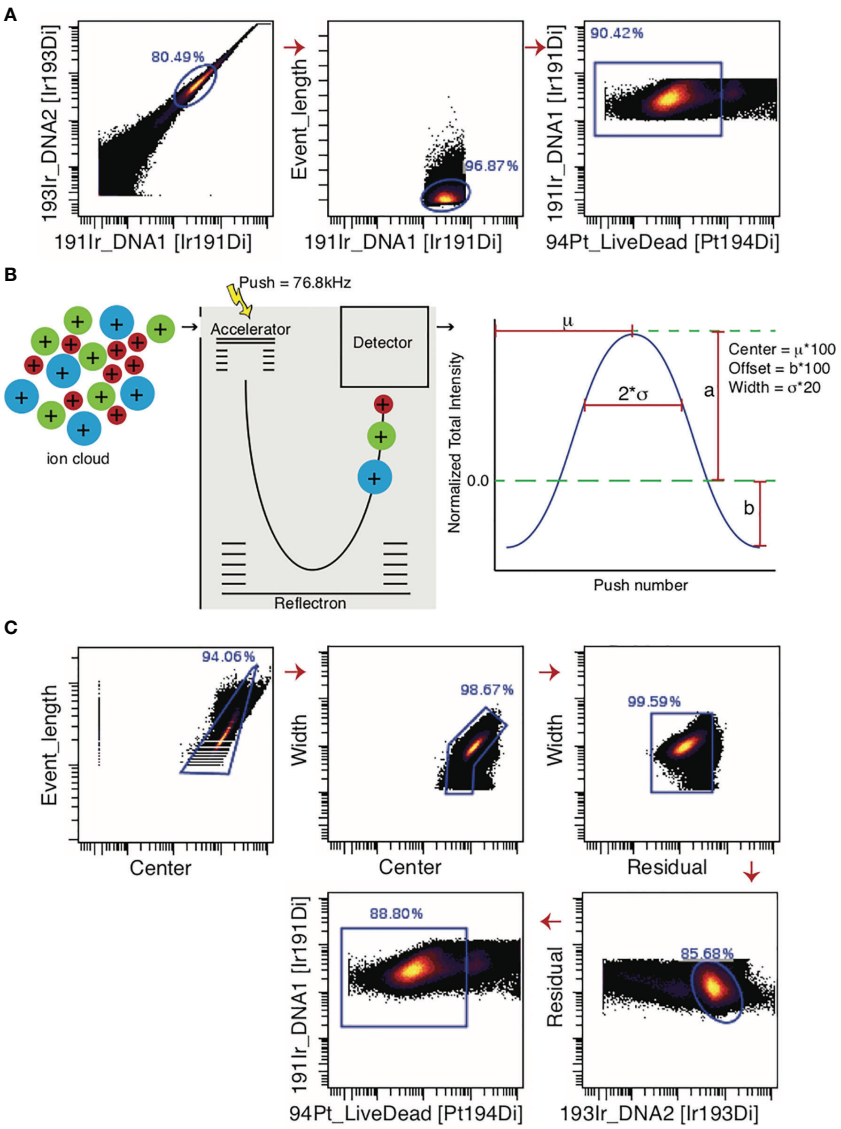

FIGURE 5 | Gating strategies for live single cell events. **(A)** For data generated by the CyTOF2® machine, the predecessor of Helios®, single cells were gated only on the bases of Event Length and the DNA intercalator. Live cells are selected as cisplatin-negative events. **(B)** In the Helios® machine, the Gaussian parameters are generated in the Time-Of-Flight chamber where ions are separated on mass-to-charge ratio. Once these ions encounter the detector, an electronic pulse is generated, which is mathematically smoothened into a bell curve. Center, Offset, and Width can be extracted from this curve. **(C)** The added Gaussian parameters greatly improve the ability to select a single-cell population. Note that if beads are not excluded through the normalization process, they must be gated out manually before gating for single live cells.

them across the TOF chamber and onto the detector. This high-voltage pulse is a push. As the energy provided to the ions is uniform, velocity varies by mass and ions with greater mass require longer times to reach the detector. Once these ions hit the detector, they generate an electronic pulse which when plotted and mathematically smoothened takes the shape of a bell curve (Gaussian distribution) from which the Gaussian discrimination parameters (center, offset, and width) are extracted (**Figure 5B**) (51, 97). The Residual parameter is extracted by calculating the difference between the actual electronic pulse plot and the smoothened bell curve (97). The improved gating strategy is shown in **Figure 5C**.

UNDERSTANDING DATA QUALITY

Once the runs are pre-processed, all events are expected to represent live single cells. Before proceeding, it is worthwhile to visualize the number of cells in each sample. This can be done in R with a simple bar graph visualizing the cell number within

each sample and comparing across samples (**Figure 6A**). If the cell count is insufficient and the population of interest is a very rare or marginal proportion of the total cells (expected to vary by experimental conditions or cell/tissue type), the experiment may have to be rerun. Even if sufficient cells exist in the input samples, if the difference in cell numbers between runs is extremely large, such variability can affect the statistical analysis. In such cases, the cells can be randomly subsampled (randomly selected from the experimental samples based on parameter matching to controls) or the experiment repeated ensuring larger cell counts.

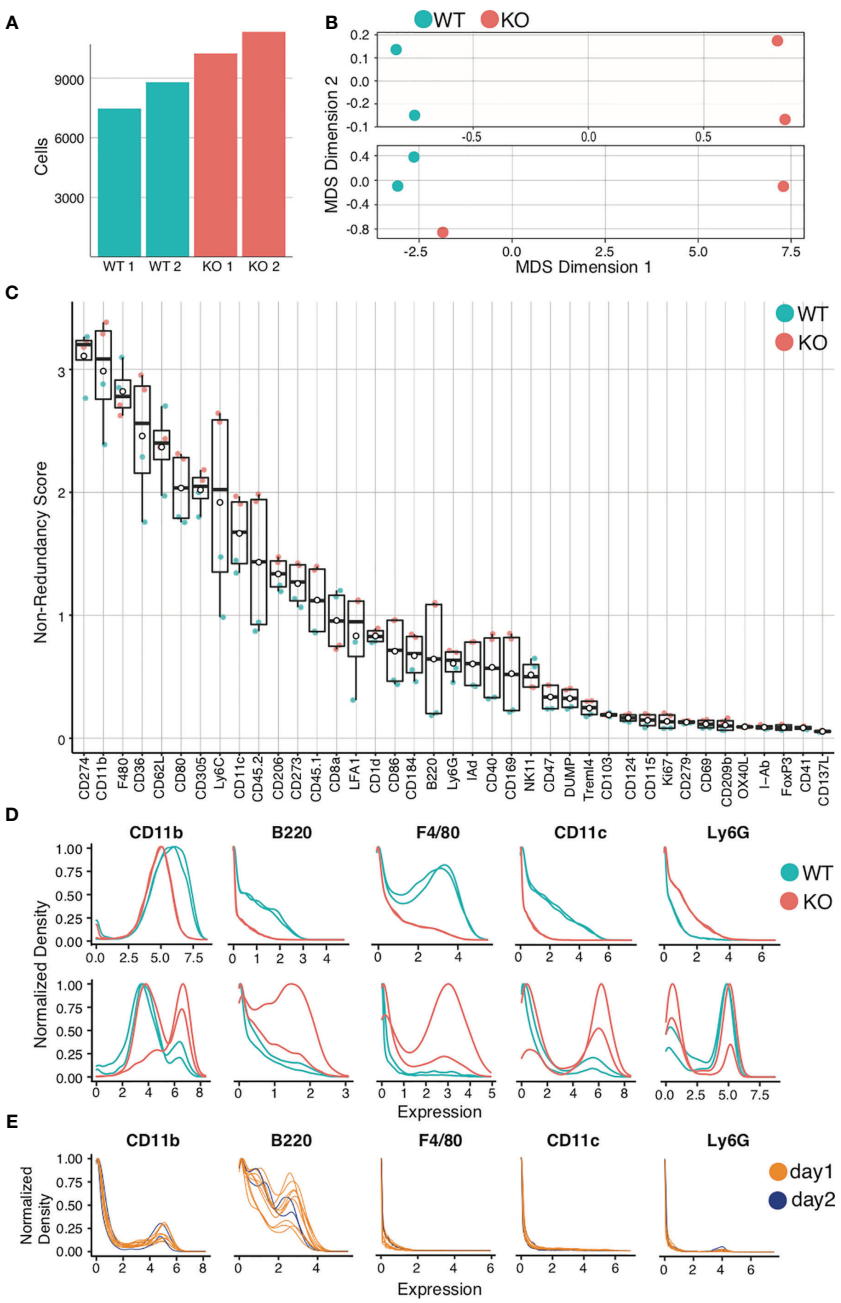

FIGURE 6 | Quality control and marker selection. Mouse bone marrow-derived dendritic cells were generated. Quality control and marker selection was performed on debarcoded/cleaned FCS output after gating to select for live dendritic cell singlets. Figures were generated in R *via* the CATALYST® package using their standard settings. **(A)** Absolute cell number comparisons for a representative analysis using wild-type (WT) and a particular strain of monogenic knockout (KO) mice. **(B)** Multidimensional Scaling (MDS) plots comparing mass cytometry runs from 2 WT and 2 KO samples are shown **(A, C)** Non-Redundancy Score (NRS) plot listing the variation between each sample for each marker in the CyTOF panel. **(D)** Histogram comparing intensity of marker expressions between the samples (Marker Expression Distribution). Due to different sample sizes and distributions, the data was normalized between 0 and 1. **(E)** Histogram demonstrating the use of spiked-in internal control samples across multiple tubes and data acquisition days.

The next step is to visualize relationships between samples. One cumulative data visualization method is Multidimensional Scaling (MDS) (98). In the sample MDS plot showing data for murine bone-marrow derived dendritic cells (BMDCs) (**Figure 6B**, *upper panel*), the controls and comparisons form distinct groups, indicating that wild-type (WT) mice are similar to one another and knockout (KO) mice are similar to one another, but that there is a significant difference between WT and KO mice. In the lower MDS plot (**Figure 6B**, *lower panel*), while the WT are similar and form a distinct group, the KO mice are dissimilar. Furthermore, one KO clusters with the WT controls. This should prompt evaluation for experimental errors requiring re-analysis. Errors can arise from variances in staining, runs on distinct days, and human error, which can be challenging to troubleshoot. For this reason, we recommend adding a barcoded internal control in each tube as a quality control ("spiking"). Errors can be identified by variation of internal controls from expected outcomes.

After critically assessing cell count and sample quality, one may proceed to analyze the specific markers for the experiment, beginning with a Non-Redundancy Score plot (NRS plot) (**Figure 6C**) (99). This graph visually represents the variation across all samples by individual marker. Ideally, the marker variation is caused by the biologic differences between experimental groups. However, large variations in cell count or in staining intensity can also be sources of variation on the NRS plot. Markers that contribute minimal or no variation between samples can be excluded from clustering. To view the marker expression in more detail, a histogram can be used to visualize the results (**Figure 6D**). Marker expression histograms are extremely useful when plotting an internal control sample spiked into a tube (by barcoding). This provides a quality control for staining reproducibility. In **Figure 6E**, the internal controls are color-coded by day of staining, demonstrating that reproducibility was not optimal.

DATA TRANSFORMATION

After bead normalization, data must be transformed to allow proper distinction between cell populations positive and negative for each marker. The most common method is the arcsinh transformation, using a cofactor of 5 (99). In R, CATALYST packages have functions that can perform this transformation (99, 100). This transformation can also be accomplished directly in Cytobank® or FlowJo®; however, this is lost when exporting.

CLUSTERING

The next step is data clustering based on group similarities between cells. This data visualization method compartmentalizes events (cells) into groups which are then used to insure the detection, characterization, and calculation of relative abundance of the different populations in the sample. Note that not all clusters/groups represent a cell population, as some clusters might represent the same population with slightly differing marker expression. For this reason, it is important to carefully study and compare the marker expression within each cluster. Several different clustering algorithms are available for mass cytometry data, differing primarily based on the criteria they use to assign a cluster designation. The highest performing algorithms are FlowSOM, X-Shift and PhenoGraph (101). If specifically looking for rare populations, X-Shift is preferable due to its statistical power to detect rare events (102).

While the algorithms themselves use a variety of models to cluster the data, they utilize the marker expression within each individual cell to distinguish the clusters based on phenotype. Therefore, it is important to determine in advance which cells to isolate and the potential cell types present in the samples, and design the experimental panel accordingly. The more distinguishing markers in the panel (markers that can be used to show a clear and meaningful difference in the subpopulations in the sample), the better the quality of the clustering will be to allow clear differentiation and ease of population identification (103). As explained in detail in the previous section (*Understanding Data Quality*), an NRS plot (**Figure 6C**) is utilized in order to select the markers most relevant for clustering; meaning markers with a low non-redundancy score will not contribute to the cluster outcome and can therefore be excluded from clustering analysis. The clustering algorithms use the data on event phenotype (using the markers selected by the analyst) to group events into clusters or "bins". Clustering is generally performed over all FCS files to allow sample/file comparability in downstream analyses. R or Python are the recommended clustering programs, as these allow much greater control over the statistical binning process. Packages such as CATALYST or Phenograph can be used, the details of which can be found in their respective workflows (99, 104). Note that apparently different clustering packages or software might ultimately utilize similar algorithms. For example, CATALYST uses the FlowSOM algorithm for clustering (105, 106). Thus, it is important to study in advance the workflow being used and identify which algorithms may best suit the output data, well in advance of the experimental run.

While some algorithms such as PhenoGraph will automatically select the optimum number of clusters to use, others like FlowSOM will require manually selection of the cluster number (k) (104, 105). One method for manual selection is initial generation of a large number of clusters (~100), followed by serial merging of particular clusters until the optimum cluster number is obtained. Again, not every cluster represents a subpopulation in the sample, and it is here that applying biological understanding is important. Whether these clusters represent a novel/separate subpopulation (creating a new cluster) or are similar to each other and therefore will need to be merged must be determined based on the marker expression levels on the different clusters. All clusters that represent a will be continually merged until only clusters that are determined to be biologically different from each other remain. The same process can be performed statistically with the help of a delta plot, where the relative change under the Cumulative Distribution Function (CDF) is plotted for each cluster value. It is no longer optimal to increase the number of clusters selected (k) when doing so results

in no/minimal change in the CDF (**Figure 7A**). A delta plot is generated using either the 'deltaPlotR' Package or the CATALYST package (107, 108). When clustering initiates, a randomly generated 'seed value' is used as the starting point of the clusters. To ensure that clustering is reproducible, one should record the seed value used to initiate clustering (177). To ensure that clustering is robust and to avoid the possibility of error due to random selection of seed value, clustering should be repeated with at least 3 random seed values and output clusters compared. These should approximate each other closely.

A good QC measure to ensure clustering has been correctly performed is to include markers for a known control cell population within the sample. When opting for internal controls, clustering could be performed on the FCS files separately in order to verify similar cluster proportions between tubes.

VISUALIZATION AND INTERPRETATION

The next step is to determine whether clusters represent distinct populations and to phenotype each cluster based on statistical analysis and graphical outputs. A combination of biological and statistical expertise become highly relevant at this stage. R packages like FlowSOM, FlowCore and CATALYST offer several different visualization functions. The most common visualization approaches are heatmaps. As seen in **Figure 7B**, the expression for each marker is aggregated by cluster. These clusters were generated from FCS files and therefore can be applied to any group used in the analysis (in this case, WT murine controls and knockouts). Heatmaps not only help to visualize markers expressed in different clusters on the merged FCS files, but also to estimate the relative expression intensity. Marker expression levels determine to which immune lineages these clusters may belong, elucidate novel cell clusters or phenotypes, and can aid in determining whether certain clusters can be merged. The heatmap parameters can be adjusted depending on use case (e.g., comparing a particular marker's expression across groups rather than comparing prevalence of clusters, etc) (178, 179).

Mass cytometry data is of necessity multi-dimensional. Clusters, marker intensity, and differing markers complicate interpretation. To address this, dimensionality reduction algorithms can be applied to concisely visualize multiparametric data in two dimensions. t-distributed Stochastic Neighbor Embedding (tSNE), Principal Component Analysis (PCA), Isometric Feature Mapping (Isomap), and Uniform Manifold Approximation and Projection (UMAP) are all common methods for high-dimensional data visualization (99, 180–183). These facilitate easy identification of the experimental group differences by visually organizing populations using distance on the plot as a surrogate for similarity between groups. The most commonly used approaches are tSNE and UMAP. UMAP, unlike tSNE, is primarily non-parametric nonlinear dimensionality reduction algorithm originally designed to preserve global data structure with smaller data sets, though more recently parametric UMAP was demonstrated to compare favorably in performance to its on-parametric counterpart while adding the benefit of learned mapping of new data. In UMAP, distances both within and between clusters are generally meaningful; whereas in tSNE, distances between clusters may not be. Therefore, UMAP is the preferred mode of visualization (181, 184). In **Figures 7C, D**, a case is presented in which both tSNE and UMAP efficiently visualize the cell clusters present in the WT control and KO mouse groups. Figures have been generated in R *via* the CATALYST package using their standard settings. One can also use Flow Core in R, or directly generate such visualizations through Cytobank.

Once clusters have been visualized and characterized, they can be annotated in R. Clustering can require hours to days to complete depending on the dimensionality reduction algorithms and the acquired total cell count per group. To prevent prolonged run times, these algorithms are ideally run on a subsample of the total population (~2000 cells). When looking for a particularly rare population, more cells can be selected for algorithm application. Because subsampling is random, a set seed function in R is used to ensure reproducibility. To ensure clusters are not lost in the sample and that subsamples are representative of the total sample, at least 3 iterations should be performed with 3 different seed values to confirm similar results (185).

Another method to visualize the relationship between clusters is the diffusion map. Clusters are arranged in order of overall similarity, creating the appearance of a gradient based on change in marker intensity per analyzed event. Diffusion maps are commonly used to study the differentiation or lineage origin of individual cells in a sample set. Another useful application of this visualization technique is for groups of cells of the same type, taken at different time points in treatment or maturation of a sample (186). When viewing diffusion maps, it is critical to remember that association does not imply causality. Viewing 2 adjacent clusters in a diffusion map indicates that they are the most similar in the sample, but not necessarily that one has differentiated from the other. Data analytics must be tempered with biological understanding of the cell type. An algorithm commonly used for this type of analysis is Wanderlust (187). Wanderlust assumes a linear trajectory and has significant limitations with complex datasets or in the case of multiple cell fates. For those datasets with potential bifurcating branches, Wishbone or Monocle 2 can be applied (188, 189).

There are several other resources that can be applied in place of or in addition to R coding such as FlowJo®, FCSExpress®, and Cytobank® (190–192). Several R scripts also offer GUIs making it significantly easier to analyze such data (e.g., Cytofkit package for Phenograph). However, R allows the maximum ability to fine-tune parameters, making it extremely attractive as an analysis platform (193). Finally, Astrolabe (Astrolabe Diagnostics, Fort Lee, NJ) offers an interactive platform in which the researchers can interact directly with a computational biologist to analyze data (194). Recently, automatic annotation algorithms have started to be developed [e.g., Automated Cell-type Discovery and Classification (ACDC)], which allows users to input

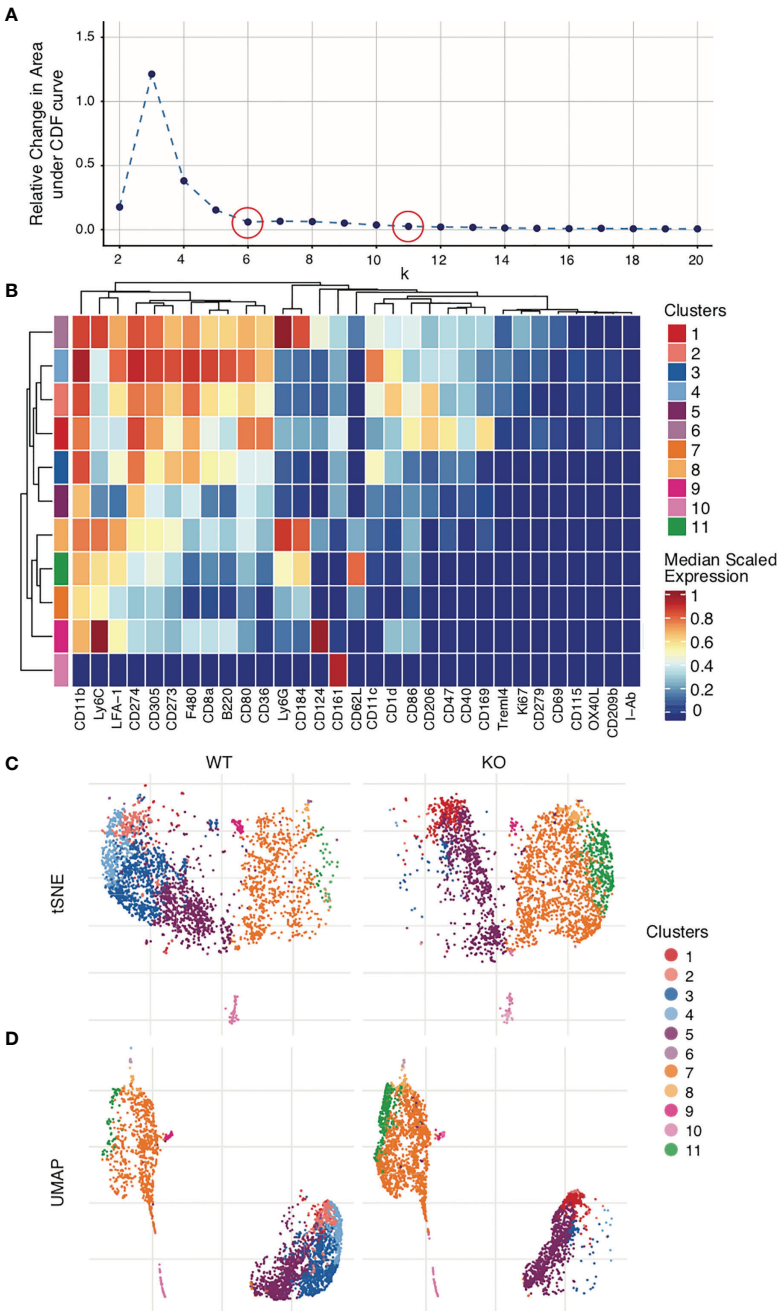

FIGURE 7 | Clustering and visualization. Mouse bone marrow-derived dendritic cells were generated. Clustering was performed on live dendritic cell singlets after Quality control and marker selection. Figures were generated in R *via* the CATALYST package using their standard settings. **(A)** Deltaplot showing relative change under the Cumulative Distribution Function (CDF) when clustering is performed using different cluster numbers. The optimal cluster number is indicated at the threshold below which a change in cluster number no longer correlates to a change under the CDF (plateau point in the graph). *red circles*, *selected cluster values.* **(B)** Clusters have been created across all FCS files. Expression levels for each marker are normalized across all clusters. The heatmap shows the normalized expression levels of each marker in each cluster in the total data set. The upper legend on the right of the heatmap indicates the clusters, coded by color to the corresponding row indicated on the far left of the heat map. The number of clusters used in the analysis was selected by deltaplot **(A)**. Markers were selected based on the NRS plot (**Figure 6C**). (C) tSNE comparison of WT vs KO samples, demonstrating clusters 3 and 4 severely diminished and cluster 11 greatly increased in KO as compared to WT. **(D)** UMAP comparison of WT vs KO.

biological parameters such as marker expression levels and apply this to identify such populations within the sample (195).

LIMITATIONS

Currently, the sensitivity of metal isotope-tagged antibodies is lower than that of the most quantum-efficient fluorophores, such as phycoerythrin (PE). The main reason for this is that chelating polymers used for antibody conjugation can only accommodate a maximum of 100 metal ions, creating a ceiling on signal intensities and making it more difficult to measure extremely weakly expressed markers (20, 21). Another major drawback of mass cytometry is the much lower acquisition flow rate as compared to flow cytometry. This is due to the dynamics in the TOF chamber, resulting in longer acquisition times. Where conventional flow cytometers can have a flow rate of up to 50,000 events/second, CyTOF2® only has a flow rate of 500 events/second (250 events/second for Helios®) (167, 196). Despite these low flow rates, mass cytometry machines are also quite prone to clogging due to the small diameters of the sample lines and nebulizer. The latter is easily resolved by passing samples through a cell strainer both before and during sample acquisition as explained in "*Sample Acquisition & Data Output*". The latest CyTOF XT® machine from Fluidigm is capable of sensing and removing clogs automatically. In addition, sample preparation and staining require extra caution with regards to possible heavy metal contaminants such as barium from dish soap or lead from distilled water. Another limitation is the high cost of metal-tagged antibodies, conjugation kits, and other reagents. Although the costs are significantly higher as compared to flow cytometry, these are still much lower than for scRNAseq (21). With increasing number of users and advancements in reagents and technology, these costs are expected to decrease. Finally, because cells are vaporized during the analysis, sorting populations of interest is not currently possible with mass cytometry. To address the latter, one can sort populations on a fluorescent-activated cell sorter for follow-up studies.

The increased number of parameters made available by mass cytometry complicates data analysis, requiring improved bioinformatics approaches for accurate interpretation and visualization of fcs files. Data analytics requires a combination of biological, statistical, and programming knowledge, making the overall process tedious for single individuals. Though packages like ParkerICI/premessa and CATALYST in R are designed to make analysis significantly more user-friendly (88, 93), without significant R programming skills, the researcher remains largely restricted to package workflow and graphing programs. More recently new options have arisen including GUIs and web interfaces that allow analysis without necessitating R programming skills. Most notable among these is Cytobank® (192).

Another potential limitation during manual cluster analysis is the potential for operator bias. This can be significantly mitigated by automating cluster identification through algorithms such as CITRUS (Cluster Identification, Characterization, and Regression) (197, 198). CITRUS automatically clusters cells in each experimental group and compares them based on inputted biological parameters as well as data from publicly available datasets.

FUTURE DIRECTIONS

Despite rapid technological advancements and vast capacities, mass cytometry has many areas ripe for future development. At the instrument level, increasing the flow rate (reducing time of analysis) without losing data quality or altering machine sensitivity are key needs. The newest mass spectrometers, have a more sensitive detector and/or improved TOF chambers than prior Helios® mass cytometers (51, 199, 200). Novel polymer chelators able to accommodate more than 100 metal ions at a time could increase signal intensities significantly and thus empower investigations of very weakly expressed molecules (tertiary antigens). Additional isotopes and new methods to purify existing isotopes at a requisite (>98%) purity and novel conjugation chemistries will allow mass cytometry to approach the theoretical 120-parameter capabilities of the Helios® (**Figure 2A**). With the recent development of compensation tools, stringency requirements for metal isotope purity may be relaxed (84, 95). By merging multiple mass cytometry panels into a single clustering analysis, the computational tool CyTOFmerge is greatly expanding the number of simultaneously analyzable parameters (201).

Annotation is one of the most difficult portions of any exploratory mass cytometry analysis. It requires a depth of biological knowledge regarding the unknown cell population, which the researcher may lack at the time of the experiment. One solution is to compare the experimental dataset to available reference datasets. This approach is seen in techniques such as Seurat, a pipeline for scRNAseq. These reference maps provide information about cellular phenotype and interactions, creating a framework to compare existing data with unknown cell populations to aid in their identification and classification. Scaffold is another example of a system for immune cells, built on flow and mass cytometry data sets. Unlike for genomic data, such reference maps are scarce for mass cytometry and represent invaluable resources for future development (202).

Another much-needed application in mass cytometry is a method similar to pseudotemporal ordering used in scRNAseq. This would support immune lineage differentiation studies, allowing clusters originating from common lineages to be identified and mapped. However, distinct from pseudotemporal ordering in scRNAseq, which uses transcription data for its analysis, this would require the use of lineage markers and other characteristic surface proteins (203).

Ultimately, the greatest limitation to more widespread application of mass cytometry is the difficulty in post-acquisition data analysis and interpretation. Automatic screening and

comparison of mass cytometry output variables through databases and existing publications represents a major step forward in understanding and annotating cells. Artificial Intelligence (AI) systems are likely to become standards in the near future to make increasingly accurate predictions and annotations based on the ever-increasing available data (204, 205). Automating the entire pipeline into a one-click analysis, with selectable output visualizations and analysis factors, will make the technique more accessible for wider applications of mass cytometry in such key expanding areas as TME responses and immunotherapies.

AUTHOR CONTRIBUTIONS

AI, AH, and AP performed the literature compilation, writing, editing, and proofreading. All authors contributed to the article and approved the submitted version.

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Next Generation Natural Killer Cells for Cancer Immunotherapy

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In recent years, immunotherapy for cancer has become mainstream with several products now authorized for therapeutic use in the clinic and are becoming the standard of care for some malignancies. Chimeric antigen receptor (CAR)-T cell therapies have demonstrated substantial efficacy for the treatment of hematological malignancies; however, they are complex and currently expensive to manufacture, and they can generate life-threatening adverse events such as cytokine release syndrome (CRS). The limitations of current CAR-T cells therapies have spurred an interest in alternative immunotherapy approaches with safer risk profiles and with less restrictive manufacturing constraints. Natural killer (NK) cells are a population of immune effector cells with potent anti-viral and anti-tumor activity; they have the capacity to swiftly recognize and kill cancer cells without the need of prior stimulation. Although NK cells are naturally equipped with cytotoxic potential, a growing body of evidence shows the added benefit of engineering them to better target tumor cells, persist longer in the host, and be fitter to resist the hostile tumor microenvironment (TME). NK-cell-based immunotherapies allow for the development of allogeneic off-the-shelf products, which have the potential to be less expensive and readily available for patients in need. In this review, we will focus on the advances in the development of engineering of NK cells for cancer immunotherapy. We will discuss the sourcing of NK cells, the technologies available to engineer NK cells, current clinical trials utilizing engineered NK cells, advances on the engineering of receptors adapted for NK cells, and stealth approaches to avoid recipient immune responses. We will conclude with comments regarding the next generation of NK cell products, i.e., armored NK cells with enhanced functionality, fitness, tumor-infiltration potential, and with the ability to overcome tumor heterogeneity and immune evasion.

Keywords: NK cell, iPSC (induced pluripotent stem cell), CAR (chimeric antigen receptor), engineering, stealth

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1 INTRODUCTION

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States, after cardiovascular disease (1, 2). Although a reduction in smoking and improvements in early detection and treatment have lowered the death rate of certain malignancies, such as non-small cell lung cancer (NSCLC), the overall survival of patients with late-stage malignancies remains poor—substantiating the need for novel therapeutic options.

Treatment regimens for cancer have long consisted of surgery, radiation, and chemotherapy; but recently, a new approach has been on the rise: cancer immunotherapy.

Inspired by the natural capacity of our immune system to recognize and prevent tumor progression, *via* the concerted action of an array of immune cells and humoral factors, multiple immunotherapies have been developed (3). Early clinical studies of the therapeutic potential of stimulating T cells in cancer patients evidenced that administration of interleukin-2 (IL-2) can lead to durable tumor regressions in advanced cancers (4). Approval of IL-2 treatment for metastatic melanoma and renal cancer by the US Federal and Drug Administration (FDA) kickstarted a new era of cancer therapy and brought the immunotherapy in the spotlight (4, 5). The immuno-therapeutic arsenal for cancer continued to expand with the approval of immune checkpoint inhibitors (ICIs), which are directed against inhibitory molecules expressed on the surface of T cells or antigen-presenting cells (APCs) such as PD-1 and PD-L1, respectively, in several indications (6). More recently, chimeric antigen receptor (CAR)-T cells have shown therapeutic efficacy in treating refractory hematological malignancies, and several CAR-T cell therapy products have been approved by the FDA (7).

Despite their efficacy in treating cancer, current CAR-T cells therapies have limitations including life-threatening adverse events such as cytokine release syndrome (CRS) and neurotoxicity; additionally, CAR-T cell therapies are potentially susceptible to antigen escape of tumor cells and can cause detrimental on-target off-tumor effects (8). Additional limitations of current CAR-T cell therapy include complex and personalized manufacturing with time-sensitive and complex vein-to-vein logistics and the need of specialized centers for its application to patients.

Natural killer (NK) cells are a population of immune effector cells with potent anti-viral and anti-tumor activity and have recently emerged as a candidate for cancer immunotherapy. The biology of NK cells could potentially overcome some of the limitations of existing CAR-T cell products. For example, NK cells can recognize and kill tumor cells without the need of prior stimulation (9, 10). In addition, the response of NK cells is not major histocompatibility complex (MHC) restricted but relies on the ligation of multiple germline-encoded receptors, which enables CAR-NK cells to recognize and eliminate tumor cells even in the event of antigen loss or downregulation. Furthermore, multiple clinical trials have shown that NK cells are well tolerated and may have a safer profile than T-cell-based therapies (11).

In this review, we discuss advances on the development of engineered NK cells for cancer immunotherapy, including the sourcing of NK cells and the move towards off-the-shelf modalities, technologies to engineer NK cells, current clinical trials using engineered NK cells, advances on the engineering of receptors, and stealth approaches to avoid host immune response. In addition, we will comment on the next generation of NK cell products, i.e., armored NK cells with enhanced functionality, fitness, tumor-infiltration potential, and with the ability to overcome tumor heterogeneity and immune evasion.

2 OVERVIEW OF NK CELLS

2.1 NK Cell Populations

NK cells belong to group 1 innate lymphoid cells (ILCs) (12–15) and are characterized by their innate capacity to swiftly detect and kill aberrant cells, such as virus-infected cells and cancer cells (9, 10, 16). NK cells exhibit potent anti-tumor responses through multiple mechanisms and are functionally similar to cytotoxic $CD8^+$ T cells.

Several populations of NK cells have been described. Most of our knowledge about human NK cells comes from peripheral blood NK (PBNK) cells, given that this population can be easily sampled. PBNK cells can be divided into two distinct subsets based on their expression of CD56 (also known as neural cell adhesion molecule 1): $CD56^{dim}$ NK cells represent a mature population characterized by their innate cytotoxic potential, and $CD56^{bright}$ NK cells represent a less mature population characterized by their immunomodulatory potential owing to their ability to secrete large amounts of cytokines upon stimulation (9, 16, 17). Additional populations of NK cells have been identified in multiple tissues where they play specialized immune functions. Tissue-resident NK cells have been observed in tissues such as the liver, gut, lungs, uterus, and kidney [reviewed elsewhere (18–21)].

Although NK cells belong to the innate immune system, certain populations of NK cells can display features attributed to the adaptive immune system such as antigen specificity and memory-like responses. Indeed, NK-cell populations mediating robust memory-like responses have been reported in the context of viral infections, contact hypersensitivity reactions, and after pro-inflammatory cytokine stimulation (22–25).

2.2 NK Cell Receptors and Target Cell Recognition

Unlike T cells, NK cells lack an antigen-specific T-cell receptor (TCR); instead, their activity is controlled by an array of germline-encoded receptors, both activating and inhibitory, that enable NK cells to sense their environment (26). The response of NK cells is modulated by the integration of signals delivered *via* activating and inhibitory receptors, and the balance of these signals determines the outcome of the interaction between NK cells and their environment, i.e., killing of target cells, secretion of cytokines, or no response.

Several activating receptors have been identified in NK cells; they recognize ligands expressed upon cell stress, viral infection, or tumor transformation. The major activating receptors involved in target cell recognition include NKG2D and natural cytotoxicity receptors (NCRs; namely, NKp46, NKp30, and NKp44). In addition, NK cells express the low-affinity Fc receptor CD16, which enables them to detect antibody-coated target cells and to exert antibody-dependent cell cytotoxicity (ADCC) (10, 26). NK cells are negatively regulated by inhibitory receptors, most of which ligate MHC class I molecules and gauge the level of expression of self-molecules on adjacent cells (10, 26–28). Human leukocyte antigen class I (HLA-I)–specific inhibitory receptors include the killer cell immunoglobulin-like receptors

(KIRs) and the lectin-like CD94-NKG2A heterodimers. The interaction between self-MHC molecules and their cognate KIRs during NK cell development provides essential inhibitory signals for NK cell maturation and contributes to their education, i.e., their acquisition of functional competency (27, 28).

NK cells can kill target cells in a perforin-dependent manner, where following the formation of a lytic immunological synapse, preformed lytic granules containing perforin and granzymes converge toward the synapse and are released into the synaptic cleft (29). Perforin molecules form pores in the postsynaptic membrane of target cells allowing granzymes to enter the target cell and activate caspases, resulting in the apoptosis of the target cell (29–31). NK cells are protected from perforin-mediated autolysis by densely packed and highly ordered presynaptic lipid membranes (32). NK cells can also kill target cells in a perforin-independent manner, *via* the expression of FAS ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (9). Additionally, NK cells have immunomodulatory potential owing to their ability to secrete cytokines, chemokines, and growth factors, including interferon gamma (IFN-γ), TNF-α, CCL5, XCL1, and granulocyte-macrophage colony-stimulating factor (GM-CSF). As such, NK cells can positively or negatively influence the anti-tumor responses by modulating innate and adaptive immune cells (30, 33, 34).

2.3 NK Cells as Prognostic Value in Cancer

NK cells are key players of tumor immunosurveillance—they have the innate ability to differentiate healthy from malignant cells and mount an immune response following recognition of transformed cells. The clinical relevance of NK cells in cancer has been investigated, both in hematological and solid malignancies.

The abundance of NK cells has been correlated with prognosis in some hematological conditions, such as chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), T-cell lymphoma, and multiple myeloma (MM) (35). However, in acute lymphoid leukemia (ALL), the presence of NK cells in the bone marrow of children at the time of diagnosis was associated with favorable response to treatment and survival (36). A retrospective study of Hodgkin's lymphoma (HL) patients showed that low numbers of infiltrating NK cells were associated with unfavorable clinical outcome (37). A prospective study of the relationship between the phenotype of NK cells at the time of ALL diagnosis and the minimal residual disease (MRD) at the end of induction chemotherapy showed that the presence of NK cells with a strong effector phenotype was associated with better leukemia control (38).

Additionally, evidence of the clinical relevance of NK cells in hematological malignancies comes from studies that linked mutations in genes essential for NK cell anti-tumor function with occurrence of cancer. As such, mutations of *PRF1* (encoding for perforin) are frequently found in patients with anaplastic large cell lymphoma (ALCL) and ALL, and mutation of *FASLG* (encoding for FasL) was observed in lymphoma patients (39–41). The secretion of IFN-γ by NK cells was found to be a positive prognostic marker in chronic myeloid leukemia (CML) (35), while reduced NK cell activity was observed in multiple malignancies, including advanced MM (42), and was associated with high-risk myelodysplastic syndrome (MDS) (43).

Concerning solid tumors, multiple studies in the early 2000s showed that increased infiltration of NK cells, based on CD57 expression, served as a positive prognosis factor in patients suffering from various malignancies. It was shown that increased infiltration of NK cells in squamous cell lung carcinoma, colorectal carcinoma, gastric carcinoma, and pulmonary adenocarcinoma correlated with better prognosis and survival of patients (44–47). In addition, in an 11-year follow-up prospective study of a cohort of Japanese general population, it was found that higher NK cell cytotoxicity was associated with reduced cancer risk, whereas low cytotoxicity was associated with increased cancer risk—in this cohort, the most frequent cancers identified were stomach, lung, and intestine (48).

More recently, a meta-analysis highlighted the important prognostic value of NK cell infiltration into a variety of solid tumors. High levels of NK cell markers in solid tumor tissues predicted favorable prognosis for solid tumor patients (49). Indeed, increased NK cell infiltration correlated with decreased risk of death; and in terms of localization, intraepithelial infiltration was more predictive of survival than NK cell infiltration into the tumor-adjacent stroma (50).

Cursons et al. showed that patients with metastatic melanoma have an improved survival rate if their tumor has a gene signature predicting NK cell infiltration, and high expression of IL-15 was associated with higher survival (51). IL-15 is an important cytokine for NK cell homeostasis and activation, and the presence of IL-15 within the TME was associated with NK cells with high anti-tumor function in head and neck squamous cell carcinoma (HNSCC) (52).

Additionally, NK cells were identified as a robust prognostic and predicative factor for chemotherapy outcome in gastric cancer (53). In metastatic melanoma patients undergoing ICI therapy, NK cell infiltration into tumor was correlated with favorable response to anti-PD-1 therapy, even in the event of tumor MHC-I downregulation (54).

Considering the association of NK cells with prognosis in several tumor malignancies and that NK cells can target tumor cells that have downregulated HLA-I molecules—an immune evasion mechanism often correlated with worse prognosis of cancer patients (55–57)—there is a window of opportunity to evaluate NK cell therapies, particularly in patients who have failed conventional immunotherapy due to tumors displaying reduced or non-existent HLA class I expression (58, 59).

3 NK CELLS FOR CANCER IMMUNOTHERAPY

3.1 Early clinical studies using NK Cells

In the early 1980s, seminal studies by Rosenberg and colleagues showed that exposure of patient-derived peripheral blood mononuclear cells (PBMCs) to IL-2 alone generated cells that

were able to recognize and kill tumor cells *in vitro* (4, 60, 61). These cells, which were named lymphokine-activated killer (LAK) cells, represented a heterogeneous mixture of T cells and NK cells. Phillips and Lanier later showed that a substantial part of the LAK anti-tumor activity was attributed to NK cells (62). Preclinical studies showed that adoptive transfer of LAK cells into tumor-bearing mice resulted in anti-tumor activity, and the concomitant administration of IL-2 with LAK cells increased *in vivo* anti-tumor activity of LAK cells (4, 63–65). These findings spurred an interest in this novel immunotherapy approach, resulting in clinical trials for advanced cancers using autologous LAK cells in combination with IL-2 infusions. However, in 1993, results from a randomized trial of 181 patients with advanced melanoma or renal cancer comparing treatment with IL-2 alone or in conjunction with LAK cells showed that the observed anti-tumor effects tended to be due to IL-2 alone (66), thereby hampering clinical studies of LAK cells.

Similarly, early clinical studies testing IL-2-activated autologous NK cells for treatment of patients with metastatic melanoma, renal cell carcinoma, relapsed lymphoma, and metastatic breast cancer were ineffective (67, 68), suggesting that in autologous settings, inhibitory signals from self-MHC molecules in tumor cells are likely to suppress NK cell function in the absence of activating signals. These investigators also noted a lack of persistence of the infused NK cells as a potential limitation.

In patients with acute myeloid leukemia (AML) undergoing hematopoietic stem cell transplant (HSCT), Ruggeri et al. showed a correlation between KIR profile and the outcome following HSCT (69), therefore suggesting that stratification of patients by their KIR ligand mismatch can select for patients with alloreactive NK cells that protect from AML relapse. Similar observations were made in additional studies, reporting decreased relapse and increased survival when patients received either HLA- or KIR-mismatched transplants (70–73).

In a landmark study in patients with poor-prognosis AML, Miller et al. found that the adoptive transfer of allogeneic IL-2-activated NK cells combined with lymphodepleting therapy resulted in a marked increase in endogenous IL-15, expansion of donor-derived NK cells, and induction of complete remissions in 26% of the patients (74). This study also highlighted the importance of lymphodepleting therapy in the creation of a cytokine milieu supportive of NK cell expansion. Using similar approaches, clinical responses were also observed when allogeneic NK cells were adoptively transferred into patients with refractory lymphoma and advanced MM (75, 76).

3.2 Clinical Trials of Engineered NK Cells

While the initial promising results from allogeneic NK cell adoptive transfer to cancer patients demonstrated the potential of NK cells, recent success of CAR-T cell therapies proved the value of targeting tumors with engineered receptors. These observations fused with the rapid progression of technologies available to engineer NK cells (**Table 1**) to set the stage for clinical trials of CAR-engineered NK cells in cancer (109).

To induce tumor-specific responses, CAR-based therapies rely on the targeting of tumor antigens (TAs) by immune effector cells. Multiple CAR approaches have been tested in preclinical studies of CAR-NK cells that target antigens including CD19, EGFR, HER2, EpCAM, GD2, Mesothelin, and HSP70 [reviewed in (77)].

In 2020, Liu et al. reported results from a phase 1/2 trial using HLA-mismatched, cord-blood derived, anti-CD19 CAR-NK cells for the treatment of relapsed or refractory B-cell malignancies and showed that 73% of patients responded, with seven of eight patients in complete remissions following therapy (110). They also reported that administration of CAR-NK cells was not associated with CRS, neurotoxicity, nor graft-versus-host disease (GvHD), therefore highlighting a safety profile of CAR-NK cell therapy.

The number of clinical trials exploring the potential of engineered NK cells for cancer therapy has steadily increased over time. In the current landscape, there are over 20 ongoing clinical trials using CAR-NK cells for hematological and solid indications (**Table 2**). One attractive CAR-NK cell strategy is the use of multiplexed induced pluripotent stem cells (iPSC)-derived NK (iNK) cells engineered to express CARs alongside additional edits, including IL-15 receptor fusion and high-affinity CD16 (114). Multiple clinical trials are evaluating iNK cells in hematological malignancies, such as MM, AML, CCL, and B-cell lymphoma, in combination with antibody therapy (daratumumab, elotuzumab, rituximab, and obinutuzumab, respectively), and iNK cells are being trialed in solid tumors, including ovarian cancer (**Table 2**).

Overall, clinical trials have demonstrated that NK cells possess potent anti-tumor effects without eliciting serious adverse effects associated with T-cell therapy, such as GvHD (115, 116), neurotoxicity (117), or cytokine release syndrome (118).

3.3 Current Sources of NK Cells for Therapy

CAR-expressing NK cells are derived from a variety of sources, including peripheral blood (PB), umbilical cord blood (CB), iPSCs, and NK cell lines (**Figure 1**). Recent reviews have described the sources of NK cells in detail, with their advantages and caveats (77, 88).

Whereas obtaining a suitable source of autologous PBNKs is a challenge because patients typically have received prior therapies, CBNKs typically exhibit a naive phenotype including lower expression of adhesion molecules (e.g., CD2, CD11a, CD18, and CD62L), CD16, KIRs, perforin, and granzyme B, resulting in decreased cytotoxicity (119, 120). NK cells can also be derived from cord-blood hematopoietic progenitor $CD34^+$ cells (121, 122). However, these sources have the limitation of poor product standardization due to the heterogeneous nature of PB and CBNKs. Genetically modifying these primary NK cells remains highly variable using currently available technologies, resulting in difficulties developing consistent and reproducible engineered NK cells (123). Although the cancer-derived NK cell line NK-92 can overcome the challenges above, the obvious safety requirement for being mitotically inactivated by irradiation creates significant limitations to potential clinical use with patients (124). Without the ability for proliferation upon infusion, their anti-tumor activity is rapidly reduced overtime when compared to alternative NK cell therapies (74, 125). Indeed, a phase I study with NK-92 cells

TABLE 1 | Technologies for the engineering of NK cells.

Technology	Description	References
Non-viral delivery		
Electroporation	Electroporation is one of the most used non-viral delivery strategies, resulting in high transfection efficiency of NK cells—particularly with mRNA. Electroporation has been used to generate functional CAR-NK cells, including CD19-, CD20-, and HER2-CAR NK cells with measurable increased cytotoxicity; and allows for co-transfection of CAR sequence with additional therapeutic nucleic acids. Among the disadvantages of electroporation, the risk of cytotoxicity and irreversible damage to the cell membrane due to high voltage, the transient expression of CAR, along with the unsuitability for large-scale manufacturing limit its clinical potential.	(77–81)
Cell squeezing	Cell squeezing is a microfluidic delivery approach in which cells are mechanically deformed as they pass through a constriction smaller than the cell diameter. The compression and shear forces result in the formation of transient holes that enable the diffusion of molecules into the cytosol. An advantages of cell squeezing is the possibility to co-transfect nucleic acids. Although the potential of this technology to engineer NK cells still needs to be further elucidated, Loo et al. recently reported that cell squeezing enables delivery of mRNA into primary NK cells with ~60% efficiency.	(82–84)
Nanoparticles	Multiple delivery approaches using nanoparticles have been developed, including lipid- and polymer-based. Nanoparticles are highly customizable with versatility for a variety of cargos, including transposons and CRISPR/Cas9 systems, and they can be designed for targeted delivery. For instances, polymer-based multifunctional nanoparticles with core-shell particles complexed with pDNA EGFR CAR can efficiently transfect NK cells and allow for monitoring of their trafficking *in vivo* through magnetic resonance and fluorescence optical imaging. Ionizable lipid nanoparticle (LNP) platforms allow stable formulation, endogenous cellular internalization, and low toxicity. Charge-altering releasable transporters (CARTs) efficiently transfect mRNA into primary human NK cells, including resting NK cells, with minimal impact on NK cell phenotype and function.	(85–87)
Viral delivery		
Viral vectors	Viral transduction allows for long-term and stable expression of transgenes—although it has an inherent risk of insertional mutagenesis. Both retroviral and lentiviral vectors have been used to engineer NK cells. Primary NK cells are resistant to transduction. To improve their transduction efficiency, NK cells can be pre-activated with cytokines or engineered K562 cells, follow multiple rounds of transduction, or incubated with reagents such as polybrene, DEAE-dextran, poly-L-lysine, fibronectin or retronectin. Additionally, pseudotyped vectors, such as Baboon envelope pseudotyped lentivirus, increase the affinity of the vector to NK cells resulting in higher transduction efficiency.	(88–93)
Gene editing		
Transposons	Transposons are "jumping" DNA elements that can change their position within the genome; the DNA transposon system involves a transposase that binds to terminal inverted repeats (TIRs) and mobilizes the DNA flanked by the TIRs. Transposons have low genotoxicity, cause less toxicity than viral transduction, and are suitable for co-delivery of multiple genes. The Sleeping Beauty (SB) DNA transposon system is capable of transposition in human cells and is currently used in several early clinical trials of CAR T cells. Using the SB system, Batchu, et al. generated mesothelin-CAR expressing NK-92 cells; and Bexte, et al. engineered primary NK cells with anti-CD19 CAR, with a safe genomic integration profile and high anti-tumor activity.	(85, 94–96)
Designer nucleases	Zinc finger nucleases (ZFN) and TALEN are the two most frequently used designer nucleases. The specificity of ZFN-mediated gene editing relies on its number of fingers, the amino acid sequence of the fingers, and the interaction of the nuclease domain. TALEN is composed of a DNA cleavage domain and a sequence-specific DNA-binding domain. Both ZFN and TALEN allow for specific editing with few off-target effects. TALEN have a simpler design than ZNF but are more difficult to deliver. The use of ZNF and TALEN have been limited in NK cells, in particular owing to the substantial protein engineering required for gene targeting.	(85, 97)
CRISPR/Cas9	The CRISPR/Cas9 system is composed of a programable single-stranded guide RNA (sgRNA) and a Cas9 endonuclease—mechanistically, the sgRNA binds to the target DNA sequence allowing the positioning of Cas9 at a specific site of the genome to make double-strand breaks, which can be followed by the integration of the desired gene cassette *via* endogenous DNA repair mechanisms. Advantages of CRISPR/Cas9 include its versatility to reach the target, and its potential for efficient and scalable manufacturing. However, CRISPR/Cas9 is less specific than ZNFs and TALENs and has a risk of off-target mutagenesis and immunogenicity. CRISPR/Cas9 system has been used to knockout *ADAM17* and *PDCD1* to improve NK cell functionality; also, it was used to develop *CISH*$^{-/-}$ iPSC-derived NK cells with improved metabolic fitness and enhanced functions. Additional CRISPR/Cas systems have been developed, such as CRISPR/Cas12a, increasing the options for CRISPR gene editing.	(98–100)
Surface engineering		
Liposomes	The functionality of NK cells can be enhanced by conjugating them with liposomes. Chandrasekaran et al. adorned liposomes with TRAIL and anti-NK1.1 *via* maleimide-thiol chemistry, allowing the ligation of the liposomes to NK cells. They showed that liposome-conjugated NK cells were retained in the tumor-draining lymph nodes, which resulted in the apoptosis of cancer cells and prevented metastasis. Also, NK cells can be engineered with drug-loaded nanoparticles. Siegler, et al. leveraging the tumor-specificity provided by CAR molecules, engineered CAR-NK cells with cross-linked multilamellar liposomal vesicles (cMLVs) containing the chemotherapeutic drug Paclitaxel (PTX). These CAR-NK cells adorned with PTX-loaded cMLVs showed enhanced antitumor efficacy in Her2- and CD19-overexpressing cancer models.	(101–103)
Antibody-cell-conjugation (ACC)	Antibody-cell-conjugation (ACC) technology enables the modification of cell surfaces with single-strand DNA (ssDNA). The modified cells are further annealed with the complementary strand-modified molecules. The ACC platform has been applied to link NK cells with transtuzumab (anti-HER2 mAb), allowing oNK cells (NK-92 cell line) to efficiently target HER2-expressing cancer cells *in vitro* and *in vivo*.	(104)
Glycoengineering	Glycoengineering allows the modification of the glycosylation of surface proteins to endow NK cells with new affinities and properties. This approach has been successfully used to the development of functional CD22-targeting NK-92 cells. Wang, et al. introduced high-affinity carbohydrate-based ligands for CD22 *via* metabolic engineering or glyco-polymer insertion. Hong, et al.,	(105, 106)

(Continued)

TABLE 1 | Continued

Technology	Description	References
	used a chemoenzymatic glycocalyx editing strategy to introduce high-affinity and specific CD22 ligands onto NK cells and further functionalized NK cells with the E-selectin ligand sialyl Lewis X to increase infiltration into the tumor microenvironment.	
Aptamers	Aptamers are short single-stranded oligonucleotides often referred as "chemical antibodies" that can specifically recognize their targets, including nucleic acids and proteins, with high affinity in a similar manner to antibodies. Yang, et al. developed aptamer-engineered NK cells (ApEn-NK) with CD30-specific aptamers and showed that ApEn-NK were able to specifically target CD30+ T-cell lymphoma. Similarly, Chen, et al. developed ApEn-NK cells with PDGC21-T-specific aptamers; they showed the ApEn-NK cells were able to recognize triple-negative breast cancer (TNBC) cells and reduce lung metastasis *in vivo* in a TNBC xenograft model.	(107, 108)

Summary of the technologies available for delivery, gene editing and surface engineering of NK cells.

TABLE 2 | List of ongoing clinical trials utilizing engineered NK cells.

TumorType	Target (s)	Disease Condition	Source of NK cells	NK cell drug candidate	Combination biological agent	Company/Sponsor	Phase	Reference
Hematologic Malignancies	CD19	ALL, CLL, NHL	Cord blood	iC9/CAR.19/ IL15 CB-NK cells	–	M.D. Anderson Cancer Center	Phase 1/2	NCT03056339
	CD33	AML	Unknown	Anti-CD33 CAR-NK cells	–	Sichuan Kelun-Biotech Biopharmaceutical Co., Ltd.	Phase 1	NCT05008575
	NKG2D ligands	AML, MDS	Peripheral blood, allogeneic	NKX101	–	Nkarta Inc.	Phase 1	NCT04623944
	CD19	NHL	Unknown, Allogeneic	CAR-NK019	–	Zhejiang University	Phase 1	NCT04887012
	BCMA	MM	Cord blood	Anti-BCMA CAR-NK cells	–	Sichuan Kelun-Biotech Biopharmaceutical Co., Ltd.	Early Phase 1	NCT05008536
	CD33 + CLL1	AML	Unknown	Anti-CD33/ CLL1 CAR-NK cells	–	Imbioray (Hangzhou) Biomedicine Co., Ltd.	Early Phase 1	NCT05215015
	CD19	ALL, CLL, NHL	Cord blood	Anti-CD19 CAR-NK cells	–	Shanghai Simnova Biotechnology Co.,Ltd.	Phase 1	NCT04796675
	BCMA	MM	NK-92 cell line	Anti-BCMA CAR-NK-92 cells	–	Asclepius Technology Company Group (Suzhou) Co., Ltd.	Phase 1/2	NCT03940833
	CD19	Leukemia, Lymphoma	Peripheral blood, allogeneic	NKX019	–	Nkarta Inc.	Phase 1	NCT05020678
	BCMA + CD38	MM	iPSC	FT576	Daratumumab (anti-CD38 mAb)	Fate Therapeutics, Inc.	Phase 1	NCT05182073, (111)
	SLAMF7 or CD38	AML, MM	iPSC	FT538	Daratumumab (anti-CD38 mAb), Elotuzumab (anti-SLAMF7 mAb)	Fate Therapeutics, Inc.	Phase 1	NCT04614636
	CD19 + CD20	B-cell lymphoma, CLL	iPSC	FT596	Rituximab (anti-CD20 mAb), Obinutuzumab (anti-CD20 mAb)	Fate Therapeutics, Inc.	Phase 1	NCT04245722
Solid Tumors	NKG2D ligands	Metastatic CRC	Unknown	NKG2D CAR-NK cells	–	Zhejiang University	Phase 1	NCT05213195
	HER-2	GC, MBC	NK-92 cell line	ACE1702	–	Acepodia Biotech Inc.	Phase 1	NCT04319757, (104, 112)
	B7-H3	OC, FTA, PPC	iPSC	FT516	Enoblituzumab (anti-B7-H3 mAb), IL-2	Masonic Cancer Center, University of Minnesota	Phase 1	NCT04630769
	PD1 ligands	NSCLC	NK-92 cell line	CCCR-NK-92 cells	–	Xinxiang medical university	Phase 1	NCT03656705, (113)
	PD-L1	Solid tumors	iPSC	FT516	Avelumab (anti-PD-L1 mAb), IL-2	Fate Therapeutics, Inc.	Phase 1	NCT04551885
	HER-2	GBM	NK-92 cell line	NK-92/5.28.z	–	German Cancer Research Center	Phase 1	NCT03383978
	5T4	Solid tumors	Unknown, Allogeneic	Anti-5T4 CAR-raNK cells	–	Shanghai East Hospital	Early Phase 1	NCT05137275

(Continued)

TABLE 2 | Continued

TumorType	Target (s)	Disease Condition	Source of NK cells	NK cell drug candidate	Combination biological agent	Company/Sponsor	Phase	Reference
	PD-L1	Pancreatic Cancer	NK-92 cell line	PD-L1 t-haNK	N-803	ImmunityBio, Inc.	Phase 2	NCT04390399
	ROBO1	Solid tumors	Unknown	ROBO1 CAR-NK cells	–	Asclepius Technology Company Group (Suzhou) Co., Ltd.	Phase 1/2	NCT03940820
	PD-L1 + PD1	GEJ, HNSCC	NK-92 cell line	PD-L1 t-haNK	Pembrolizumab (anti-PD1 mAb), N-803	National Cancer Institute	Phase 2	NCT04847466

List of active clinical trials (recruiting, enrolling by invitation, active not recruiting) involving engineered NK cells, obtained from https://clinicaltrials.gov/ on February 8, 2022. ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; FTA, fallopian tube adenocarcinoma; GBM, glioblastoma; GC: gastric cancer; GEJ, gastroesophageal junction cancer; HNSCC, head and neck squamous cell carcinoma; MBC, metastatic breast cancer; MDS, myelodysplastic syndromes; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; OC, ovarian cancer; PPC, primary peritoneal cavity cancer.

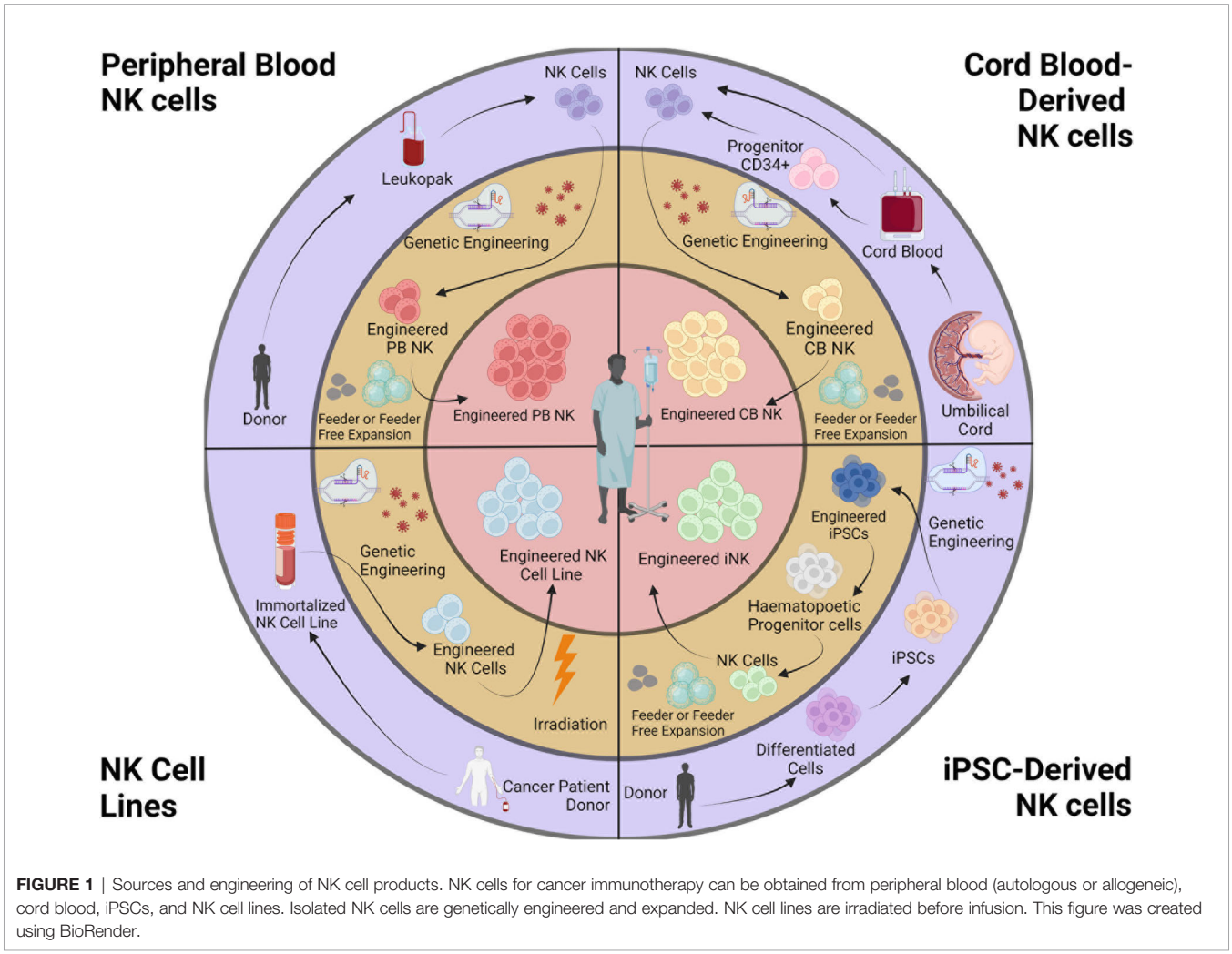

FIGURE 1 | Sources and engineering of NK cell products. NK cells for cancer immunotherapy can be obtained from peripheral blood (autologous or allogeneic), cord blood, iPSCs, and NK cell lines. Isolated NK cells are genetically engineered and expanded. NK cell lines are irradiated before infusion. This figure was created using BioRender.

transduced with a CAR targeting CD33 were tested in patients with relapsed or refractory AML but showed no durable responses partly due to lower persistence and efficacy due to irradiation prior to treatment (126).

On the contrary, human iPSCs are a source for cell therapy that can be genetically engineered *via* established methods, expanded and produced indefinitely in a homogenous and limitless manner (127, 128), and can be differentiated into iNK cells (129). iPSC-derived NK cells have inherent safety considerations; indeed, given that iPSC can proliferate indefinitely, careful analysis of the final iNK product needs to be undertaken to ensure that it is free from residual iPSCs (130).

These benefits can be translated into a highly versatile and standardized off-the-shelf iNK cell therapy to treat various malignancies (110, 131), with the possibility to make multiple precision edits at the single-cell level to produce banks of homogeneous NK-cell products with improved persistence, tumor targeting, homing, and functionality (132) (**Figure 2**).

4 DEVELOPING THE NEXT GENERATION OF NK PRODUCTS

4.1 Engineered Receptors for Targeting Tumors

Engineering CARs onto an immune cell redirects their specificity onto a particular antigen. The first proof of concept for antigen specificity stemmed from combining the signaling of a T cell with the antigen specificity of an antibody *via* fusing the variable regions of an antibody with the constant region of TCR (133, 134). This CAR technology was developed further by utilizing the commonly utilized antigen-binding domain known as single-chain variable fragment (scFv; containing the variable heavy and light chains from an antibody) leading to "first-generation" CAR constructs. These have proven to be capable of eliciting tumor-specific cytotoxicity, as demonstrated by human epidermal growth factor receptor 2 (HER2)-specific CARs using scFv recognizing HER2 fused to a CD3ζ signaling domain for activation (signal 1) (135, 136). However, these first-generation CARs failed to elicit significant anti-tumor responses due to limited persistence (137) and expansion *in vivo* (138).

Endogenous TCR cellular signaling requires both the TCR together with costimulatory (signal 2) or accessory molecules to elicit a robust response, which can be adapted into a single CAR molecule *via* molecular engineering. By taking advantage of the modular nature of CAR receptor technology, this allowed for the continual refinement and modification of these engineered proteins to optimize functionality, leading to improved second- and third-generation CARs containing one or two costimulatory

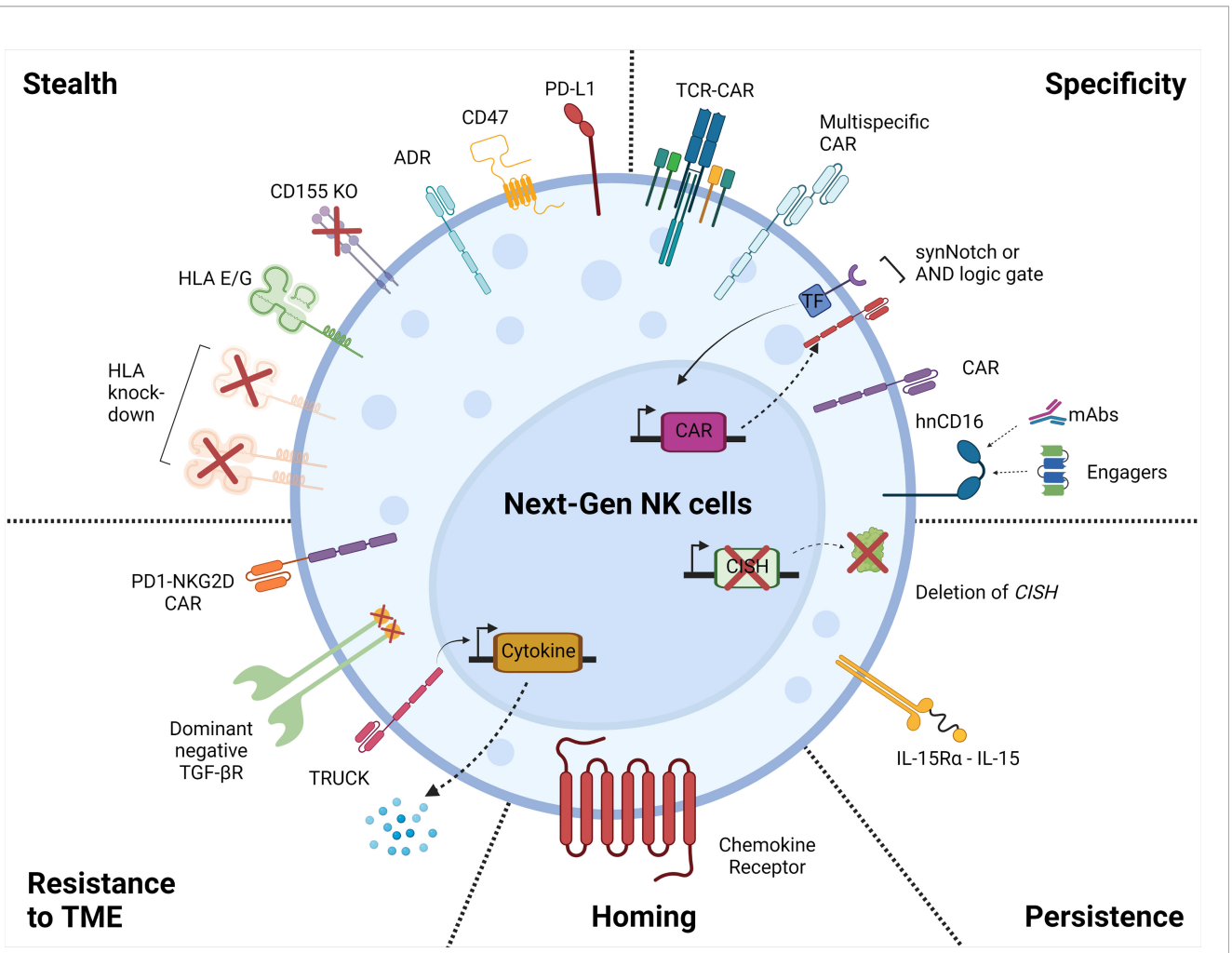

FIGURE 2 | Next generation NK cell products. Illustration of the attributes of the next-generation NK cell products with increased tumor-targeting specificity, persistence, homing, resistance to the tumor microenvironment, and with stealth capabilities. This figure was created using BioRender.

domains, respectively. Despite promising results for various hematological malignancies (110, 139–143), the therapeutic efficacy of CAR-T approaches was limited in both hematological (143, 144) and solid tumors (145, 146). These limitations have been related to diminished cytotoxicity, inefficient trafficking, and infiltration of tumors in an immunosuppressive environment where heterogeneity in tumor target expression can often occur. Initiatives to overcome these shortcomings have led to various strategies including engineering fourth-generation CARs to produce and release a transgenic product, such as pro-inflammatory cytokine IL-12, which is constitutively or inducibly expressed upon CAR activation (147, 148). T cells that are transduced with these fourth-generation CARs are generally referred to as T cells redirected for universal cytokine-mediated killing (TRUCKs) and will be further discussed in later sections.

The development of next-generation NK cells for cancer therapy has been facilitated by the emergence of sophisticated technologies enabling the engineering of NK cells (**Table 1**) in terms of delivery (viral vectors, electroporation, cell squeezing, and nanoparticles), gene editing (transposons, designer nucleases, and CRISPR/Cas systems), and surface engineering (liposomes, antibody–cell conjugation, glycoengineering, and aptamers).

4.2 "Armoring" of NK Cells Through Enhanced Functionality

Engineering genetic modifications to augment persistence and functionality can be easily performed using a single-cell iPSC engineering platform. This was demonstrated in iNK cells with the addition of a modified high-affinity version of CD16a (149). NK cells naturally express CD16a, which binds the Fc portion of immunoglobulin G (IgG) attached to target cells and induces ADCC. Engagement of CD16a alone is sufficient for inducing NK cell activation, leading to secretion of various inflammatory cytokines and chemokines for the recruitment and activation of other immune cells (150–152). However, CD16a undergoes rapid proteolytic cleavage upon stimulation mediated by ADAM17 (153–155). Substitution of serine at position 197 located in the middle of the cleavage region with proline (S197P) effectively prevents cleavage of the intact and functional receptor (156). In addition, the binding affinity of CD16a varies between allelic variants (157). This led to the discovery of a high-affinity CD16a variant with valine at position 158 (158V) (158); patients with this variant had greater objective responses and progression-free survival when treated with mAb therapy such as rituximab (159), cetuximab (160), or trastuzumab (161). Engineered iNK cells with a high-affinity non-cleavable version of CD16a (hnCD16) displayed enhanced ADCC effector function when combined with anti-tumor mAbs (131).

NK cells do not naturally produce signal 3 cytokines such as IL-2, IL-7, or IL-15, which is in contrast to polyclonal CAR-T cell therapies that, upon CAR engagement, produce significant amounts of IL-2 that supports their expansion (162). Because NK cells rely on signal 3 for expansion, survival, and cytotoxicity, investigators have focused on ways to provide this signal to NK cell therapies, either exogenously or by design in the NK cell product. Optimizing CAR signaling with the addition of signal 3, which includes the transgenic expression of cytokines or a chimeric cytokine receptor, further enhances cytotoxicity and persistence of NK cells. The TME is often deficient in signal 3, thus hampering the potential degree of anti-tumor response of cell therapies. As previously mentioned, fourth-generation TRUCK CAR-T cells have been engineered to minimize systemic toxicity and to induce targeted accumulation of cytokines at the tumor site by providing signal 3. To enhance both NK durability and host immune responses, initial clinical trials focused on the administration of cytokines such as IL-2 and IL-15 for safety and efficacy. However, IL-2 administration resulted in severe systemic toxicity (163), which prompted the focus of investigations onto IL-15. In contrast to IL-2, treatment with IL-15 did not promote expansion of inhibitory Tregs (164) and activation-induced cell death (AICD) of T cells (165). Clinical trials combining NK cell therapy and IL-15 cytokine support resulted in limited anti-tumor responses in patients due to a short half-life after delivery (166, 167). To enhance and maintain the therapeutic effectiveness of IL-15, a membrane-bound IL-15/IL-15 receptor fusion (IL-15RF) was generated, resulting in increased persistence, proliferation, and enhanced cytotoxicity in iNK cells (131, 168). Deletion of cytokine-inducible SH2-containing protein (CIS, encoded by *CISH*), a negative regulator of IL-15 signaling, further improved cytotoxic effector function, metabolic fitness, and *in vivo* persistence of iNK cells (98, 169).

4.3 Strategies to Overcome Tumor Heterogeneity and Immune Evasion

Heterogeneous antigen expression in tumor cells led to the development of CAR-T cells targeting simultaneously two or more tumor-associated antigens either by a tandem (170–172) or split CAR construct configuration (173). Indeed, both anti-CD19 and anti-CD22 CAR-T cell therapies have shown impressive efficacy; however, patients have shown reduced antigen density at relapse, suggesting tumor-antigen-specific downregulation as a mechanism for immune escape (140, 174). Currently, the optimal strategy for multi-targeting is being investigated between tandem or separate CAR constructs in T cells regarding safety and efficacy of these constructs (170, 172, 175). However, having a heterogeneous mixed product resulting from transducing T cells with either a tandem or bicistronic CAR has proven to be a complex manufacturing procedure and makes mechanistic investigations difficult. This could potentially be overcome by utilizing a renewable source for consistent and selective gene editing (131). In another example, targeting B-cell maturation antigen (BCMA) has shown extremely promising and potentially durable objective response rates in MM (139). However, gamma-secretase (GS)-mediated cleavage of BCMA releases soluble BCMA fragments that have been shown to be capable of inhibiting BCMA-CAR function, leading to the testing of combinatorial therapy with GS inhibitors to prevent antigen escape (176). Targeting pan-cancer antigens

such as CD276 (B7-H3) has also shown tremendous success in treating various solid malignancies; however, their success is largely dependent on the level of surface antigen density (177). Although multi-targeting or combinatorial cleavage inhibition may address tumor immune evasion through antigen loss, it does not address other resistance mechanisms such as inhibition by engagement of PD-L1 on tumor cells (178) or navigating through the immunosuppressive TME.

While these multi-specific CAR strategies are developed to target tumor-associated antigens—which may be also expressed in healthy tissues—they could result in severe on-target off-tumor toxicities similar to those seen in specific cases with some CAR-T cell therapy trials (179–181). Strategies to circumvent this issue have led to engineering approaches to insert logic gates into the cells, resulting in activation in the presence of combinations of target antigens. Utilizing a split CAR model by separating signal 1 and signal 2 into different CARs, each with a binder for a different antigen, has now led to the development of an AND logic gate (182). Although intracellular CD3ζ domain for signal 1 by itself is sufficient for signaling, Kloss et al. modified the CD3ζ domain to make it insufficient to produce an activating signal without the co-stimulation by a secondary CAR with signal 2 (183). Another strategy of utilizing AND logic gating is by inducing expression of the primary tumor-targeting CAR only in response to a secondary antigen such as the SynNotch system (184). To overcome the potential off-target recognition of healthy cells, inhibitory NOT logic gates can be engineered to ignore cells that express a particular antigen that is expressed in healthy tissue but not in tumors. The NOT logic CAR was initially conceived by fusing the intracellular domain of PD1 or CTLA4 with a targeting domain recognizing antigens expressed on healthy tissues, thus allowing the primary tumor-targeting CAR to selectively kill tumor cells (185). Naturally, for any of these approaches to work requires identification of combinatorial expression patterns that are truly unique to tumors. In addition, a distinct separation between healthy and tumor cells must be present; otherwise, unintended activation or inactivation may still potentially occur. The recent improvements in engineering multiple edits into cell therapy products demonstrate the benefits of logic circuitry if the appropriate antigen combinations can be identified.

Beyond the use of exquisite, but synthetic, logic circuitry for efficient tumor targeting comes augmenting cells with CARs based on natural cytotoxicity ligands native to NK cells to target heterogeneous tumors. Several groups have demonstrated the use of NKG2D-based CAR therapy with unique intracellular signaling domains, such as a second-generation CD3ζ/CD28 (186) or NK-inspired DAP12 (187), to target various tumor and immunosuppressive cells. Alternatively, NKp46 (188), NKp44 (189), and NKp30 (190) have each been fused to generate second-generation CAR-T cells that further demonstrates the benefit of tumor recognition capability of natural NK ligands for anti-tumor efficacy. This concept was taken further by utilizing an scFv targeting B7H6, a ligand for NKp30, to create a CAR that targets multiple tumor cells while demonstrating an impressive safety profile, since B7H6 is not constitutively expressed on healthy tissues (191). Indeed, utilizing NK ligands for the recognition of heterogeneous tumor targets provides a selective but effective way to overcome evasion by tumor cells to T-cell therapies. Alternatively, NK-insensitive cancer cell lines that are non-targetable by NKG2D-mediated cytotoxicity have resulted in the generation of NK cells expressing a TCR-CAR (192). This allows the TCR-CAR to redirect cytotoxic effector cells such as NK-92 cells by endowing them the ability to recognize tumor cells typically elusive to NK cell detection. Indeed, expressing the TCR in NK cells could greatly enhance the range of targetable antigens by enabling recognition of tumorigenic neoantigens within the intracellular proteome. However, this strategy can potentially give rise to TCR-mediated GvHD. There is also a limitation due to the diversity of MHC alleles and the likelihood that a particular TCR will only recognize a neoantigen when it is presented by a specific MHC allele. Thus, TCRs would have to be selected to recognize a neoantigen presented in a wide range of MHC alleles to minimize GvHD. One potential clone, MC.7.G5, appeared to recognize a currently unknown cancer-specific molecule presented by the non-polymorphic protein MHC-related 1 (MR1) (193). Since MR1 is a member of a family of non-classical MHC proteins, this TCR should enable recognition of cancer cells in a wide range of patients while minimizing off-target effects. Indeed, further investigation will be required for TCR-CARs to assess whether their off-target potential is worth their clinical impact.

4.4 Targeting Negative Regulators

Successful treatment of solid tumors has been elusive in part due to the immunosuppressive nature of the TME. Tumor cells can evade immune surveillance by secreting or promoting the secretion of transforming growth factor beta (TGF-β) (194). Because of its suppressive role in the TME, TGF-β has been targeted to boost cell therapy anti-tumor response. TGF-β mediates downregulation of NKG2D, NKp30, TRAIL, and DNAM1 receptors on activated NK cells (195, 196). To shield adoptive NK cell therapies from the suppressive effects of TGF-β, introduction of a dominant negative form of TGF-β type II receptor (TGF-βRII) efficiently blocked TGF-β signaling and maintained cell surface expression of receptors and cytotoxicity in NK and T cells (197–199). Elegant strategies embracing the inhibitory cytokine and converting it into a potent stimulatory signaling have been created by rewiring the recognition domain into a second-generation CAR-T cell to orchestrate upregulation of cytokine production and proliferation (200). Similarly, expressing a CAR with a TGF-βRII extracellular and transmembrane domains combined with the intracellular domain of NKG2D on NK-92 cells converted the immunosuppressive signal into increased cytotoxicity while preventing downregulation of NKG2D surface expression (201). This strategy has also been applied to other inhibitory receptors such as PD-1, generating a PD-1 CAR with NK-tailored endodomains such as NKG2D or DAP10/NKG2D to mediate cytotoxicity by NK cells against solid malignancies in the TME (202, 203).

4.5 Enhancing Homing to Navigate the Tumor Microenvironment

In addition to improving effector function within a hostile immunosuppressive environment, NK cell trafficking and retention within tumor sites is essential for optimal anti-tumor efficacy. Inducing expression of CCR7 on NK cells enhanced migration and homing to the lymph node-associated chemokine CCL19 for CD16 and rituximab-mediated ADCC against hematological malignancies (78, 204). Augmenting CAR-NK cells targeting the glioma antigen epidermal growth factor variant III (EGFRvIII) with CXCR4 expression conferred enhanced chemotaxis to U87-MG glioblastoma cells that secrete CXCL12/SDF-1α, a CXC chemokine that binds to receptors CXCR4 and CXCR7 (205). Furthermore, inducing expression of CXCR1 in NK cells with a NKG2D CAR were shown to significantly increase anti-tumor responses in subcutaneous and intraperitoneal xenograft models along with an intravenous injection model against established peritoneal ovarian cancer xenografts (206).

4.6 Stealth Approaches to Avoid Elimination by Host Immune Responses

4.6.1 Host-Versus-Graft Immunity: A Barrier for Sustained Therapeutic Activity of Allogeneic NK Cells

NK cells have an advantage over T cells for allogeneic cell therapies because they bypass the risk of GvHD driven by αβ T-cell receptors. However, host-versus-graft (HvG) immune rejection by recipient immune cells and antibodies could limit the expansion and/or persistence of allogeneic NK cells, thus impairing their efficacy. Allogeneic HvG is the rejection of non-self donor cells due to genetic polymorphisms between the donor and recipient. In humans, this response is primarily directed against polymorphic HLA genes (major mismatch), polymorphisms in non-HLA proteins leading to "non-self" peptides presented on shared HLA alleles (minor mismatch), and red blood cell antigens (such as ABO and Rh antigens). Since NK cells do not express red blood cell antigens (207), strategies to avoid HvG for NK cell therapies are focused on avoiding rejection due to major and minor mismatch mechanisms, which are driven by CD8+ T cells, CD4+ T cells, antibodies, and to a lesser extent NK cells (208, 209).

Decades of experience in allogeneic transplantation have shown that immune rejection can occur rapidly after cell or organ transplantation, sometimes within hours if patients have pre-existing antibodies against donor HLA types (210). Allogeneic, haplo-identical (50% HLA-matched) NK cells administered to patients pre-treated with lymphodepletion usually do not persist over a month, sometimes even being eliminated within several days in lower intensity lymphodepletion regimens (74, 75, 211–213). The loss of transferred NK cells tends to coincide with the return of patient lymphocytes to baseline levels, and one study has confirmed *de novo* generation of an anti-donor T-cell response 9–14 days after NK cell transfer (75). Occasionally allogeneic donor NK cells can engraft and persist long-term in patients, which may be related to the enhanced levels of IL-15 after intense lymphodepletion (74), or the transgenic inclusion of IL-15 in the donor NK cells (110). However, another study showed that systemic IL-15, intended to support donor NK expansion, actually accelerated their loss by stimulating patient CD8+ T cells that may have eliminated the transferred NK cells (212). One approach to mitigate HvG rejection is to further deplete the immune system of the patient, for instance by adding anti-CD52 antibodies to the lymphodepletion regimen. Because CD52 is expressed in all lymphocytes (including NK cells), an engineering strategy to remove CD52 in the transferred cell therapy is required. This method has been used for allogeneic CAR-T cells and could conceivably be extended to allogeneic NK cells, but the deep and sustained depletion of the patient's immune system can lead to severe toxicities (214). Other researchers have proposed suppressing the HvG response by engineering the cell therapy product with a 4-1BB-targeting CAR that can eliminate activated recipient lymphocytes (215). This alloimmune defense receptor (ADR) has the potential benefits of not requiring additional lymphodepleting agents and enhancing co-stimulation of the transferred cells; however, the safety and feasibility of targeting 4-1BB+ endogenous cells in patients is still unknown.

4.6.2 Stealth Engineering: Evasion of Patient CD8+ T Cells and NK Cells

Many investigators have instead focused on engineering NK cells to be immunologically silent and evade the HvG response, sometimes termed "stealth." HLA class I contains the polymorphic HLA-A, HLA-B, and HLA-C surface proteins. These molecules are heterodimers that consist of two polypeptide chains: the polymorphic HLA-encoded α chain and β2-microglobulin (β2M). HLA class I molecules are expressed in all nucleate cells and are the anchors to present intracellular peptides to CD8+ T cells (216). If the NK cell therapy is from a donor that does not share all the HLA-A, B, and C alleles of the patient, the patient's CD8+ T cells will recognize the donor's HLA molecules as foreign and mount a rejection response (major mismatch). Even if the donor and patient are perfectly HLA matched, patient CD8+ T cells can respond to non-self-peptides presented on the shared HLA class I molecules (minor mismatch). Thus, one mechanism to avoid both major and minor CD8+ T cell HvG is to delete or silence β2M because it is required for the surface expression of all HLA class I molecules. It has been shown that β2M-knockout (KO) NK cells do not induce an allogeneic CD8+ T cell response (217), a finding which has been shown with other cell types, including iPSCs and their derivatives (218, 219).

However, targeting β2M expression creates a problem because HLA class I molecules have an important function as inhibitory ligands for NK cells (220). HLA-C and certain HLA-A and HLA-B alleles are ligands for KIRs. HLA-E, a non-polymorphic HLA class I molecule expressed on all healthy cells, is a ligand for the NKG2A/CD94 receptor. HLA class I interactions with KIRs and NKG2A/CD94 play a major role in self-tolerance of NK cells, such that when these interactions are lost, the balance between activating and inhibitory signals on NK cells is shifted towards activation, resulting in "missing-self" lysis

of target cells (26). In addition to potentially making a cell therapy product susceptible to patient NK cells, HLA class I reduction in an NK cell therapy could induce fratricide that limits the expansion and/or survival of the modified NK cells.

One strategy to solve this problem is to force expression of molecules that can inhibit NK cells, such as a transgenic HLA-E-β2M fusion protein (217). Not all NK cells express NKG2A, so other investigators have combined the HLA-E-β2M evasion strategy with genetic deletion of CD155, a ligand for the activating receptor DNAM-1, which further reduces the number of patient NK cells that are triggered by β2M-KO cell therapies (219). This finding was described with iPSC-derived T cells, so whether CD155-KO would also be relevant for NK cell therapies is an open question. Overexpression of HLA-G has also been proposed as a strategy to limit patient NK cell activation, which may also help suppress effector CD8+ T cell responses (221). Another strategy proposed to prevent NK missing-self responses of HLA class I deficient cells is to overexpress CD47 (222). CD47 is a transmembrane protein with a well-described role as a "don't eat me" signal due to its binding to signal regulatory protein α (SIRPα) on myeloid cells (223) and high CD47 expression on tumor cells is thought to protect tumor cells from immune responses (224). Recently it was found that IL-2 stimulated NK cells upregulate SIRPα and can be inhibited through high levels of CD47 expression on β2M-KO target cells (225). Altogether, the concept to express ligands for inhibitory receptors is a promising strategy to evade patient NK cells but requires careful evaluation to ensure that trans-inhibition does not limit the function of the NK cell therapy product.

An alternative approach to avoid CD8+ T cell HvG while minimizing the induction of NK "missing-self" is to specifically delete the HLA-A, HLA-B, and HLA-C genes while leaving β2M and HLA-E intact to engage NKG2A on patient NK cells (218). This strategy still results in a loss of inhibitory KIR signaling, so it could be enhanced with additional immunosuppressive molecules like PD-L1 and CD47. In another modification of this approach, only HLA-A and HLA-B are deleted, thus providing HLA-C-driven KIR signaling in addition to HLA-E-driven NKG2A signaling (226). Although this approach opens the door to allogeneic CD8+ T cell responses to HLA-C, 12 separate banks could be made that each retain a common HLA-C allele to allow matching with >90% of patients.

4.6.3 Stealth Engineering: Evasion of Patient CD4+ T Cells and Myeloid Cells

Activated NK cells express HLA class II, which, like HLA class I, is also highly polymorphic. During an allogeneic encounter, CD4+ T cells become activated through major or minor mismatch with HLA class II, leading to both enhancement of allo-reactive CD8+ T cells and direct cytotoxicity by CD4+ T cells. Additional genome editing of NK cell therapies could include the disruption of the MHC II trans-activator (CIITA) gene, a required component for HLA class II gene transcription. Several research groups have described using CRISPR technology to generate CIITA-KO hypoimmunogenic iPSC lines, either alone or in combination with β2M-KO (218, 219, 225, 227). Importantly, a cell therapy deficient for both HLA class I and II (e.g., β2M KO plus CIITA KO) will completely avoid host CD8+ and CD4+ T cell responses, in addition to evading anti-donor HLA antibodies that may exist or be generated in the patient.

Myeloid cells are crucial members of the innate immune system, where they are the first responders against infection. However, much is still unknown about the full contribution of these cells during an allogeneic response in humans; for instance, do they specifically respond to allogeneic non-self, or do they simply facilitate host T and B cell responses to allogeneic cells? A study done by Dai et al. showed that naive myeloid cells, specifically monocytes and macrophages, retain innate immune memory after the first encounter with allogeneic cells (228). This specific memory was acquired by the binding of the paired Ig-like receptor-A (PIR-A) on murine monocytes and macrophages with the MHC class I molecule on allogeneic cells. The human ortholog of PIR-A is the leukocyte IgG-like receptor (LILR) family, which contains 11 functional genes that encode five activating (LILRA1, 2, 4–6) receptors, five inhibitory (LILRB1-5) receptors, and one soluble protein (LILRA3) (229, 230). Barkal et al. showed that binding of MHC class I with the inhibitory receptor LILRB1 suppresses macrophage phagocytic activity (231). Given that some LILR genes are highly polymorphic (232), an intriguing possibility is that human myeloid cells may respond to allogeneic cells more vigorously than autologous cells and potentially acquire memory capabilities. Whether this mechanism could limit the persistence of allogeneic NK cell therapies is unknown, but one method proposed to prevent myeloid cell phagocytosis is the overexpression of CD47 (225).

Overall, there are many proposed strategies to avoid the HvG response, and their efficacy will need to be determined in clinical trials. Importantly, better characterization of patient immune responses against administered allogeneic NK cell therapies will facilitate improved stealth approaches in the future.

5 CONCLUSION

NK cells have numerous features that make them a promising cell therapy strategy for the treatment of cancer. Many trials using adoptively transferred allogeneic NK cells have demonstrated their favorable safety profile, so the main challenge for NK cell therapies is to enhance their efficacy to the level expected from CAR-T cells. A seminal study has demonstrated that allogeneic NK cells engineered to express a CAR and IL-15 have encouraging anti-tumor activity in lymphoid malignancies (110), but obstacles still remain for scalability, activity in solid tumors, and reliable persistence. Engineered iNK cells offer a highly scalable, off-the-shelf cell therapy. Modifying iNK cells with cytokine signaling and immune-evasion modules will boost their expansion and persistence, further improving clinical benefit from these therapies. Because NK cells integrate multiple receptor–ligand interactions to recognize and destroy target cells, they are naturally suited to limit the chance of tumor antigen escape, synergize with antibody therapeutics, and incorporate logic gates

that can fine-tune the specificity of their response. Rapid advances in single-cell sequencing and CRISPR screening promise to deepen knowledge of NK cell signaling networks, enabling future improvements in NK cell therapies that build upon the advantageous biology of NK cells.

AUTHOR CONTRIBUTIONS

MA and UM-N contributed equally to this work. FR, MA, and UM-N wrote the manuscript. NF created the illustrations. All authors contributed to the article and approved the submitted version.

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