Review

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Beyond CAR T cells: exploring alternative cell sources for CAR-like cellular therapies

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Abstract: Chimeric antigen receptor (CAR)-T cell therapy has led to remarkable clinical outcomes in the treatment of hematological malignancies. However, challenges remain, such as limited infiltration into solid tumors, inadequate persistence, systemic toxicities, and manufacturing insufficiencies. The use of alternative cell sources for CAR-based therapies, such as natural killer cells (NK), macrophages (MΦ), invariant Natural Killer T (iNKT) cells, γδT cells, neutrophils, and induced pluripotent stem cells (iPSC), has emerged as a promising avenue. By harnessing these cells' inherent cytotoxic mechanisms and incorporating CAR technology, common CAR-T cell-related limitations can be effectively mitigated. We herein present an overview of the tumoricidal mechanisms, CAR designs, and manufacturing processes of CAR-NK cells, CAR-MΦ, CAR-iNKT cells, CAR-γδT cells, CAR-neutrophils, and iPSC-derived CAR-cells, outlining the advantages, limitations, and potential solutions of these therapeutic strategies.

Keywords: CAR-T cells; CAR-iNKT cells; CAR-macrophages; CAR-NK cells; CAR-neutrophils; CAR-γδT cells

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1 Introduction

Cancer presents one of the leading causes of premature mortality globally (Bray et al. 2021). Although conventional treatment strategies such as surgery, radiation, and chemotherapy are widely used and readily available, they display significant limitations (Hayes 2021). A crucial cornerstone in cancer therapy is immunotherapy, which is based on modulating the immune system to recognize and destroy malignant cells (Esfahani et al. 2020). Next to cytokine therapy, monoclonal antibodies, and immune checkpoint inhibitors (ICIs), cell-based immunotherapies have emerged as a promising immunotherapeutic approach, including dendritic cell (DC) vaccines and adoptive cell transfer (ACT) (Hayes 2021; Zhang and Zhang 2020). In contrast to small molecules or antibodies, immune cells can receive various signals, adapt to the surrounding environment, interact with other cells, and respond through complex signaling pathways as true living drugs (Hayes 2021). Their dynamic properties, longer persistence as well as the induction of an immunological memory give cellular

immunotherapies a clear advantage over conventional drugs. A milestone in cellular immunotherapy has been CAR T cell therapy, in which T cells are redirected against specific tumor antigens via the insertion of an antigen-targeting receptor.

CAR are fully synthetic receptors comprising four main components (Figure 1): (1) an extracellular antigen binding domain, which most often consists of the single-chain variable fragment (scFv) of a monoclonal antibody, (2) a spacer or hinge region, (3) a transmembrane domain and (4) one or more intracellular signaling domains (Sterner and Sterner 2021). CAR constructs have evolved through different generations, each representing advancements in the design of the intracellular CAR signaling (Larson and Maus 2021). First-generation CAR-T cells harbor a single CD3ζ signaling domain and often exhibit limited persistence and

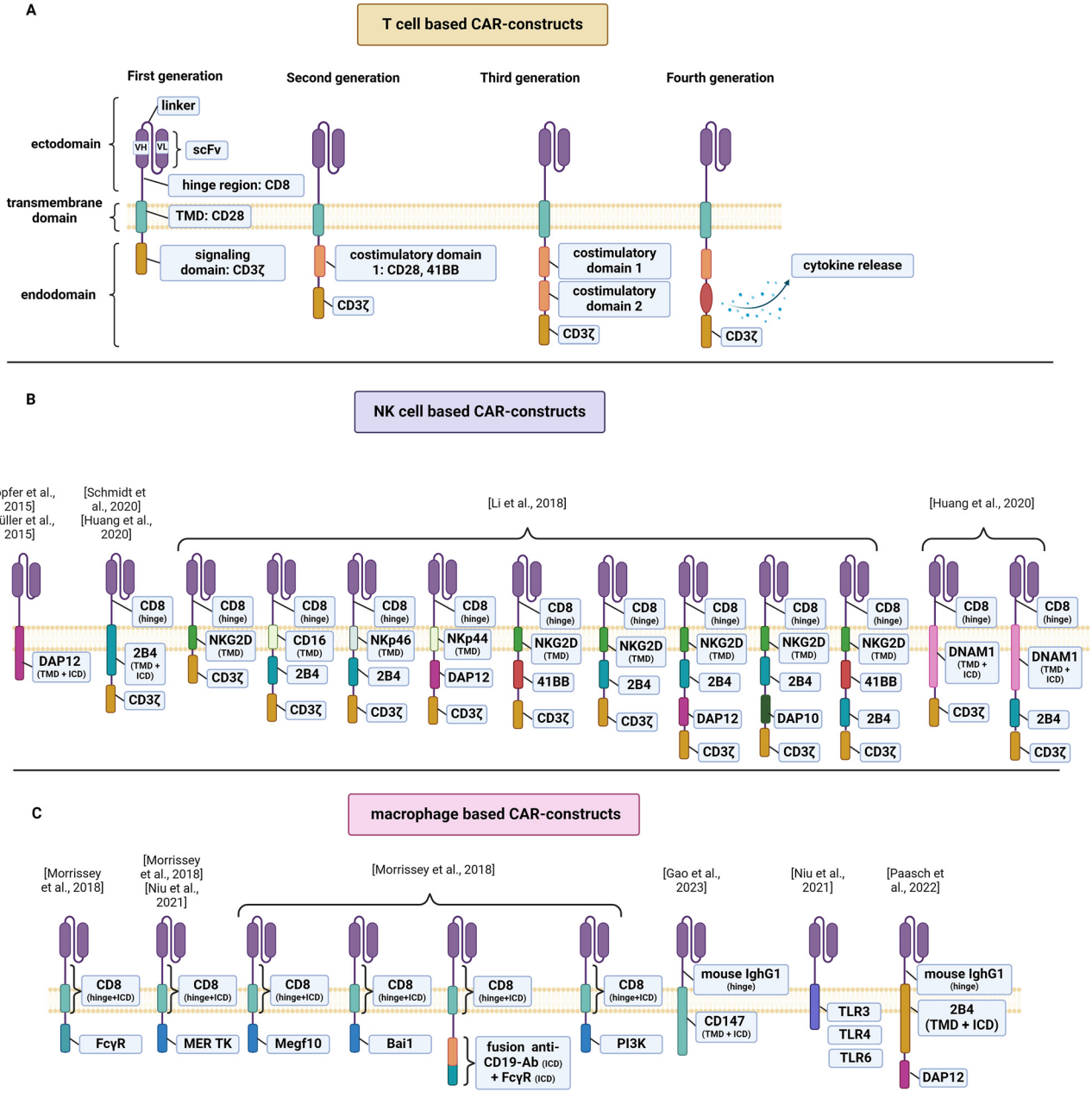

Figure 1: Schematic diagram of CAR structure. (A) Overview of the structural components of first, second, third and fourth generation CAR for T-cells. (B) Overview of CAR for CAR-NKs that incorporate signaling domains adapted to innate intracellular signaling pathways of macrophages. (C) Overview of CAR for CAR-MΦ that incorporate signaling domains adapted to innate intracellular signaling pathways of macrophages. Single chain variable fragment, scFv; transmembrane domain, TMD; intracellular domain, ICD.

efficacy (Brocker and Karjalainen 1995). Second-generation CAR-T cells incorporate an additional co-stimulatory domain, such as CD28 or CD137 (4-1BB) which enhance T-cell activation, proliferation, and persistence. Third-generation CAR-T cells incorporate two costimulatory domains and fourth-generation CAR-T cells are engineered to secrete specific cytokines or immunomodulatory molecules upon target engagement (Larson and Maus 2021).

CAR-T cell therapy has demonstrated outstanding clinical efficacy in distinct hematological malignancies (Kochenderfer et al. 2012, 2015, 2017; Maude et al. 2018, Neelapu et al. 2017; Schuster et al. 2017) leading to the FDA-approval of six CAR-T cell products: Abecma® (idecabtagene vicleucel, anti-BCMA-CAR), Breyanzi® (lisocabtagene maraleucel, anti-BCMA-CAR), Kymriah® (tisagenlecleucel, CD19-CAR), Tecartus® (brexucabtagene autoleucel, anti-CD19-CAR), Yescarta® (axicabtagene ciloleucel, anti-CD19-CAR) and Carvykti® (ciltacabtagene autoleucel, anti-BCMA-CAR). Despite the remarkable success achieved by CAR-T cell therapy in hematology, substantial challenges remain to be addressed (Majzner and Mackall 2019; Sterner and Sterner 2021) (Table 1). A prevalent hurdle is antigen escape-the down-regulation of targeted antigens on tumor cells-hampering the effective recognition and elimination of tumor cells by CAR-T cells (Majzner and Mackall 2018). T cell exhaustion and senescence further limit the efficacy of CAR-T cell therapy. Especially in solid tumors, poor T cell trafficking and infiltration into the tumor site substantially limit the efficacy of CAR-T cell therapy (Majzner and Mackall 2019; Sterner and Sterner 2021). Physical barriers, including cancer-associated fibroblasts (CAFs) and a dense extracellular matrix (ECM) hinder CAR-T cell penetration into solid tumors. Once inside the tumor, CAR-T cells often fail to persist due to the immunosuppressive tumor microenvironment (TME), characterized by hypoxia, acidity, nutrient deficiency, and immunosuppressive cells, such as myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), tumor associated neutrophils (TANs) and regulatory T cells (Tregs). "On-target off-tumor" toxicity and severe adverse effects, including cytokine release syndrome (CRS), hemophagocytic lymphohistiocytosis/macrophage activation syndrome (HLH/MAS), and immune effector cell-associated neurotoxicity syndrome (ICANS), limit the value of CAR-T cell therapy as well (Neelapu et al. 2018). Current approaches involve corticosteroid treatment, IL-6 blockade by tocilizumab (Neelapu et al. 2018) as well as IL-1 receptor (IL-1R) blockade by Anakinra (Jatiani et al. 2020). Finally, the risk of graft-versus-host disease (GvHD) associated with allogeneic CAR-T cell therapy necessitates the use of autologous sources. However, the insufficient quantities and compromised quality of autologous T cells from heavily pretreated cancer patients place limitations on CAR-T cell manufacturing and affect the quality of the CAR-T cell products (Ceppi et al. 2018; Sanber et al. 2021).

The remarkable clinical impact achieved with CAR-T cell therapy, along with the challenges it presents, highlights the necessity of expanding the scope of cellular immunotherapies. Up to this point, a large share of approaches has explored T cells as cellular therapeutics, which have spearheaded the outlined development. However, basic immunology supported by translational work on the tumor environment teaches that immune cells are not created equally and come with distinct functional properties and abilities. In other words, limitations incomed by T cells may not show for myeloid or other lymphoid cells, because of their inborn functionalities. Therefore, exploring alternative cellular sources emerges as a promising strategy to overcome many of the aforementioned limitations. In this review, we offer a comprehensive overview of the latest advancements in CAR-based therapies, including CAR-Natural Killer cells (CAR-NK), CAR-macrophages (CAR-MΦ), CAR-iNKT cells, CAR-γδ T cells CAR-neutrophils, and CAR-cells derived from induced pluripotent stem cells (iPSC). By encompassing these diverse therapeutic approaches, we aim to provide an understanding of the current landscape and prospects in the field of anti-tumoral cellular immunotherapy.

2 NK cells

2.1 Properties

NK cells belong to the heterogenous group of innate lymphoid cells (ILCs) (Chiossone et al. 2018) and play a pivotal role in orchestrating the body's immunological defenses and mediating anticancer immunity. They possess the ability to discriminate between self and non-self and to directly recognize and lyse target cells in an HLA-unrestricted manner with no need for prior sensitization unlike T cells (Chiossone et al. 2018; Wang et al. 2015; Wu et al. 2020). NK cells lack genetically rearranged antigen-specific receptors but detect antigens through a variety of germline-encoded activating and inhibitory receptors (Chiossone et al. 2018). NK cell activating receptors, such as the natural cytotoxicity triggering receptor 3 (NKp30), the natural cytotoxicity triggering receptor 2 (NKp44), and the natural cytotoxicity receptor 1 (NKp46), detect viral, bacterial, or stress-induced ligands (Shin et al. 2023). The activating receptor natural killer group 2 D (NKG2D) recognizes MHC class I polypeptide-related sequences A and B (MICA and MICB) as well as UL16 binding proteins (ULBP1-6), which are often expressed on rapidly dividing tumor cells (Chang et al. 2013;

Table 1: Overview of advantages and limitations of CAR-based therapies and strategies to overcome some challenges.

	Advantages (⊕)	Limitations (⊖) and solutions (💡)
CAR-T cells	⊕ Sufficient number of circulating T cells ⊕ Successful clinical outcomes for treating hematologic malignancies ⊕ Easy genetic engineering/established protocols	⊖ Tumor antigen heterogeneity and tumor antigen loss 💡 Bispecific CAR-T cells ⊖ Poor T cell trafficking and tumor infiltration 💡 Chemokine receptor-expressing CAR-T cells 💡 locoregional administration 💡 In vivo CAR delivery ⊖ Limited persistence in immunosuppressive TME 💡 Combination with ICIs 💡 ICI-secreting CAR-T cells 💡 CAR-T cells targeting MDSCs, M2 macrophages, Tregs ⊖ Toxicity: CRS, neurotoxicity, HLH 💡 IL-6 blockade, IL-1R blockade, 💡 Steroids ⊖ GvHD risk ⊖ On-target, off-tumor toxicity ⊖ T cell exhaustion 💡 Immune checkpoint inhibition
CAR-NK cells	⊕ HLA-independent antigen recognition → allogeneic approach without GvHD risk ⊕ Safety profile: • Limited production of CRS-related cytokines • Limited lifespan (2 weeks) • Limited OTOT due to self-identification of healthy cells via germline-encoded inhibitory receptors ⊕ Various cell sources (PB-NK, UB-NK, NK-92, iPSC) → allows streamlining of manufacturing ⊕ CAR-independent cytotoxicity via innate activating NK receptors → resistance to antigen-downregulation	⊖ Limited persistence in the absence of cytokine support: 💡 Insertion of membrane-bound IL-15 💡 Insertion of IL-15SA/IL-15RA fusion protein 💡 Induction of ectopic IL-15 💡 Production by NK cells 💡 CISH knockout ⊖ Poor tumor infiltration 💡 Arming NKs with chemokine receptors ⊖ Immunosuppressive TME 💡 Soluble PD-1 (sPD1) secreting NKs 💡 CAR-NKs targeting MDSCs, Tregs, M2 macrophages 💡 Insertion of dominant-negative TGFβ-receptor 💡 TGFβ knockout 💡 Decoy-resistant IL-18 💡 Inhibition of HIF1 signaling
CAR-MΦs	⊕ Several cell sources (peripheral blood cells, iPSCs, HSPCs) → allows streamlining of manufacturing ⊕ Good tumor infiltration in solid tumors ⊕ Diverse cytotoxicity mechanisms: phagocytosis, ADCC, antigen presentation, ROS, cytokine secretion ⊕ ECM degradation through MMP → migration of further immune cells ⊕ Low GvHD risk → allows use of allogeneic sources	⊖ Difficult genetic engineering: 💡 Viral vectors: adenoviral transduction 💡 Non-viral: nanobiomaterial assisted mRNA/pDNA insertion 💡 iPSC/HSPC as a starting material ⊖ Risk of polarisation to M2 macrophages: 💡 Adenoviral transduction 💡 IFNγ gene insertion 💡 M1 polarisation of CAR-M with IFN-γ ⊖ Potential for CRS toxicity (IL-6, IFNγ)
CAR-neutrophils	⊕ Diverse cytotoxicity mechanisms: phagocytosis, ADCC, antigen presentation, ROS, NETosis, cytokine secretion ⊕ Abundant immune cell population in the TME	⊖ Risk for polarisation to N2 phenotype through immunosuppressive TME 💡 CAR-insertion promotes 💡 Resistance to N2 polarization ⊖ Difficult genetic engineering (tightly controlled NA defence system) 💡 HPSCs as a starting material
CAR-iNKTs	⊕ HLA-independent antigen recognition → allogeneic approach without GvHD risk ⊕ Safety profile: • Limited production of CRS-related cytokines • No on-target, off-tumor toxicity detected ⊕ Good infiltration in solid tumors due to high chemokine receptor expression ⊕ CAR-independent target cell recognition: via TCR, DNAM-1, NCR ⊕ Reshaping of TME: lysis of TAMs, M2 to M1 polarization, eradication of MDSCs ⊕ Activation of further immune cells: B, T, DC, NK cells, macrophages	⊖ Low abundance in peripheral blood 💡 Use of more available sources: iPSCs, HSCs ⊖ Immune escape of tumor cells through CD1d downregulation 💡 Treatment with ATRA ⊖ Limited persistence: 💡 Coexpression of IL-15 💡 Stimulation with IL-21 💡 41BB as signaling domain
CAR-γδT cells	⊕ HLA-independent antigen recognition → allogeneic approach without GvHD risk ⊕ Safety profile: • Limited production of CRS-related cytokines • Limited lifespan ⊕ CAR-independent target cell recognition: TCR, NKRs, NCRs ⊕ Multiple cytotoxicity modalities: ADCC, granzyme B and perforin secretion, TRAIL and Fas/FasL interaction ⊕ Recruitment of further immune cells: antigen cross presentation, stimulation of DCs	⊖ Low abundance in peripheral blood ⊖ Polarization to an immunosuppressive γδTregs and γδ17T cells ⊖ CAR-Vδ2+ T cells: susceptibility to tonic signaling, AICD and exhaustion 💡 Use of δ2+ T 💡 Use of CCRs ⊖ Short lifespan ⊖ Poor T cell trafficking and tumor infiltration

Spear et al. 2013). NK cell inhibitory receptors, such as the killer cell immunoglobulin-like receptors (KIRs) recognize the classical HLA class-I molecules (HLA- A, -B, and -C) expressed on healthy cells and thereby ensure self-tolerance, a concept known as "missing-self" hypothesis (Yokoyama and Kim 2006). NK cells execute their cytotoxic function by releasing granules containing granzyme B and perforin (Smyth et al. 2005). Moreover, serial killing is possible via the NK cell death ligand receptors CD95/Fas and TNF-α-related apoptosis-inducing ligand (TRAIL) (Prager et al. 2019). Furthermore, NK cells can engage in antibody-dependent cellular cytotoxicity (ADCC) mediated by CD16, an Fc-receptor that recognizes antibodies bound to target cells (Wang et al. 2015). Apart from direct cytotoxicity, NK cells also contribute to immune regulation and augment anti-tumor responses through cytokine and chemokine secretion as well as recruitment of further immune cells to the TME, such as DCs (Böttcher et al. 2018). Interestingly, while traditionally considered part of the innate immune system, studies have demonstrated that NK cells can acquire immunological memory, a hallmark of adaptive immunity (Wu et al. 2020). "Trained immunity" is formed through direct antigen contact (Sun et al. 2009; Vivier et al. 2011) or by cytokine stimulation with interleukin-12 (IL-12), interleukin-15 (IL-15) and interleukin-18 (IL-18) (Cooper et al. 2009). Cytokine induced memory like (CIML) NK cells display a recall response akin to adaptive immune cells, demonstrated by their heightened secretion of IFN-γ and granzyme B upon restimulation with cytokines or tumor cells (Cooper et al. 2009; Keppel et al. 2013; Ni et al. 2012; Uppendahl et al. 2019) as well as their prolonged persistence (Keppel et al. 2013; Ni et al. 2012). In a study by Ni et al. (2012), IL-12/-15/-18 preactivated human NK cells delayed tumor growth of established tumors in xenogeneic mouse models and displayed rapid proliferation *in vivo*, which was dependent on endogenous IL-2 produced by CD4+ cells. Surprisingly, these preactivated NK cells were still detectable 3 months after adoptive transfer in mice that had rejected the tumors and had remained tumor free (Ni et al. 2012). In a phase I clinical trial, adoptively transferred CIML NK cells successfully expanded in AML patients, exhibited increased functionality and lead to complete remissions in 4 out of 11 evaluable patients (Romee et al. 2016). However, the preactivated NK cells were only detectable up to 14 days in the patients' peripheral blood (Romee et al. 2016). The incorporation of CAR into NK cells allows to harness the natural cytotoxicity and immunomodulatory functions of these cells and further enhance their ability to specifically recognize and target tumor cells.

2.2 CAR design for NK cells

The basic structure of CAR used in CAR-NK cells is generally similar to that of CAR-T cells, with at times differences in the signaling and costimulatory domains. Most of the studies involving CAR-NK cells have utilized CAR domains optimized for T-cell signaling, namely CD3ζ as an initial signaling domain and CD28 or 4-1BB as costimulatory domains. There also have been efforts to tailor CAR to the intracellular signaling pathways of NK cell activation by incorporating NK-cell-specific domains, such as the DNAX-activation protein 12 (DAP12) (Li et al. 2018; Müller et al. 2015; Töpfer et al. 2015), the DNAX-activation protein 12 (DAP10) (Chang et al. 2013), 2B4 (CD244) (Cifaldi et al. 2023; Huang et al. 2020; Li et al. 2018; Xu et al. 2019b), the DNAX accessory molecule 1 (DNAM1/CD266) (Huang et al. 2020), NKG2D (Li et al. 2018), CD16 (Li et al. 2018), NKp44 (Li et al. 2018) or NKp46 (Li et al. 2018) (Figure 2). DAP12 participates in signal transduction involving NK activation and DAP10 is involved in the signal transduction of NKG2D (Billadeau et al. 2003). CAR-NK cells signaling via DAP12 have demonstrated an increased specific cytotoxicity *in vitro* compared to NK cells with the conventional first-generation CAR signaling via CD3ζ (Töpfer et al. 2015). The incorporation of 2B4, a member of the signaling lymphocyte activation molecule (SLAM) family (Schmidt et al. 2020), as a costimulatory domain in CAR-NK cells led to a remarkable increase in NK cell activation, degranulation, IFN-γ and TFN-α secretion and anti-tumor cytotoxicity, compared to CAR-NK cells signaling via 41BB (Xu et al. 2019b). Similarly, screening of 9 different anti-Mesothelin CAR-NK cells with NK cell-specific stimulatory domains showed that CAR-NK cells incorporating the costimulatory domains 2B4 outperformed NK cells transduced with third generation T-cell based CAR in terms of cytotoxicity and activation (Li et al. 2018). Moreover, a further group demonstrated that 2B4 has a favorable role in the expansion of CAR-NK cells (Huang et al. 2020). CAR-NK cells signaling via DNAM1 demonstrated improved proliferation compared CD28-signaling CAR-NK cells, whereas co-expression of the signaling domains DNAM1 and 2B4 led to a maximal expansion and persistence of CAR-NK cells. Screening of 9 different anti-Mesothelin CAR-NK cells with NK cell-specific stimulatory domains showed that CAR-NK cells incorporating the costimulatory domains 2B4 outperformed NK cells transduced with third generation T-cell based CAR in terms of cytotoxicity and activation (Li et al. 2018). Furthermore, Li et al. could show that the transmembrane domain also influences CAR-NK cell mediated cytotoxicity, with CAR-NK cells incorporating the transmembrane domain of NKG2D leading to higher target cell lysis compared to the transmembrane domains

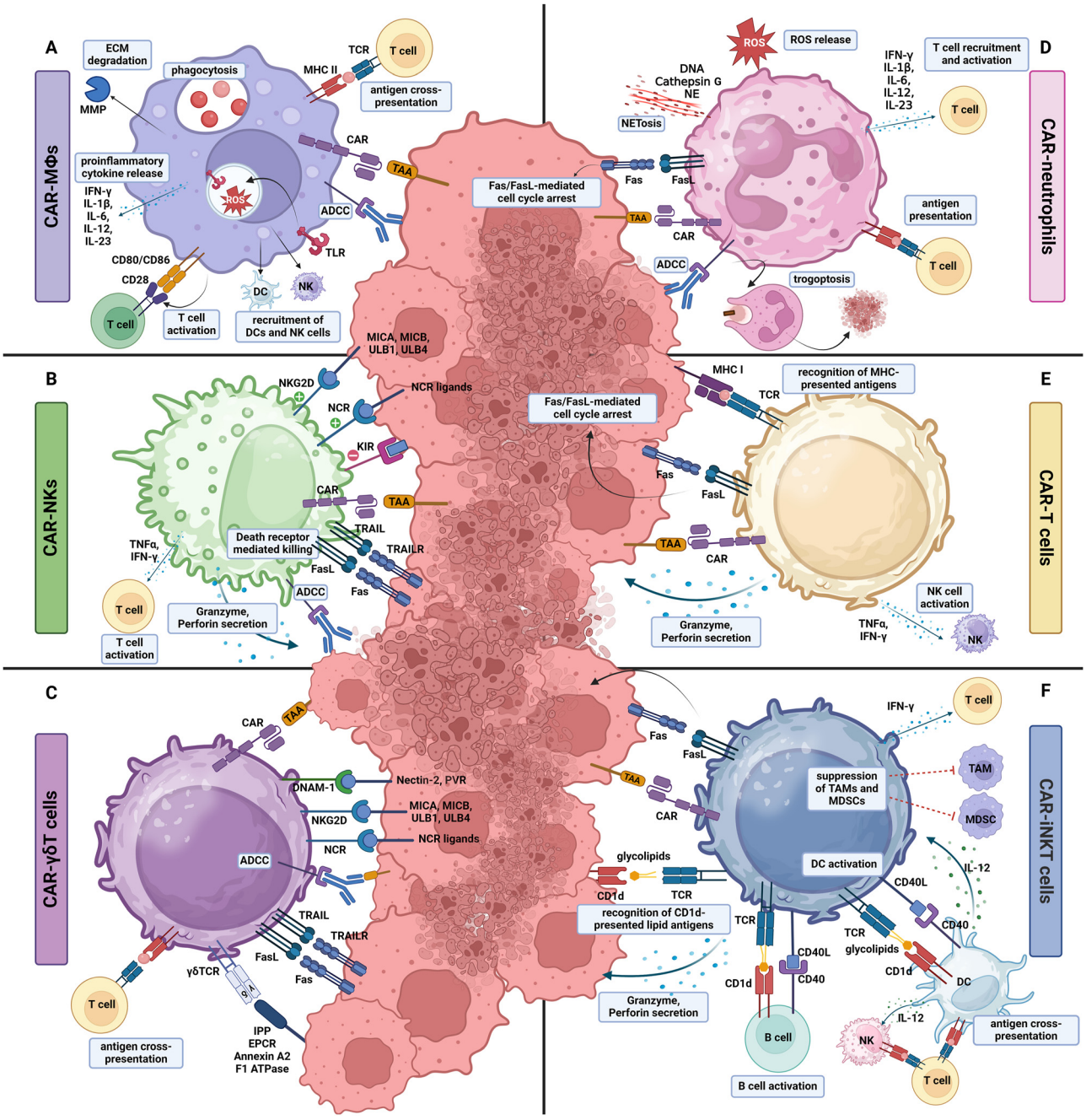

Figure 2: Killing mechanisms of CAR-cells. (A) CAR-macrophages infiltrate solid tumors through secretion of MMP and ECM degradation. They can recognize tumor cells via their CAR and CAR-independently via innate receptors (TLR). Apart from phagocytosing tumor cells and participating in ADCC, they can release proinflammatory cytokines, recruit further immune cells (NKs, DCs) and activate T cells through antigen presentation or interaction with CD28. (B) CAR-NK cells recognize cancer cells via their CAR and activating NK cell receptors. They induce anti-tumor toxicity through granzyme B/perforin secretion and death receptor mediated killing (FasL, TRAIL), participate in ADCC and activate T cells through cytokine secretion. (C) CAR-γδT cells recognize tumor cells via their CAR, but also through their γδTCR and coreceptors NKG2D, DNAM-1 and NCRs (NKp30, NKp44, NKp46). They can mediate anti-tumor toxicity through TRAIL and Fas/FasL pathways, granzyme B/perforin secretion, ADCC and antigen cross presentation to CD8+ and CD4+ T cells. (D) CAR-neutrophils recognize cancer cells via their CAR and their innate receptors and can mediate anti-tumor toxicity through FasL/Fas interaction, ADCC, NETosis, release of ROS, and trogoptosis. They can furthermore activate T cells through antigen-presentation and cytokine secretion. (E) CAR-T cells can recognize cancer cells via their CAR or TCR and mediate anti-tumor toxicity through granzyme B/perforin secretion and Fas/FasL-interaction. They can activate NK cells through cytokine secretion. (F) CAR-iNKT cells recognise TAA via their CAR and CD1d presented phospholipids via their TCR and mediate anti-tumor toxicity through granzyme B/perforin and the Fas/FasL pathway. They can stimulate T cells via IFN-γ and induce DC maturation through CD40/CD40L interaction. Activated DCs can thereafter cross-present antigens to T cells and activate iNKT, NK and T cells via IL-12. iNKT can also reduce the immunosuppressive activity of TAMs and MDSCs.

of NKp46, NKp44 or CD16 (Li et al. 2018). In summary, a multitude of CAR designs endow NK cell with tumor-targeting properties that may be exploited therapeutically but a definite answer on the optimal NK-related CAR design is still missing.

2.3 Manufacturing of CAR-NK cells

Different genetic engineering methods can be applied for CAR delivery into NK cells (Schmidt et al. 2020). Similar to CAR-T cells, the main viral methods applied for CAR-NK cells are lentiviral and retroviral transduction. Primary benefits of this approach are the possibility of inserting larger genes as well as the permanent gene integration into the cells. Lentiviral transduction seems to be more favorable, as it allows gene integration also in non-replicating primary NK cells (Naldini et al. 1996). Nevertheless, viral transduction of NK cells is challenging due to their innate defense mechanisms against foreign genetic material via pattern recognition receptors, such as Toll-Like Receptors (TLR) (Littwitz et al. 2013; Schmidt et al. 2020). Commonly used non-viral transfection methods for CAR-NK cells are mRNA or DNA electroporation, which result in only a short period of CAR-expression[44]. The transient expression of the construct provides a favorable safety profile but also requires the direct administration of the cell product (Ingegnere et al. 2019; Littwitz et al. 2013). Furthermore, although DNA and mRNA electroporation are simple and cost-efficient, they are associated with poor cell viability (Batista Napotnik et al. 2021). To optimize cell viability in nonviral transfection, Wilk et al. developed readily synthesized and inexpensive nonviral charge-altering releasable transporters (Wilk et al. 2020). The nonviral transposon systems Sleeping Beauty and PiggyBac have also been used by a few groups to successfully manufacture CAR-NK cells (Batchu et al. 2019; Du et al. 2021; Wang et al. 2018). CRISPR/Cas9 technology has been mainly adopted to knockout or insert genes to improve CAR-NK performance (Daher et al. 2021; Gurney et al. 2022, Levy et al. 2021; Ureña-Bailén et al. 2022) but was also used by a group for safe-harbor locus CAR-insertion (Naeimi Kararoudi et al. 2020).

2.4 Cell sources for CAR-NK cell generation

Due to their limited risk for GvHD, NK cells can be obtained from various autologous or allogeneic sources, including peripheral blood (PB-NK), umbilical cord blood (UB-NK), induced pluripotent stem cells (iPSC), human embryonic stem cells (hESCs) and established NK cell lines, such as the NK cell non-Hodgkin lymphoma cell line NK-92 (Laskowski et al. 2022; Zhang et al. 2023c). The advantages and limitations of each cell source have been extensively reviewed elsewhere (Laskowski et al. 2022; Shevtsov and Multhoff 2016).

2.5 CAR-NK cells against hematologic malignancies – preclinical and clinical studies

Most initial studies of CAR-NK cells focused on hematological malignancies. Anti-CD19 CAR-NKs from different cell sources and with different signaling domains have shown effective anti-tumor cytotoxicity against B-cell malignancies *in vitro* and *in vivo* (Boissel et al. 2009; Imai et al. 2005; Liu et al. 2020b; Luanpitpong et al. 2021; Ravi et al. 2020). In a study of Ravi et al., anti-CD19-CAR-NK-92 cells showed potent anti-lymphoma activity against Rituximab and Obinutuzumab resistant B cell lymphoma cells (Ravi et al. 2020). Apart from granule-mediated tumor cell apoptosis, anti-CD19 CAR-NK cells also participated in IFN-γ signaling and secretion of CCL3, an important chemotactic mediator essential for the recruitment of NK cells, T cells, macrophages and DCs to the TME (Ravi et al. 2020). These multimodal cytotoxicity mechanisms render CAR-NKs more potent than other therapy modalities such as monoclonal antibodies, as shown in a study of anti-CD20 CAR-NK cells against primary CLL samples (Boissel et al. 2013). Augmenting anti-CD19 CAR-NK cells with the human CXCR4 gene improved homing of NK cells to the bone marrow (Jamali et al. 2020). CRISPR-Cas9 gene knockout of NK cell immune checkpoints further enhanced anti-CD19 CAR-NK cell cytotoxicity (Ureña-Bailén et al. 2022). Following the trend of bispecific CAR-T cells, anti-CD19/CD22 bispecific CAR-NK cells against B cell lymphoma (Kim et al. 2023) and anti-BCMA/CD19 CAR-NK cells against multiple myeloma (MM) (Roex et al. 2022) demonstrated superior efficacy to CAR NKs targeting a single antigen. In a phase I/II clinical trial, 8 out of 11 patients with CD19-positive leukemia or lymphoma had an objective response to therapy with anti-CD19-CAR NK cells following lymphodepleting chemotherapy (Liu et al. 2020a). One notable aspect of this trial was the safety profile of CAR-NK cell therapy. The administration of CAR-NK cells did not result in the development of cytokine release syndrome (CRS), neurotoxicity, or graft-versus-host-disease (GvHD) (Liu et al. 2020a). Furthermore, the infused CAR-NK cells demonstrated expansion and persistence in the patients' bodies, albeit at low levels, for at least 12 months. However, all patients available at follow up eventually went on to receive additional or concomitant treatment, leaving the final assessment of long-term CAR-NK cell activity uncertain.

The treatment of acute myeloid leukemia (AML) remains a challenge in the field of CAR-T cell therapy (Haslauer et al. 2021). CAR-NK cells may be an attractive alternative therapeutic strategy due to the recognition of AML blasts by innate activating NK cell receptors, such as NKG2D (Baragaño Raneros et al. 2019; Gurney and O'Dwyer 2021). CAR NK cells targeting CD33 (Albinger et al. 2022; Zhang et al. 2023b), CD123 (Morgan et al. 2021), FLT3 (Oelsner et al. 2019) and CD38 (Gurney et al. 2022) were able to effectively kill AML cells *in vitro* and *in vivo*. CD38-CAR NK cells also raise the concern of fratricide, as CD38, an immunotherapeutic target in MM and AML, is also expressed on primary NK cells. Gurney et al. managed to overcome this risk through a CD38 knockout prior to anti-CD38-CAR expression (Gurney et al. 2022). Unfortunately, in a phase I clinical trial of anti-CD33 CAR-NK cells against relapsed or refractory (R/R) AML, no notable clinical efficacy was demonstrated (Tang et al. 2018), while no significant adverse effects were observed.

Lastly, CAR-NK cells have emerged as a promising alternative to CAR-T cells for the treatment of T-lymphoid malignancies due to their ability to overcome the challenge of fratricide, which occurs when CAR-T cells target both normal and malignant T cells sharing the same antigens (Alcantara et al. 2018). Due to their innate repertoire of inhibitory receptors, NK cells can selectively target and eliminate malignant T cells, while sparing healthy T cells. Anti-CD5 CAR-NK (Chen et al. 2017; Voynova et al. 2022), anti-CD7 CAR-NK (You et al. 2019) and anti-CD4 CAR-NKs cells (Pinz et al. 2017) have shown specific cytotoxicity against T cell malignancies in preclinical studies.

2.6 CAR-NK cells against solid tumors – clinical and preclinical studies

There has been increasing interest in exploring the potential of CAR-NK cells for solid tumor therapy as well. CAR-NK cells targeting the epidermal growth factor receptor (EGFR) (Liu et al. 2020c), the human epidermal growth factor receptor 2 (HER2) (Portillo et al. 2021; Uherek et al. 2002; Xia et al. 2023), Mesothelin (Li et al. 2018), EpCAM (Zhang et al. 2018b), the epidermal growth factor receptor variant III (EGFRvIII) (Müller et al. 2015) or the prostate stem cell antigen (PSCA) (Töpfer et al. 2015), have demonstrated potent antigen-specific cytotoxicity in preclinical studies. To our knowledge, there are two main preclinical studies benchmarking CAR-NK against CAR-T cells: In the study of Portillo et al., HER2-targeting CAR-NK cells exhibited superior cell-mediated tumor cell killing *in vivo* (Portillo et al. 2021), while the research of Li et al. demonstrated an equivalent ability of Mesothelin-targeting CAR-NK and CAR-T cells to reduce tumor burden (Li et al. 2018). Furthermore, CAR-NK cells could be a potential alternative therapy for specific therapy-resistant tumor entities. For instance, EGFR-targeting CAR-NK cells have shown potential as an alternative treatment for triple negative breast cancer (TNBC) (Liu et al. 2020c) and anti-CEA CAR-NK cells could effectively treat 5-FU-resistant CEA-expressing colorectal cancer cells (Shiozawa et al. 2018) in preclinical studies. Clinical trials have been initiated to evaluate the safety and therapeutic potential of CAR-NK cells in solid tumors (Table 2).

2.7 Advantages

CAR-NKs can circumvent some of the challenges associated with T cell-based immunotherapy (Table 1). Due to the CAR-T cell associated risk of GVHD, manufacturing of CAR-T cells depends mainly on autologous T cells, rendering many patients ineligible for therapy, considering that their immune cells are often diminished or dysfunctional due to heavy pretreatment. A unique advantage of NK cells is the HLA-independent recognition of target antigens, which allows their use in an allogeneic setting without the risk of GVHD (Miller et al. 2005). The ability to generate CAR-NK cells from diverse cell sources simplifies the manufacturing process and enhances the accessibility of CAR-NK cell therapy. Furthermore, the intrinsic capacity of NK cells to recognize tumor cells via their native receptors makes them resistant to antigen escape, as they can bypass the need for antigen-specific targets. A further significant advantage of CAR-NK cells over CAR-T cells is their reduced risk of adverse effects, as demonstrated in clinical trials of haploidentical NK cell transfer (Bachanova et al. 2014; Curti et al. 2011; Geller et al. 2011; Iliopoulou et al. 2010; Miller et al. 2005) and two trials of CAR-NK cell transfer (Liu et al. 2020a; Tang et al. 2018). The low risk of CAR-NK cells for neurotoxicity, and CRS can be attributed to their distinct cytokine profile (Li et al. 2018). Namely, in an ovarian cancer xenograft model, anti-Mesothelin CAR-T or CAR-NK cells exhibited similar anti-tumor cytotoxicity, however the CAR-T cell treated mice experienced significant body weight loss, severe visceral hemorrhage and ischemia and demonstrated increased TNF-α and IL-6 levels, eventually leading to markedly poorer survival (Li et al. 2018). Early clinical trials confirm the safe cytokine profile of CAR-NK cells. Namely, the levels of IL-6, TNF-α and IFN-γ remained stable in patients treated with anti-CD19 CAR-NK cells (Liu et al. 2020a). In a further phase I clinical study on anti-CD33 CAR-NK-92 cells, IL-6 and IL-10 were drastically increased on day 6, but quickly changed back to normal, whereas the levels of IL-17A, IL-2, IL-4, TNF-α and IFN-γ did not rise (Tang et al. 2018). Additionally, the

Table 2: Overview of clinical trials on CAR-NK cells, CAR-macrophages, CAR-γδT cells and CAR-iNKT cells. B-cell lymphoma, BCL; relapsed/refractory, R/R; small-cell lung cancer, SCLC; non-small-cell lung cancer, NSCLC; non-Hodgkin lymphoma, NHL; B-cell non-Hodgkin lymphoma, B-NHL; myelodysplastic syndrome, MDS; acute lymphoblastic leukemia, ALL; acute myeloid leukemia, AML.

CAR-cell	Target	Tumor entity	Identifier	Cell source	Phase	Status
NK cells	CD19	B-lineage ALL	NCT00995137	PB-NK from haplo-identical donor	I	Completed, no results posted
		R/R CD19+ lymphoid malignancies	NCT02892695	Not disclosed	I/II	Unknown
		R/R ALL/ CLL/B-NHL	NCT03056339	CB-NK	I/II	Completed, no results posted
		R/R B-NH	NCT03824951	iPSC	early I	Unknown
		R/R B-NH	NCT03690310	iPSC	early I	Unknown
		R/R B-NHL/ B-ALL	NCT05020678	PB-NK	I	Recruiting
		BCL, CLL	NCT04245722	iPSC	I	Ongoing, not recruiting
		NHL	NCT04555811	iPSC	I	Ongoing, not recruiting
		NHL	NCT05472558	CB NK	I	Recruiting
		R/R NHL	NCT04639739	Not disclosed	early I	Not yet recruiting
		R/R B-cell NHL	NCT04887012	Not disclosed	I	Recruiting
		R/R B Lymphoid Malignancies	NCT04796675	CB-NK	I	Recruiting
		R/R B-cell Acute Lymphoblastic Leukemia (B-ALL) and in combination with Rituximab	NCT05379647	Allogeneic PB-NK	I	Recruiting
		R/R B-NHL	NCT05336409	iPSC	I	Recruiting
		R/R B-NHL, R/R AML	NCT04023071	iPSC	I	Ongoing, not recruiting
		DLBCL	NCT05673447	Not disclosed	early I	Not yet recruiting
		B-cell Malignancies	NCT05645601	Not disclosed	I	Recruiting
		B cell malignancies	NCT05654038	Not disclosed	I/II	Recruiting
		B-cell maliganncies	NCT05410041	Not disclosed	I	Recruiting
		ALL	NCT05563545	Not disclosed	I	Completed
	CD19/CD22	R/R B-NHL	NCT03824964	Not disclosed	early I	Unknown
	CD19/CD70	NHL	NCT05667155	CB NK	I	Not yet recruiting
	CD70	AML, MDC, B-cell Lymphoma	NCT05092451	CB NK	I/II	Not yet recruiting
	CD5	Hematological maliganncies	NCT05110742	CB NK	I/II	Not yet recruiting
	NKG2DL	AML, MDS	NCT04623944	PB NK	I	Recruiting
		Solid tumors	NCT03415100	PB NK	I	Unknown
		Solid tumors	NCT05528341	NK-92	I	Recruiting
		AML	NCT05247957	Not disclosed	NA	terminated
		Metastatic colorectal cancer	NCT05213195	Not disclosed	I	Recruiting
	CD7	Leukemia, Lymphoma	NCT02742727	NK-92	I/II	Unknown
	CD22	R/R B-NHL	NCT03692767	Not disclosed	I	Unknown
	CD33	R/R AML	NCT02944162	NK-92	I/II	Unknown
		R/R AML	NCT05008575	Not disclosed	I	Recruiting
	CD33/CLL1	AML	NCT05215015	Not disclosed	early I	Recruiting
	BCMA	R/R MM	NCT03940833	NK-92	I/II	Unknown
		R/R MM	NCT05008536	CB-NK	I	Recruiting
		MM	NCT05652530	Not disclosed	early I	Recruiting
		MM	NCT05182073	iPSC	I	Recruiting
	CD123	AML	NCT05574608	Not disclosed	early I	Recruiting
	MUC1	MUC1 Positive R/R Solid Tumor	NCT02839954	iPSC	I/II	Unknown
	HER2	Recurrent HER2-positive glioblastoma	NCT03383978	NK-92	I	Recruiting
	PSMA	Castration-resistant prostate cancer	NCT03692663	iPSC	early I	Recruiting
	Mesothelin	Ovarian cancer	NCT03692637	PB NK	early I	Unknown
	ROBO1	Advanced solid tumours; pancreatic cancer	NCT03931720	iPSC	I/II	Unknown
		Pancreatic cancer	NCT03941457	NK-92	I/II	Unknown
	DLL3	SCLC	NCT05507593	not disclosed	I	Recruiting
	MICA/ MICB	Advanced solid tumors	NCT05395052	iPSC	I	Active, not recruiting

Table 2: (continued)

CAR-cell	Target	Tumor entity	Identifier	Cell source	Phase	Status
	CLDN6	Advanced solid tumors	NCT05410717	Not disclosed	I/II	Recruiting
	5T4	Advanced solid tumors	NCT05137275	Not disclosed	early I	Recruiting
		Advanced Solid Tumors	NCT05194709	Not disclosed	early I	Recruiting
	PD1	Recurrent/metastatic gastric or head and neck cancer	NCT04847466	NK-92	II	Recruiting
		Advanced NSCLC	NCT03656705	NK-92	I	Recruiting
		Advanced solid cancers	NCT04050709	NK-92	I	Active, not recruiting
MΦ	HER2	HER2 overexpressing solid tumor	NCT04660929	Autologous macrophages	I	Recruiting
	HER2	Breast cancer	NCT05007379	Not disclosed	I	not yet recruiting
	GPC3	Solid tumors	NCT05164666	Not disclosed	I	Recruiting
	Mesothelin	Advanced or metastatic solid tumors	NCT05164666	Autologous macrophages	I	Recruiting
γδT cells	CD7	T-ALL	NCT04702841	γδT cells	early I	Unknown
	NKG2DL	Advanced solid tumors or hematological malignancies	NCT05302037	Allogeneic γδT cells	I	Not yet recruiting
		R/R solid tumors	NCT04107142	Haplo-/allogeneic γδT cells	I	Unknown
	CD20	B cell malignancies	NCT04735471	Allogeneic γδT cells	I	Recruiting
	CD19	B-cell Lymphoma, ALL, CLL	NCT02656147	Allogeneic γδT cells	I	Unknown
		R/R NHL	NCT05554939	Allogeneic γδT cells	I	Recruiting
	CD123	AML	NCT05388305	γδT cells	I	Recruiting
	CD123	AML	NCT04796441	γδT cells	I	Unknown
iNKT cells	CD19	R/R B-NHL, ALL, CLL	NCT05487651	Allogeneic NKT cells	I	Recruiting
		R/R B cell malignancies	NCT03774654	Allogeneic NKT cells	I	Recruiting
		ALL, B cell lymphoma	NCT04814004	Autologous NKT cells	I	Recruiting
	GD2	R/R neuroblstoma	NCT03294954	Autologous NKT cells	I	Recruiting

natural ability of NK cells to discriminate between healthy and malignant cells via their germline-encoded inhibitory receptors, reduces their on-target off-tumor toxicity as they can specifically target tumor cells while sparing normal cells. In the study of Portillo et al. benchmarking CAR-T against CAR-NK cells, the latter did not mediate off-tumor-on-target toxicity, when cultured with non-malignant human lung epithelial cells expressing basal levels of HER2 (Portillo et al. 2021). Nevertheless, several research groups have incorporated switchable mechanisms to eliminate NK cells in case of toxicity, such as the pharmacologically inducible caspase-9-based suicide gene, which allows for controlled elimination of NK cells if necessary (Liu et al. 2018; Oelsner et al. 2019).

2.8 Challenges and solutions

While CAR-NK cells present a viable therapeutic candidate for alleviating certain limitations and safety concerns linked to CAR-T cell therapy, they are not exempt from encountering their own set of challenges. A major drawback is the limited persistence of NK cells in the absence of cytokine support (IL-2 and IL-15). Expression of a membrane-bound form of IL-15 (mIL-15) in human PB-NK cells has been shown to augment NK cell survival and expansion *in vitro* and *in vivo* without the need of additional exogenous cytokines (Imamura et al. 2014). Other approaches include the insertion of a IL-15 receptor fusion construct into NK cells, comprising of an IL-15 superagonist and the IL-15 receptor (IL-15SA/IL-15RA) (Kim et al. 2016) or genetic engineering of CAR-NK cells to ectopically produce IL-15 (Liu et al. 2018). Greater *in vivo* persistence and cytotoxic function of NK cells was achieved by knocking out the CISH gene, which encodes the Cytokine-inducible Src homology 2-containing (CIS) protein, a key negative regulator of IL-15 signaling (Daher et al. 2021). The hostile tumor microenvironment (TME) presents another challenge. For instance, the interleukin-18-binding protein (IL18BP), abundantly present in the TME, neutralizes IL-18 required for NK activation (Dinarello et al. 2013). To counteract this, a decoy-resistant IL-18 has been developed to enhance NK cell activity and maturation. Additionally, efforts have been made to overcome the immunosuppressive effects of TGF-β in the TME, such as using dominant negative TGF-β receptors (Yvon et al. 2017) or knocking out the TGF-β-induced miR-27-5p (Yvon et al.

2017), resulting in increased NK cell cytotoxicity *in vivo*. CAR-NKs have been developed against components of the immunosuppressive TME, such as CSF1R-CAR-NK cells (Zhang et al. 2018a) targeting M2 like tumor associated macrophages (TAMs), or NK-CARs equipped with NKG2Dz (Parihar et al. 2019) that circumvent suppression via MDSCs. Modulating the regulatory checkpoint ADAM17, responsible for CD16 shedding, has shown potential in blocking CD16a shedding and enhancing ADCC (Romee et al. 2013), which is often compromised in the immunosuppressive TME. Poor trafficking to solid tumors poses a further challenge of adoptive NK cell therapy. Arming CAR-NK cells with chemokine receptors, such as CCR7 and CXCR2 has shown improved homing of NK cells to the tumor bed (Kremer et al. 2017; Ng et al. 2020).

3 Macrophages

3.1 Properties

Macrophages are cells of the innate immune system that predominantly arise in the bone marrow from common myeloid progenitors (CMP), which develop into monocytes, move into the bloodstream and ultimately cross the walls of capillaries into the connective tissue (Davies et al. 2013). Tissue-resident macrophages can recognize conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) on pathogens through germline-encoded pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), scavenger receptors, and C-type lectin receptors (Akira et al. 2001; Taylor et al. 2005). Upon detection of microbial components or altered self-molecules on malignant cells, macrophages can eliminate target cells through phagocytosis and the release of reactive oxygen and nitrogen species (ROS/iNOS) (Fang 2011). Moreover, as professional antigen-presenting cells (APC), macrophages can cross-present antigens on MHC class II molecules and stimulate CD4+ T helper cells (Th), thereby activating adaptive immunity. Via their Fc-receptors, they can also participate in ADCC. TAMs represent a predominant immune cell population in the TME (up to 50 %) in various types of cancer (van Ravenswaay Claasen et al. 1992). They are attracted to tumors via chemokines (Huynh et al. 2023) and act as a double-edged sword by undergoing distinct polarization states (Jayasingam et al. 2019). Classically activated or M1 like macrophages, exhibit tumoricidal activities, release pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12, and IL-23) and upregulate markers associated with antigen presentation (major histocompatibility complex class II (MHC-II), CD80 and CD86) (Jayasingam et al. 2019). On the other hand, alternatively activated or M2 macrophages display an immunoregulatory function, producing anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-β), and supporting tumor progression and metastasis (Jayasingam et al. 2019). Adoptive transfer of unmodified macrophages has failed to control tumor growth (Andreesen et al. 1998), highlighting the need for additional signals to effectively combat tumors. In an early attempt to redirect macrophages against tumor cells, the high affinity Fc-receptor for IgG CD64 was fused with a scFv against CEA resulting in antigen specific cytotoxicity (Biglari et al. 2006). Following this innovative approach, several groups have tried to enhance the inherent anti-tumor capabilities of macrophages by equipping these cells with a CAR (Table 2).

3.2 CAR design

The CAR constructs utilized in CAR-macrophages (CAR-MΦ) share the fundamental components seen in CAR T cells. Several research groups have applied conventional T cell signaling domains into CAR-MΦ leading to relevant functionality (Klichinsky et al. 2020; Pierini et al. 2020; Zhang et al. 2020; Zhang et al. 2023a). In an effort to adapt the CARs to innate signaling pathways of macrophages, several signaling domains have been tested, such as the Fc receptor for IgG (FcγR) (Zhang et al. 2020), DAP12 (Paasch et al. 2022), multiple EGF-like domains 10 (Meg10) (Morrissey et al. 2018), the proto-oncogene tyrosine-protein kinase (MerTK) (Morrissey et al. 2018; Niu et al. 2021), TLRs (Niu et al. 2021), or the brain-specific angiogenesis inhibitor 1 (Bai1) (Morrissey et al. 2018) (Table 3 and Figure 2). CAR-macrophages bearing the signaling domains CD3ζ or FcγR, exhibited similar function, which could be explained by the homology of the CD3ζ to FcεRI-γ, a canonical signaling molecule for antibody-dependent cellular phagocytosis (ADCP) (Zhang et al. 2020). Interestingly, incorporating DAP12 as a signaling domain in CAR-MΦ decreased phagocytosis rates compared to conventional signaling domain CD3ζ (Paasch et al. 2022). This discrepancy could potentially be attributed to the different immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3ζ (three ITAMs) and DAP12 (one ITAM). Notably, a similar correlation between CAR performance and intracellular ITAM signaling domains has been previously observed in CAR-T cells (James 2018). Screening of several murine phagocytic receptors showed that CAR-MΦwith MerTK or Bai1 did not bind or phagocytose their target, whereas CAR-MΦ signaling via ITAM-containing intracellular domains CD3ζ, Megf10 or FcRγ were capable of phagocytosing beads and cancer cells (Morrissey et al. 2018). However, Niu et al. presented contradictory results, showing that CAR-MΦ with the MerTK activation domain displayed

the highest tumor cell toxicity (Niu et al. 2021). Interestingly, the signaling domain influences the capacity of CAR-MΦ for whole-cell engulfment instead of trogocytosis or nibbling (Morrissey et al. 2018). Based on the theory that whole-cell engulfment strongly correlates with phosphoinositide 3-kinase (PI3K) signaling, a tandem CD19-CAR was developed, in which the portion of the CD19 cytoplasmic domain recruits the p85 subunit of PI3K (Morrissey et al. 2018). The tandem CAR performed a 3-fold increase in whole-cell engulfment, compared to macrophages with the signaling domains FcγR or Megf10 (Morrissey et al. 2018). Similar to CAR-NK cells, the ideal design of a CAR tailored to macrophages remains unknown.

3.3 Manufacturing of CAR-macrophages

One of the major challenges of CAR-MΦ manufacturing is transgene delivery into myeloid cells. As innate immune cells, monocytes and macrophages can detect foreign nucleic acids and respond via inflammatory programs and apoptosis (Bartok and Hartmann 2020). Due to this tightly controlled nucleic acid defense system, macrophages are resistant to genetic manipulation (Bartok and Hartmann 2020). Lentiviral transduction methods, commonly employed for CAR-T cells, presents challenges when applied to monocytes and macrophages due to the myeloid-specific HIV-1 restriction factor which hinders efficient reverse transcription. A solution would be the use of lentiviral particles derived from HIV-1 with the viral accessory protein Vpx, which facilitates degradation of the myeloid-specific HIV-1 restriction factor (Bobadilla et al. 2013; Laguette et al. 2011). Adenoviral transduction with the chimeric Adenovirus 5-fiber 35 vector (Ad5f35) has emerged as a highly effective method for gene delivery into macrophages based on the expression of CD46 on macrophages and monocytes, which serves as the docking protein for adenoviruses like Ad35 (Gaggar et al. 2003). A unique advantage of adenoviral transduction is that it not only yields sufficient gene insertion, but it also activates the inflammasome (Lam et al. 2014; Muruve et al. 2008) and locks macrophages in a pro-inflammatory M1 like phenotype (Klichinsky et al. 2020). To facilitate non-viral gene delivery, several groups have employed nanobiomaterials, such as lipid nanoparticles (LPN), which can complex mRNA in a stable core, protect it from degradation and aid intracellular delivery (Cullis and Hope 2017). A significant benefit of employing nanobiomaterials lies in their capacity for CAR delivery into macrophages *in vivo* (Gao et al. 2023; Kang et al. 2021). Intratumorally injected nanoparticles loaded with plasmid-DNA (pDNA) encoding an anti-HER2 CAR, were able to locoregionally transfect macrophages in a GBM patient-derived xenograft model (Gao et al. 2023). In a further study, macrophages could be successfully transfected with mRNA using an LPN-based delivery system, resulting in CAR-macrophages with significant anti-tumor toxicity (Ye et al. 2022b). While there is limited information on the persistence of CAR-expression on macrophages following mRNA-delivery, transfection of T cells with mRNA typically results in CAR expression lasting up to 1 week (Parayath et al. 2020). Despite the transient expression of proteins after mRNA transfection, repetitive mRNA delivery using nanobodies can still yeild sustained CAR expression within host lymphocytes, resulting in comparable *in vivo* cytotoxicity to retrovirally transduced CAR-T cells (Parayath et al. 2020). This transient nature of mRNA-based CAR-delivery presents a potential solution to the drawbacks of viral transduction, such as permanent CAR-expression associated with toxicity, random insertion into the host genome, and low titers in nondividing cells (Parayath et al. 2020; Ye et al. 2022b).

3.4 Cell sources for CAR-MΦ

Proof-of-concept studies for CAR-MΦ can be performed in model cell lines such as the human leukemia monocyte cell line THP-1 (Klichinsky et al. 2020) or the murine macrophage cell line Raw 264.7 (Zhang et al. 2019) or with primary/immortalized murine bone marrow derived macrophages (BMDM) (Liu et al. 2022). For preclinical and clinical applications, peripheral blood CD14+ monocytes can be isolated from peripheral blood mononuclear cells (PBMC) and differentiated to macrophages in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). iPSC present a renewable, allogeneic source for CAR-MΦ therapies, which is discussed below.

3.5 Efficacy of CAR-macrophages

Although the majority of CAR-MΦ research remains in the preclinical stage, it showcases promising efficacy in combating both hematological malignancies and solid tumors. Phagocytosis of cancer cells expressing the CAR-target, is one of the main ways CAR-macrophages mediate cytotoxicity. CD19-targeting CAR-macrophages performed specific engulfment and elimination of CD19+ beads as well as Raji B cells *in vitro* (Morrissey et al. 2018). However, CAR-MΦ mainly exerted trogocytosis or nibbling of the target cells, instead of whole-cell engulfment. The inhibition of the CD47-mediated "do not eat me signal" through a CD47-targeting antibody, has been shown to enhance phagocytosis rates (Chen et al. 2022;

Table 3: Overview of the preclinical studies on CAR-macrophages, CAR-iNKT cells, CAR-γδT cells and CAR-neutrophils. CD8-hinge domain, CD8H; CD28 transmembrane domain, CD28TM; CD8 transmembrane domain, CD8TM; CD28 intracellular domain, CD28ICD; CD3ζ transmembrane domain, CD3ζTM; CD3ζ intracellular domain, CD3ζICD; hematopoietic stem and progenitor cells, HSPCs; castration resistant prostate cancer, CRPC; non-small-cell lung cancer, NSCLC; triple negative breast cancer, TNBC; diffuse large B cell lymphoma, DLBCL; Chondroitin sulfate proteoglycan 4, CSPG4; human Fc-region, hFc.

Cell type	CAR-target	Target cell	Macrophage source	Tested CAR constructs	CAR insertion method	References
MΦ	CD19 CD22	Raji B cells	J774A.1 macrophages	Anti-CD19-scFv-CD8H-CD8(TM)-MegF10Anti-CD22-scFv-CD8H-CD8(TM)-MegF10Anti-CD19-scFv-CD8H-CD8(TM)-FcRγAnti-CD19-scFv-CD8H-CD8(TM)-FcRγ-PI3KAnti-CD19-scFv-CD8H-CD8(TM)-Bai1Anti-CD19-scFv-CD8H-CD8(TM)-MerTKAnti-CD19-scFv-CD8H-CD8(TM)-CD3ζ	Lentiviral transduction	(Morrissey et al. 2018)
	ALK	ALK-expressing neutroblastoma	Macrophages	Anti-ALK-scFv-CD8H-CD28(TM)-CD28(ICD)-IFNγ	Non-viral vector delivery of nanocomplexes consisting of CAR-IFNγ-vector and jetPEI--macrophage (MPEI)	(Kang et al. 2021)
	CD133	GBM	THP-1Bone marrow-derived macro-phagesRAW 264.7 macrophages	Anti-CD133-scFv-CD8H-CD8(TM)-CD3ζ	Nonviral vector delivery via nanoporter (NP) hydrogel	(Chen et al. 2022)
	CD19	B cell lymphoma	RAW264.7Bone marrow derived macrophages (BMDMs)	Anti-CD19-scFv-CD8H-CD8(TM)-41BB-CD3ζ	Non-viral vector, lipid nano-particle (LNP)	(Ye et al. 2022a)
	HER2	SKOV3 ovarian cancer cell line	Peripheral blood monocytes	Anti-HER2-scFv-CD8H-CD28(TM)-CD3ζ	Adevoviral transduction	(Klichinsky et al. 2020)
	HER2	HER2-positive CT26	Murine BM derived macrophages	Not disclosed	Adenoviral transduction	Pierini et al. (2020)
	CD19	CD19-positive K562 cells	THP-1	CD19-CD8H-CD28(TM)-CD3ζ	Lentiviral transduction	(Klichinsky et al. 2020)
	Msln	Mesothelin-positive K562 cells	THP-1	Anti-Mesothelin-scFv-CD8H-CD28(TM)-CD3ζ	Lentiviral transduction	(Klichinsky et al. 2020)
	HER2	HER2-positive K562 cells	THP-1	Anti-HER2-scFv-CD8H-CD28(TM)-CD3ζ	Lentiviral transduction	(Klichinsky et al. 2020)
	HER2	Human HER2-overexpressing 4T1 cells	Raw264.7 macrophages	Anti-HER2-scFv-mouseIghG1(hinge)-mouseCD147	Not mentioned	(Zhang et al. 2019)
	CCR7	CCR7-expressing immunosu-pressive cells of the TMETumor models: 4T1-Luc	Raw264.7 macrophages	CCL19-CD8H-MerTKCCL19-CD8H-41BB-CD3ζCCL19-CD8H-TLR2CCL19-CD8H-TLR4CCL19-CD8H-TLR6	Lentiviral transduction	(Niu et al. 2021)
	CEA	CEA-positive HT1080 cells	THP-1HSPCs	Anti-CEA-scFv-IghG1(hinge)-2B4-DAP12Anti-CEA-scFv-IghG1(hinge)-CD28(TM)-CD28(ICD)-CD3ζ	Lentiviral transduction	(Paasch et al. 2022)
		CEA-positive cell lines CRE8 and MKN45K	Peripheral blood monocytes	Anti-CEA-scFv-hFc-CD64(TM)-CD64(ICD)	Adenoviral transduction	(Biglari et al. 2006)
	GD2	CHLA-20-AkaLuc-GFP neuroblas-toma cellsWM266-4-AkaLuc-GFP melanoma cells	hPSC	Anti-CD2-scFv-CD28TM-CD28(ICD)-OX40-CD3ζ	CRISPR knock-in of the CAR in a safe-harbor locus	(Zhang et al. 2023)

Table 3: (continued)

Cell type	CAR-target	Target cell	Macrophage source	Tested CAR constructs	CAR insertion method	References
	CD19	CD19-positive K562 cells	iPSC	Anti-CD19-scFv-CD8H-CD3ζ	Lentiviral transduction	(Zhang et al. 2020)
	Msln	OVCAR3 ovarian cancer cell-sASPC1 pancreatic cancer cellsHO8910-Luc ovarian cancer cells	iPSC	Anti-Msln-scFv-CD8H-CD3ζ	Lentiviral transduction	(Zhang et al. 2020)
	HER2	Luciferase-positive GL261-H glioblastoma cells	Murine macrophages	Anti-HER2-scFv-CD8H-CD8(TM)-CD3ζ	Non-viral vector,locoregional administration of nano-particles loaded with pDNA	(Gao et al. 2023)
NKT	GD2	Neuroblastoma cell lines	Human iNKT	Anti-GD2-scv-hinge-CD28(TM)-CD28(ICD)-CD3zAnti-GD2-scv-hinge-CD28(TM)-41BB-CD3zAnti-GD2-scv-hinge-CD28(TM)-CD28ICD-41BB-CD3z	Retroviral transduction	Heczey et al. (2014)
	GD2	Neuroblastoma cell lines	Human iNKT	Anti-GD2-scFv-CD8TM-CD28-CD3ζ-2A-IL15	Retroviral transduction	(Xu et al. 2019)
	CD19	CML cell line K562, plasma cell leukemia cell line ARH-77, MM cell lines KMS-12-BM, NCI-H929 and U266	Human iNKT	Anti-CD19-scFv-CD28-CD3ζAnti-CD19-scFv-CD28-OX40-CD3ζ	Lentiviral transduction	(Rotolo et al. 2018)
	CD19	CML cell line K562, Burkitt lymphoma cell lines Raji and Daudi	Human iNKT	Anti-CD19-scFv-IgG1(hinge)-CD8(TM)-41BB-CD3ζ	Retroviral transduction	(Tian et al. 2016)
	TCRVβ1/2/9	T cell lymphoma cell line JRT3-T3.5	Human iNKT	Anti-TCRVβ1-scFv-CD8H-CD28(ICD)-CD3ζAnti-TCRVβ2-scFv-CD8H--CD28(ICD)-CD3ζAnti-TCRVβ9-scFv-CD8H-CD28(ICD)-CD3ζ	Lentiviral transduction	(Rowan et al. 2023)
	BCMA CD38	MM cell lines UM9, MM1.s and MM1.s-CD1d	Human iNKT	Anti-BCMA-scFv-CD8TM-41BB-CD3ζAnti-BCMA-scFv-CD8TM-41BB–CD3ζAnti-CD38-scFv-CD28TM-CD28(ICD)-CD3ζAnti-CD38-scFv-CD28TM-CD28(ICD)-CD3ζ-41BBL	Retroviral transduction	(Poels et al. 2021)
	CSPG4CEA		Human iNKT	Anti-CSPG4-scFv-CD28-CD3ζAnti- CEA-scFv-CD28-CD3ζ	mRNA electroporation	(Simon et al. 2018)
γδT	GD2,CD19	neuroblastoma cell line LAN1 erythroleukemia cell line K-562, Burkitt lymphoma cell lines Raji and Daudi, ALL cell line Reh	Human primary γδ2+ T cells	Anti-GD2-scFv-IghG1(hinge)-CD3ζ(TM)-CD3ζ(ICD)Anti-CD19-scFv-IghG1(hinge)- CD3ζ(TM)-CD3ζ(ICD)	Retroviral transduction	(Rischer et al. 2004)
	GD2	Neuroblastoma cell lines LAN1 and SK-N-SH	Human primary Vδ1+ and Vδ2+T cells	Anti-GD2-scFv-CD28(TM)-CD28(ICD)-CD3ζ	Retroviral transduction	(Capsomidis et al. 2018)
	MCSP	Melanoma cell lines Mel526 and A375	Human primary γδ2+ T cells	Anti-MCSP-scFv-CD28(TM)-CD28(ICD)-CD3ζ	mRNA electroporation	(Harrer et al. 2017)
	CD20	Mantle cell lymphoma cell line Mino, Burkitt lymphoma cell line Raji, DLBCL cell line WILL-2	Human primary Vδ1+ T cells	Anti-CD20-scFv-CD8H-CD8TM-41BB-CD3ζ	Retroviral transduction	(Nishimoto et al. 2022)
	CD19	B-ALL cell lines REH, Kasumi-2, Daudu, Nalm-6, EL4	Human γδT cells	Anti-CD19-scFv -CD8H-CD28(TM)-CD28(ICD)-CD3ζ	Sleeping Beauty transposon system	(Deniger et al. 2013)

Table 3: (continued)

Cell type	CAR-target	Target cell	Macrophage source	Tested CAR constructs	CAR insertion method	References
	PSCA	Patient derived CRPC	Human γδ2+ T cells	Anti-PSCA-scFv-CD28TM-CD28(ICD)-CD3ζ	Retroviral transduction	(Frieling et al. 2023)
	CD5CD19	Jurkat cells, Molt-4 and 697	Human γδ2+ T cells	Anti-CD5-scFv-cmyc-CD28(TM)Anti-CD19-scFv-CD8H-CD28(TM)	Lentiviral transduction	(Fleischer et al. 2020)
	Folate receptor	Human breast cancer cell line MCF-7, human TNBC cell lines MDA-MB-231, MDA-MB-436 and MDA-MB-468	Human γδ2+ T cells	Anti-Mov19-CD8H-CD8TM-CD28-CD3ζ-F2A-CCL19-F2A-IL7	Lentiviral transduction	(Ye et al. 2022)
	HLA-G	Patient derived NSCLC and TNBC,NSCLC cell line H1975, TNBC cell line MDA-MB-231	Human γδ2+ T cells	HLA-G-Nb -CD8H-CD8TM-41BB-CD3ζ(incorporation of an additional ITAM copied from a DAP12 fragment into the C-terminal residues of CD3ζ)	mRNA electroporation	(Huang et al. 2023)
Neutrophils	CLTX	Glioblastoma cell line	hPSCs	SP-CLTX-IgG4(hinge)-CD4TM-CD3ζSP-CLTX-IgG4(hinge)-CD32aTM--CD3ζSP-CLTX-IgG4(hinge)-CD32aTM-CD32aITAM-CD3ζSP-CLTX-IgG4(hinge)-CD32aTM-CD32aITAM	Nanodrug assisted in vivo transfection	(Chang et al. 2023)
	CLTX	Glioblastoma cell line	hPSCs	SP-CLTX-IgG4(hinge)-CD4TM-CD3ζ	Cas9-medited homologous recombination	(Chang et al. 2022)

Morrissey et al. 2018; Yang et al. 2019). In the study conducted by Kichinsky et al. (2020), adenovirally transduced anti-HER2 CAR-MΦ exhibited antigen-specific phagocytosis as well as time- and dose-dependent killing of HER2-positive tumor cells and were able to cross-present intracellular tumor antigens to T helper (Th) cells. Through adenoviral transduction, the macrophages were not only locked in an M1 like phenotype, but they were also able reshape the TME by shifting TAMs from an M2 to an M1 like phenotype and recruiting dendritic and cytotoxic T cells (Klichinsky et al. 2020). *In vivo*, the anti-HER2 CAR-MΦ persisted up to 62 days and prolonged the overall survival in models of SCOV3 lung metastasis and peritoneal carcinomatosis in NSG mice (Klichinsky et al. 2020). Further research groups have demonstrated that macrophages, via antigen cross-presentation of intracellular tumor antigens to T cells, can initiate epitope spreading, thereby augmenting the anti-tumor potential of CAR-MΦ (Klichinsky et al. 2020; Pierini et al. 2020). More specifically, treatment of CT26-bearing mice with murine anti-HER2 CAR-MΦ led to higher numbers of intratumoral CD4+ and CD8+ T cells and to an increase in T cell responsiveness to the CT26 antigen gp70 (Pierini et al. 2020). Other immunomodulatory functions of CAR-MΦ include the recruitment of CD4+ and CD8+ T cells, NK cells and dendtritic cells to the TME (Pierini et al. 2020; Gao et al. 2023), as well as the repression of Tregs, M2 macrophages and MDSCs (Gao et al. 2023). Furthermore, Liu et al. demonstrated the superiority of a combinational CAR-T cell/CAR-MΦ cancer immunotherapy to a CAR-T cell or CAR-MΦ monotherapy (Liu et al. 2022). The mechanism underlying this synergistic effect was shown to be a feedback loop in which the cytokines secreted by the CAR-T cells (IFNγ, IL-1β, CXCL1, MIP-1, IL-6, MCP-1) increase the expression of costimulatory ligands CD80 and CD86, which serve as ligands for the T-cell receptor CD28. Innovative strategies have emerged to develop CAR-MΦ that not only target tumor antigens but also components of the immunosuppressive TME. For instance, CCL19-expressing CAR-MΦ successfully bind CCR7 and disrupt the migration of CCR7-positive immunosuppressive cells into the TME, leading to enhanced tumor cell toxicity, prolonged survival, and prevention of metastasis in preclinical models of tumor-bearing mice (Niu et al. 2021).

3.6 Clinical trials

Currently, four phase I clinical trials are registered, testing the clinical efficacy of CAR-macrophages against of solid tumors (Table 2). One of the trials (NCT04660929) assesses the safety, tolerability and efficacy as well as the efficacy of autologous

anti-HER-2 CAR-MΦ against HER-2 expressing solid tumors. Another trial (NCT04405778) focuses on the treatment of adults with previously treated GPC3-positive solid tumors using anti-GPC3 CAR-MΦ. Additionally, Mesothelin-targeting CAR-MΦ are being evaluated in patients with advanced or metastatic solid tumors (NCT05164666).

3.7 Advantages

Macrophages possess two unique advantages over T and NK cells: higher migration to the tumor bed and infiltration into the TME (Mantovani et al. 2022). Through secretion of matrix metalloproteases (MMP) macrophages are able to degrade the TME and thereby penetrate solid tumors to exert their cytotoxic functions. Degradation of the TME also enables the T cell infiltration, therefore circumventing the hurdle of poor tumor infiltration of T cells (Zhang et al. 2019). By incorporating CD147 as a signaling domain, the expression of MMP on CAR-MΦ can be enhanced, promoting ECM degradation and T cell recruitment (Zhang et al. 2019). Macrophages also exhibit some unique cytotoxic properties over T cells, such as phagocytosis and ADCC. Additionally, through antigen cross presentation to T cells and induction of epitope spreading, CAR-MΦ have the ability to broaden the immune response beyond the primary CAR-targeted antigen. Moreover, CAR-MΦ have been proven capable of reprogramming the tumor microenvironment (TME) by polarizing M2 macrophages towards an M1 like phenotype and recruiting further immune cells, such as cytotoxic T cells, NK and dendritic cells (Gao et al. 2023; Pierini et al. 2020). Unlike T cells, they can be generated from numerous reliable sources (peripheral blood, UCB, bone marrow (BM), hESCs, hematopoietic stem and progenitor cells (HSPCs), iPSC, THP1) which can streamline CAR-MΦ manufacturing. Compared to CAR-T cells, CAR-MΦ could potentially display a more attractive safety profile due to their limited time in circulation, this however remains to be evaluated in clinical trials.

3.8 Limitations and solutions

Although CAR macrophages hold immense potential as a novel immunotherapeutic approach, their low proliferation is a limiting factor. Unlike T cells, macrophages do not naturally expand *in vivo*, necessitating repeated infusions to maintain an adequate number for effective immunosurveillance. Therefore, developing standardized protocols for efficient and cost-effective production at large scales is crucial for the widespread clinical application of CAR-MΦ. Additionally, achieving successful gene transfer into macrophages remains technically challenging. Adenoviral transduction or the use of iPSC as a starting material are viable strategies (Zhang et al. 2020). A further challenge facing CAR-MΦ therapy is the risk for M2 polarization mediated by the dynamic nature of the TME. An M1 polarization can be guaranteed through adenoviral transduction (Pierini et al. 2020) or cytokine treatment of CAR-MΦ prior to infusion (Zhang et al. 2020). Lastly, some studies note the secretion of IL-6 by activated CAR-MΦ (Liu et al. 2022; Paasch et al. 2022). The link between IL-6 and CRS in CAR-T cell therapy raises safety concerns regarding CAR-MΦ, highlighting the need for additional assessment in ongoing clinical trials.

4 Invariant Natural Killer T (iNKT) cells

4.1 Properties

Natural Killer T cells (NKT cells) present a heterogenous innate-like subset of αβT cells that shares characteristics with both T and NK cells. Invariant Natural Killer T (iNKT) cells are the main subtype of NKT cells, comprising approximately 0.05 % of the circulating T cells (Wolf et al. 2018). In contrast to conventional T cells, the TCR of iNKT cells contains an invariant TCR-α chain Vα24-Jα18 paired with a limited repertoire of TCRβ chains (Wolf et al. 2018). The positive correlation of a high NKT cell infiltration with an improved survival in various tumor entities (Lundgren et al. 2016; Metelitsa et al. 2004; Tachibana et al. 2005; Tang et al. 2021a), underlines the key role of iNKT cells in cancer immunosurveillance. Tumor homing of iNKT cells is mediated via their rich repertoire of chemokine receptors, including CCR1, CCR2, CCR4, CCR6 and CXCR3 (Kim et al. 2002). iNKT cells can recognize their targets mainly via their TCR, which binds antigens in an MHC-independent but CD1d-restricted manner (Wolf et al. 2018). CD1d is a non-polymorphic MHC I-like molecule that presents glycosphingolipids and membrane phospholipids rather than peptide antigens and is expressed in APC, thymocytes and several malignant cells (Metelitsa 2011). The α-galactosylceramide (α-GalCer) was the first identified CD1d-presented lipid antigen on APC and tumor cells, which activates iNKT cells and induces their expansion *in vivo* and *in vitro* (Metelitsa 2011). Tumor recognition is also mediated by the recognition of stress-induced ligands via their NK activating receptor NKG2D (Kuylenstierna et al. 2011), which plays an important role in targeting tumors that lack of downregulate CD1d expression as a immune-evasion

mechanism (Metelitsa 2011). Upon target recognition, iNKT cells mediate toxicity by secreting perforin and granzyme B as well as through TRAIL and Fas/FasL pathways. Apart from direct cytolytic activity, iNKT cells can recruit macrophages, neutrophils, T and B cells to the tumor site through secretion of IL-2, IL-4, IL-27, TNF-α and IFN-γ (Sag et al. 2017). iNKT cells can induce DC cells to upregulate CD40, CD80 and CD86 and produce IL12, which itself increases IFN-γ production by iNKT cells, leading to a positive feedback loop for Th1 immunity (Keller et al. 2017). The iNKT cell-mediated maturation of DC and the following DC-mediated transactivation of NK cells, lead to an increased antigen cross-presentation to T cells, inducing naïve T cells to undergo expansion and differentiation, leading to persistent antitumor immunity (Fujii et al. 2003). Additionally, iNKT cells exhibit notable immunomodulatory potential, highlighted by their ability to lyse TAMs in an CD1d-dependent manner (Song et al. 2009), reprogram M2 like TAMs to M1 like TAMs (Paul et al. 2019) and reduce the immunosuppressive activity of MDSCs (Ko et al. 2009).

In vivo stimulation of iNKT cells with aGelCer (Chang et al. 2005; Giaccone et al. 2002, Ishikawa et al. 2005), adoptive transfer of iNKT cells (Kunii et al. 2009; Motohashi et al. 2006), as well as genetic engineering of iNKT cells to express a recombinant TCR (rTCR) (Luo et al. 2011), have led to potent antitumor responses in preclinical and clinical studies. Therefore, scientists have proceeded to arm iNKT cells with CAR, to maximally harness their therapeutic potential.

4.2 CAR-design and manufacturing of CAR-iNKT cells

CAR-iNKT cells are typically generated using either autologous or allogeneic iNKT cells. However, it is worth noting that iNKT cells can also be derived from alternative sources such as HSC (Zhu et al. 2019) and iPSC (Yamada et al. 2017). Notably, recent studies have demonstrated that iPSC-derived iNKT cells performed anti-tumor cytotoxicity and cytokine production similar to their primary counterparts (Yamada et al. 2017). Various approaches are employed to expand iNKT cells, primarily capitalizing on the responsiveness of their TCR to α-GalCer loaded onto CD1d molecules and the stimulation of iNKT cells via IL-2, IL-7 and IL-15. Most research groups opt for co-culturing iNTK cells with PBMCs (Heczey et al. 2014), artificial APCs or DCs (Poels et al. 2021) that are loaded with α-GalCer. Additionally, IL-21 has been demonstrated to selectively protect CD62L-positive iNKT cells, which exhibit a favorable profile in terms of persistence and proliferation and a higher resistance to activation induced cell death (AICD) (Tian et al. 2016). As for the CAR constructs, research groups are employing the same signaling domains used for conventional CAR-αβT cells, with 41BB having a favorable impact on the cytotoxic potential of CAR-iNKT cells (Heczey et al. 2014; Poels et al. 2021). Namely, Heczey et al. observed that in contrast to CD28 or CD3ζ, the incorporation of the costimulatory domain 41BB shifted the cytokine profile of iNKT cells towards an Th1 phenotype (Heczey et al. 2014). A similar Th1 polarization was shown in iNKT cells equipped with 4-1BB signaling anti-BCMA and anti-CD38 CAR (Poels et al. 2021). In terms of genetic engineering methods, most research groups utilize retroviral transduction to introduce CAR into iNKT cells (Heczey et al. 2014; Poels et al. 2021; Tian et al. 2016; Xu et al. 2019a). Lentiviral transduction (Rowan et al. 2023) or mRNA electroporation (Du et al. 2016; Simon et al. 2018) also yield sufficient CAR-expression levels.

4.3 Preclinical studies on CAR-iNKT cells

Preclinical studies have tested CAR-iNKT targeting various antigens, including CD19 (Tian et al. 2016), GD2 (Heczey et al. 2014; Xu et al. 2019a), the chondroitin sulfate proteoglycan 4 (CSPG4) (Simon et al. 2018), BCMA (Poels et al. 2021), CD38 (Poels et al. 2021) or TCRVβ (Rowan et al. 2023). Heczey et al., the pioneer of CAR-iNKTs, introduced different second and first generation anti-GD2 CAR into iNKT cells (Heczey et al. 2014). Regardless of the CAR design, CAR-iNKT cells were able to kill GD2-positive neuroblastoma cells *in vitro* and *in vivo* by engaging both their CAR and their TCR (Heczey et al. 2014). Interestingly, introducing the IL-15 gene into the anti-GD2 CAR-iNKT cells, enhanced their expansion, reduced expression levels of exhaustion markers, increased multi-round *in vitro* tumor cell killing, enhanced *in vivo* persistence and improved tumor control (Xu et al. 2019a). The combination of CAR-iNKT cells with the administration of αGalCer has been shown to exhibit synergistic anti-tumor activity by enabling CAR-iNKT cells to eliminate target cells through dual targeting of the CAR-antigen and lipid-loaded CD1d. More specifically, in two studies, TCRVβ- or CSPG4-targeting CAR-iNKT cells, significantly enhanced cytotoxicity in tumor bearing mice when combined with αGalCer administration (Rowan et al. 2023; Simon et al. 2018). Unfortunately, CD1d-dependent antitumor toxicity is limited in tumors that downregulate CD1d (Metelitsa 2011). To address the issue of low CD1d expression in CLL, one group treated CLL cells with all-trans retinoid acid (ATRA), which upregulated CD1d expression and led to a significantly higher cytotoxic effect of CAR-iNKT cells compared to CAR-T cells (Rotolo et al. 2018). *In vivo*, CAR-iNKT

cells present an advantage over CAR-T cells due to their ability to better infiltrate peripheral tissues, as demonstrated in neuroblastoma bearing mice treated with anti-GD2 CAR-iNKT cells (Heczey et al. 2014).

4.4 Clinical studies on CAR-iNKT cells

Although there are no completed clinical trials involving CAR-iNKT cells, interim clinical data of two studies by Kuur Therapeutics have been reported (NCT03294954, NCT03774654). Anti-GD2 CAR IL15-expressing iNKT cells against relapsed or resistant neuroblastoma in children were found to be safe in the 10 enrolled patients, among which one patient achieved complete remission, one had a partial response and three showed stable disease (Heczey et al. 2020). In two evaluable B cell lymphoma patients, allogeneic anti-CD19 CAR-iNKT cells resulted in one complete remission and one partial remission without evidence of CRS, ICANs, or GvHD.

4.5 Advantages

One of the main benefits of iNKT cells as a platform for CAR-therapy lies in their inherent ability to efficiently infiltrate solid tumors, thanks to their natural chemotactic and migratory properties. This was demonstrated in a study benchmarking CAR-iNKT cells against CAR-T cells: In B cell lymphoma bearing mice, anti-CD19 CAR-iNKT cells outperformed CAR-T cells by exhibiting better brain infiltration and eradicating intracranial metastasis (Rotolo et al. 2018). Furthermore, their minimal risk of causing GvHD (Rotolo et al. 2018) due to their MHC-independent antigen recognition makes them suitable for an off-the-shelf allogeneic approach. Moreover, iNKT cells possess multiple mechanisms for targeting tumors. They can employ their native TCR, NK cell activating receptors, or utilize CAR, providing three distinct targeting strategies. This versatility allows them to target tumors regardless of the downregulation of MHC, CD1d- or CAR antigen-expression. In addition to their cytotoxic capabilities, iNKT cells can reshape the TME by abolishing immunosuppressive cells and recruiting other immune cells. Despite their role in innate immunity, they can also enhance T cell responses and promote the development of tumor-specific immune memory (Fujii et al. 2003). As for their safety profile, CAR-iNKT seem to carry a lower risk of toxicities, although further evaluation in clinical trials is required. Compared to conventional CAR-T cells, CAR-iNKT cells secrete lower levels of CRS-related cytokines (Poels et al. 2021; Simon et al. 2018) and mediate no on-target, off-tumor toxicity in in vitro cocultures (Poels et al. 2021).

4.6 Limitations and solutions

CAR-iNKT cell therapy, while promising, presents certain limitations that restrict its widespread adoption in clinical applications. The relatively low abundance of iNKT cells in peripheral blood necessitates extensive ex vivo expansion to achieve the necessary quantities for clinical-scale CAR-iNKT cell production. This, however, could be dealt with by using more available cells as a starting material, namely iPSCs or HSCs (Yamada et al. 2017; Zhu et al. 2019). Furthermore, the constrained persistence of iNKT cells *in vivo* presents an additional obstacle to fully realizing their therapeutic potential. Optimal selection of a CAR signaling domain (Heczey et al. 2014), the co-expression of IL-15 (Xu et al. 2019a) or the stimulation of CAR-iNKT cells with IL-21 (Tian et al. 2016) appear to bolster their persistence.

5 γδT cells

5.1 Properties

γδT cells constitute a distinctive subset of innate T cells that account for approximately 1–10 % of the circulating T cell population. In contrast to the conventional αβT cells, their TCR is composed of a γ and a δ chain. Based on their specific γ and δ chains, γδT cells can be further subcategorized, with Vγ9Vδ2T (Vδ2+ T) cells being the most prevalent subtype in the peripheral blood and Vδ1+ T cells being the predominant type in epithelia and solid tumors (Silva-Santos et al. 2015). γδT cells have emerged as crucial contributors to anti-tumor immune responses and recent research underscores their presence within tumors as a strong predictor of favorable outcomes (Gentles et al. 2015). A distinguishing feature of the γδTCR is its ability for MHC-independent antigen recognition. The TCR of Vδ2+ T cells can identify transformed cells that express stress-induced surface proteins, including endothelial protein C receptor (EPCR), annexin A2, F1-ATPase and phosphoantigens (pAgs). The most described pAg recognized by the Vδ2+ TCR is isopentenyl phosphate (IPP), produced as a result of the overstimulation of the mevalonate pathway in transformed cells (Miyagawa et al. 2001). Interestingly, FDA-approved amino bisphosphonates (N-BP) such as pamidronate or zoledronate (ZOL), used for treating osteoporosis and bone metastasis, disrupt phosphoantigen-processing enzymes, thereby increasing intracellular IPP levels in tumor cells and activating Vδ2+T cells. This interaction has been harnessed to expand γδT cells *in vitro* and explored in clinical trials to enhance susceptibility to γδTCR-mediated recognition and cell killing (Dieli et al. 2007; Lang et al. 2011). The

TCR of Vδ1+ T cells recognizes the MHC I associated molecules MICA and MICB, as well as CD1d presented lipid antigens. Tumor cell recognition by γδT cells also relies on co-stimulatory receptors usually associated with NK cells (Simões et al. 2018), such as NKG2D and DNAM-1 and NCR (NKp30, NKp44 and NKp46) (Liu and Zhang 2020; Simões et al. 2018). Upon identifying their target, γδT cells can eliminate tumor cells via granzyme B and perforin release as well as through the activation of TRAIL and Fas/FasL pathways. Additionally, γδT cells can engage in ADCC (Liu and Zhang 2020). Furthermore, through their capacity to cross-present antigens, γδT cells activate CD4+ and CD8+ T cells, therefore bridging the gap between innate and adaptive immunity (Brandes et al. 2005; Muto et al. 2015). By secreting IFN-γ and TNF-α, γδT cells can also stimulate further immune cells, such as macrophages and DC, thus enhancing the anti-tumor response. Although both Vδ1+ and Vδ2+ T cell subtypes are endowed with potent anti-tumor cytolytic function, Vδ1+ generally outperform Vδ2+ T cells due to their high tropism for tumor tissues, naturally more naïve memory phenotype and reduced susceptibility to activation-induced cell death (AICD) compared to their counterparts (Siegers and Lamb 2014). Paradoxically, most clinical trials have focused on Vδ2+ T cells, given their relative abundance in peripheral blood and ease of expanding using ZOL. Currently evaluated γδT cell based cancer immunotherapies in clinical trials include treatment of cancer patients with aminobisphosphonates (Dieli et al. 2007; Lang et al. 2011) or synthetic phosphoantigens (Gertner-Dardenne et al. 2009) as well as the adoptive transfer of autologous γδT cell cells (Bennouna et al. 2008; Kobayashi et al. 2007). With the adoptive transfer of γδT emerging as a safe and promising immunotherapeutic strategy (Liu and Zhang 2020), scientists are trying to enhance the therapeutic potential of these cells by equipping them with CAR.

5.2 Engineering and generation of CAR γδT cells

Due to their low abundance in the peripheral blood, the generation of sufficient numbers of γδT cells for clinical use is challenging. The most widely used protocol relies on ZOL and IL-2 stimulation, which is limited to the expansion of Vδ2+ T cells (Capsomidis et al. 2018; Harrer et al. 2017, Nishimoto et al. 2022; Rischer et al. 2004). Another approach introduced by Aehnlich et al. involves stimulation with low dose IL-2 and high-dose IL-15, resulting in successful long-term expansion of Vδ2+ cells with enhanced cytotoxicity (Aehnlich et al. 2020). Exclusive Vδ1+ T cell expansion can be achieved using immobilized agonistic monoclonal antibodies (Nishimoto et al. 2022). PBMC stimulation with Concanavalin A allows the expansion of both Vδ1+ and Vδ2+ subsets (Capsomidis et al. 2018), whereas expansion of the full repertoire of γδT cels can be achieved via stimulation with artificial antigen presenting cells (aAPC) expressing anti-γδTCR antibodies (Fisher et al. 2014).

The CAR-structure and signaling domains employed for CAR-γδT cells are identical to those used for αβT cells. Two notable exceptions are the deployment of a non-signaling CAR (NSCAR) (Fleischer et al. 2020) and the design of a chimeric receptor lacking the CD3ζ (Fisher et al. 2019) domain, which are both discussed below. In general, the genetic engineering techniques applied to γδT closely resemble those used for CAR-αβT cell generation. The predicted shorter lifespan of infused γδT cells offers the opportunity to use more transient engineering approaches. The primary approach is gammaretroviral transduction (Capsomidis et al. 2018; Nishimoto et al. 2022; Rischer et al. 2004) or lentiviral transduction (Ye et al. 2022a), while other groups have employed electroporation (Harrer et al. 2017; Huang et al. 2023) or the Sleeping Beauty Transposon System (Deniger et al. 2013).

5.3 Efficacy of CAR-γδT cells

In contrast to CAR-αβT cells, the research landscape surrounding CAR-γδT cells remains relatively underexplored. The inception of CAR-γδT cells can be traced back to the work of Rischer et al. in 2004, who generated GD2-and CD19-targeting CAR-Vδ2+ T cells (Rischer et al. 2004). Remarkably, both CAR-Vδ2+ T cells mediated antigen-specific lysis and IFN-γ secretion. Despite the ALL-cell line Raji considered γδT cell resistant, it demonstrated a high susceptibility to anti-CD19 CAR-γδ2+ T cell mediated killing, suggesting that CAR expression could overcome immune escape of target cells. Building on this approach, further groups designed CAR-γδT cells targeting antigens, such as GD2 (Capsomidis et al. 2018), CD19 (Deniger et al. 2013), CD5 (Fleischer et al. 2020), CD20 (Nishimoto et al. 2022), the Melanoma-associated chondroitin sulfate proteoglycan (MCSP) (Harrer et al. 2017) or the Prostate stem cell antigen (PSCA) (Frieling et al. 2023).

While Vδ2+ T cells are typically used for CAR-insertion, recent investigations have scrutinized the potential of Vδ1+ T cells as CAR carriers. A direct comparison of Vδ1+ and Vδ2+ T cells transduced with an anti-GD2 CAR, revealed that Vδ1+ T cells maintained a “naïve memory” (TM) phenotype, while Vδ2+ cells adopted a predominantly “T effector memory” (TEM) phenotype marked by higher expression of exhaustion markers TIM-3 and PD-1 (Capsomidis et al. 2018). Similar

observations were made in a further study, where anti-CD20 CAR-Vδ1+ T cells maintained a non-exhausted state *in vitro* and *in vivo*. This is a promising finding, since less differentiated memory T cell phenotypes have been associated with heightened proliferative potential following CAR activation (Kaartinen et al. 2017; Xu et al. 2014). Nevertheless, direct *in vitro* comparison of CAR-Vδ1+ to CAR-Vδ2+ cells revealed equivalent anti-tumor cytotoxicity and migration capacity of the two γδT cell subsets (Capsomidis et al. 2018). Based on the association of tonic signaling and exhaustion with CD3ζ signaling, Fischer et al. employed an alternative CAR design which lacks the TCR signal transduction elements and provides an AND gate mechanism (Fisher et al. 2019). These so called chimeric costimulatory receptors (CCR) lacking CD3ζ and incorporating DAP10 instead, provide only costimulation and rely on the γδTCR to provide CD3ζ signals. CCR expressing γδT cells avoided tonic signaling and were enabled for full signaling and cytotoxic responses in the presence of both antigen and CCR stimuli (Fisher et al. 2019).

In general, CAR-γδT cells have been shown to recognize their target cells and mediate anti-tumor toxicity in a CAR-dependent manner but also via their innate receptors, namely their TCR and NK cell associated co-receptors, such as NKG2D. This was highlighted by the ability of anti-CD20 CAR-γδT cells to effectively lyse NHL cells despite their low CD20 expression (Nishimoto et al. 2022). In a further study anti-CD19 CAR-γδT cells were also able to effectively target and lyse CD19 antigen negative leukemia cells (Rozenbaum et al. 2020). In addition to direct cytotoxicity, CAR-γδT cells can augment the adaptive immune response via cross presentation of tumor associated antigens (TAAs) to CD8+ T cells (Capsomidis et al. 2018). Furthermore, leveraging the inherent anti-tumor toxicity of γδT cells is particularly advantageous in the context of T cell malignancies, offering a potential solution to mitigate the fratricide risk often associated with conventional CAR-αβT cells. Notably, a study demonstrated a rise in anti-cancer cytotoxicity when arming γδT cells with an CD5 targeting non-signaling CAR (NSAR), whereas such enhancement was not witnessed with αβT cells (Fleischer et al. 2020). The NSCAR enables γδT cells to approach target cells, after which the innate cytotoxic mechanisms of γδT cells can be activated, with NKG2D engagement hypothesized as the primary mechanism of toxicity. More complex genetic engineering approaches have further expanded the therapeutic potential of CAR-γδ T cells. For example, CAR-γδT cells targeting folate receptors have been designed to secrete interleukin-7 (IL-7) and chemokine C–C motif ligand 19 (CCL19), resulting in increased infiltration of DC and αβT cells into tumor tissues (Ye et al. 2022a). Another research group engineered anti-HLA-G CAR-γδT cells secreting PDL1/CD3e BITEs, which were able to enhance cytotoxic killing responses by triggering bystander effector cells (Huang et al. 2023).

Building upon the promising results from clinical trials of N-BP therapy and adoptive γδT cell transfer, Frieling et al. tested the combination therapy of CAR-γδT cells with ZOL (Frieling et al. 2023). CAR-γδT cells achieved a significant reduction in tumor viability and an increased cytokine secretion in metastatic castrate-resistant prostate cancer (mCRSC) bearing, ZOL-pretreated mice (Frieling et al. 2023). While there are no completed clinical trials on CAR-γδT cells yet, eight phase I clinical trials are registered focusing on hematological malignancies as well as solid tumors (Table 2).

5.4 Advantages

γδT cells offer distinct advantages over conventional αβT cells as a platform for CAR-based therapy. Their versatile anti-cancer properties render γδT cells as strong contenders for CAR-cell therapy, resolving some of the caveats of CAR-αβT cell therapy. When comparing the CAR-dependent killing capacity of the two cell types, two studies have revealed equivalent killing capacity of CAR-γδT and CAR-αβT cell (Capsomidis et al. 2018; Harrer et al. 2017), while in a further study anti-CD20 CAR-Vδ1+ T cells exhibited markedly swifter kinetics in eradicating CD20-positive target cells compared to CAR-αβT cells (Nishimoto et al. 2022). The extensive repertoire of innate receptors on CAR-γδT cells offers them a clear advantage over CAR-αβT cells, as the former do not depend solely on their CAR for target recognition and killing (Nishimoto et al. 2022). Furthermore, their endogenous receptor arsenal also renders CAR-γδT cells more resistant to cancer immune evasion: cancer cells with MHC downregulation or CAR-antigen downregulation can still be detected in an MHC-independent and CAR-independent manner via engagement of the γδTCR or other costimulatory receptors. This was illustrated in the study by Rosenbaum et al., in which compared to CAR-αβT cells, anti-CD19 CAR-γδT cells displayed increased cytotoxicity against CD19-knockout target cells (Rozenbaum et al. 2020). Beyond mediating direct cytotoxicity, γδT cells can also participate in ADCC, cross-present antigens (Capsomidis et al. 2018), and orchestrate interactions with other immune cells in the TME (Ye et al. 2022a). Moreover, as the first line of cancer immunosurveillance, γδT cells can sense tumors with low mutational loads but with already acquired metabolic changes, such as expression of IPP or EPCR, which allows them to attack cancer cells early in their transformation (Gober et al. 2003). Additionally, due to their low risk of GvHD, γδT cells enable cellular immunotherapy in an allogeneic setting. Lastly, these cells could also have a potentially better

safety profile and decreased risk for CRS when compared to their αβ counterparts. This was demonstrated in a comparative assessment between anti-MCSP CAR-γδT cells and anti-MCSP CAR-αβT cells, where similar cytotoxicity against melanoma cell lines was observed, with Vδ1+ T cells producing lower levels of the CRS-associated cytokine IL-2 (Harrer et al. 2017). Although there are no completed clinical trials on CAR-γδT cells, adoptive transfer of γδT cells has been well tolerated in early clinical trials (Abe et al. 2009; Lin et al. 2020; Xu et al. 2021; Wilhelm et al. 2014).

5.5 Limitations and solutions

Nonetheless, γδT cells do not stand without limitations. In contrast to αβT cells, γδT cells constitute a relatively minor fraction of PBMCs, necessitating substantial expansion efforts to attain clinically relevant cell numbers. Furthermore, once expanded and administered to patients, γδT cells exhibit a considerably lower persistence compared to αβT cells (Brandes et al. 2005) which could potentially undermine the maintenance of the body's long-term antitumor response. In a comparative study, anti-CD19 CAR-αβT cells lead to more drastic reduction of the leukemic burden in a mouse xenograft model than anti-CD19 CAR-γδT cells, which was mainly attributed to the limited persistence of the CAR-γδT cells in the spleen and bone marrow (Rozenbaum et al. 2020). Furthermore, within the TME, γδT cells can undergo a functional polarization towards an exhausted and immunosuppressive phenotype (Wu et al. 2014). Two immunosuppressive phenotypes have been described for Vδ1+ T cells. The first involves γδ17 T cells, which secrete IL17 and induce MDSC recruitment and production of transforming growth factor beta (TGFβ) by macrophages (Wu et al. 2014). The second phenotype comprises γδT regulatory cells (Tregs) which promote tumor growth through secretion of IL-10 and TFG-β (Wu et al. 2014). Finally, the susceptibility of Vδ2+ T cells to AICD, tonic signaling, and exhaustion seems to curtail their therapeutic efficacy. One approach would be to use Vδ1+ T cells. Another approach is to apply costimulatory chimeric receptors (Fisher et al. 2019).

6 Neutrophils

6.1 Properties

Neutrophils account for 50–70 % of circulating leukocytes in humans and are critical components of the innate immune system. Their primary function is to combat pathogens, achieved through their remarkable phagocytic capabilities, allowing them to engulf and eliminate invading pathogens. Neutrophils also produce a variety of antimicrobial substances, including reactive oxygen species (ROS), antimicrobial peptides, and enzymes, further contributing to microbial eradication. Additionally, they can form neutrophil extracellular traps (NETs), extracellular network structures composed of neutrophil elastase (NE), cathepsin G and DNA-histone protease complex that can entrap and neutralize pathogens through a process called NETosis (Brinkmann et al. 2004). Beyond their antimicrobial functions, neutrophils are increasingly recognized for their interactions with tumor cells. Through induction by the TME, tumor associated neutrophils (TANs) undergo phenotypic and functional remodeling can be categorized into protumorigenic N2 neutrophils and anti-tumorous N1 TANs (Que et al. 2022). While N2 neutrophils promote cancer cell proliferation, angiogenesis, and metastasis (Que et al. 2022), N1 TANs can mediate anti-tumor cytotoxicity through the release of ROS and NETs (Schedel et al. 2020) and a Fas/Fas ligand pathway-mediated cell cycle arrest. Target cell death is further facilitated through a specific form of antibody-dependent cell-mediated cytotoxicity (ADCC) known as trogoptosis, in which neutrophils ingest a fraction of the antibody-opsonized plasma membrane of cancer cells (Matlung et al. 2018). N1 TANs are able to engage the adaptive immune system as well, by recruiting and activating CD8+ T cells via pro-inflammatory cytokine secretion (Governa et al. 2017; Sionov et al. 2015). Notably, studies analyzing colorectal cancer samples indicate that neutrophils enhance the responsiveness of CD8+ T cells by lowering the threshold of T cell receptor signaling (Governa et al. 2017). Although neutrophils are not typically associated with antigen presentation, a study involving early-stage human lung cancer demonstrated the presence of a subset of TANs capable of cross-presenting tumor antigens and stimulating T cell responses (Singhal et al. 2016).

6.2 CAR-neutrophils

So far, there has been a single group that successfully assessed the incorporation of CAR into neutrophils (Chang et al. 2022, 2023). In a recent study, Chang et al. developed CAR-neutrophils by utilizing the Chlorotoxin (CLTX)-T-CAR, which specifically targets Glioblastoma (GBM) peptides and signals via CD3ζ (Chang et al. 2022). To overcome the hurdle of genetically engineering neutrophils, the researchers inserted the CLTX-T-CAR into a safe harbor locus of hESCs, which were subsequently differentiated into neutrophils. These CAR-neutrophils rapidly formed immunological synapses with their targets and exhibited enhanced phagocytosis, ROS release and NETosis.

Interestingly, in contrast to their physiological counterparts, CAR-neutrophils maintained an N1 phenotype even under immunosuppressive TME conditions *in vivo* and *in vitro*. Building upon these promising findings, CAR-neutrophils were loaded with nanodrugs comprising silica nanoparticles carrying chemotherapeutic or radiation drugs (Chang et al. 2023). After target cell recognition, CAR-neutrophils successfully released the intracellularly located nanodrugs. This novel combination of chemotherapy and cellular immunotherapy effectively slowed tumor growth in a GBM mouse model. This innovative approach opens new possibilities for redirecting neutrophils against cancer.

6.3 Advantages

Although there is very limited research on CAR-neutrophils, they seem to present some distinct benefits. Constituting a substantial portion of circulating leukocytes, neutrophils are conveniently accessible for therapeutic applications. Furthermore, they possess a multifaceted array of tumoricidal mechanisms, with NETosis being notably potent. Their innate ability to infiltrate peripheral tissues could be particularly beneficial in the setting of solid tumors resistant to CAR-T cells.

6.4 Disadvantages

Nonetheless, neutrophils, owing to their inherent nature, exhibit a relatively abbreviated lifespan, potentially constraining the duration of their therapeutic impact and necessitating recurrent infusions. While both studies investigating CAR-neutrophils have exhibited the preservation of an N1 phenotype, there remains a concern regarding the potential for N2 polarization within the immunosuppressive TME, which could counteract their therapeutic efficacy. Overall, while promising, the use of neutrophils as CAR carriers demands ongoing research and optimization to overcome these limitations and fully exploit their therapeutic potential.

7 Induced pluripotent stem cells (iPSC)

7.1 Properties

Human pluripotent stem cells (hPSC) are able to proliferate indefinitely and differentiate into cells of all three germ layers: ectoderm, mesoderm and hemogenic endothelium (Yamanaka 2020). Due to these outstanding properties, they have become a potential alternative platform for producing blood cells for clinical use. Two main types of hPSC are being explored: human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) (Yamanaka 2020). iPSC are somatic cells that have been reprogrammed to an embryonic-like state and have the potential to differentiate into specialized cell types derived from any primary germ layer. Although any somatic cell type could be reprogrammed to iPSCs, fibroblasts and blood cells are mostly used as a starting material. In 2006, Takahashi and Yamanaka described a method for reprogramming mouse fibroblasts into pluripotent stem cells by introducing genes encoding 4 transcription factors: OCT4, SOC2, KLF4 and MYC (Takahashi and Yamanaka 2006). A year later, iPSC were generated from human fibroblasts (Yu et al. 2007). Starting from iPSC, cellular immunotherapies, such as iPSC-derived CAR-T cells (CAR-iT) (Jing et al. 2022), CAR-NK cells (CAR-iNK) and CAR-macrophages (CAR-iMΦ) have been developed.

7.2 Advantages

Several qualities of iPSC render them highly suitable as the starting material for cellular immunotherapies. Firstly, their unlimited self-renewal enables large-scale production for clinical applications, which is not the case with primary cells (Yamanaka 2020). Additionally, as a clonal population, iPSC present a reproducible and consistent cell source that allows the manufacturing of standardized cell products. The availability of iPSC from a diverse range of donors further eases HLA matching. Moreover, in contrast to primary cells, iPSC are readily amenable to genetic engineering, enabling multiple complex genetic alterations to augment the efficacy of cellular therapies. Another notable advantage is the rigorous quality control of iPSC-derived products. Presently, several iPSC cell banks exist that undergo extensive characterization to ensure their safety and suitability for clinical use (Umekage et al. 2019). This allows the isolation of individual singe-cell derived engineered iPSC clones as well as the identification of off-target genomic alterations through whole-genome sequencing (Goldenson et al. 2022). Taken together, these factors position iPSC as the ideal candidate for the development of an “off-the-shelf” cellular immunotherapy.

7.3 iPSC derived CAR-NK cells (CAR-iNK)

iPCS derived NK cells (iNK cells) demonstrate phenotypic and functional similarities to primary NK cells. iPSC-to-NK differentiation protocols are broadly divided into 2D and 3D

systems (Lyadova et al. 2021). In both systems, iPSC are first differentiated to hematopoietic progenitor cells, which thereafter are cultured in specific cytokines and inducing factors. In 2D systems, iPSC are co-cultured with mouse bone marrow stromal cells, which act as feeder cells, promoting hematopoietic differentiation. Then, CD34-positive hematopoietic stem cells (HSC) are sorted and transferred to a second feeder cell line, where cytokines are added to promote differentiation to NK cells (Ni et al. 2011; Woll et al. 2009). In 3D feeder-free systems iPSC are left in suspension and spontaneously form embryonic bodies (EB) which are 3D cell structures capable of differentiating into all 3 germ layers. EB can be transferred to a stromal cell line (Ueda et al. 2020) or be directly differentiated to HSCs in feeder-free conditions (Hermanson et al. 2016; Ueda et al. 2020). Centrifuging iPSC to form spin-EBs is a novel approach, which allows a synchronized and more efficient differentiation (Knorr et al. 2013).

CAR-iNK cells can be generated by insertion of a CAR construct in iPSC and subsequent iPSC differentiation to NK cells and exhibit similar therapeutic efficacy to their primary counterparts. Notably, CAR-iNK cells targeting GPC3 (Ueda et al. 2020) or EGFR (Ingegnere et al. 2019) demonstrated potent specific anti-tumor cytotoxicity both *in vitro* and *in vivo* (Li et al. 2018; Ueda et al. 2020). Easy genetic engineering in the iPSC stage allows CAR-insertion into a safe harbor locus to ensure safety (Tang et al. 2021b) and avoid insertional mutagenesis. Furthermore, iPSCs allow more intricate genetic engineering, leading to CAR-NK cell products with enhanced performance. For instance, NK cell persistence was amplified through knocking out the CISH gene in the iPSC-stage, which encodes for the cytokine-inducible homology 1-containing protein, a negative regulator of IL-15 signaling (Zhu et al. 2020). Several more complex engineered CAR-iNK products have been developed by Fate Therapeutics, including FT596 (Bachanova et al. 2020; Goodridge et al. 2019), FT576 (Bjordahl et al. 2019; Goodridge et al. 2020), FT536 (Goulding et al. 2022) and FT573 (Zorko et al. 2022). FT576 is a CAR-iNK cell product that incorporates 4 genetically encoded functional attributes: in addition to a BCMA-targeting CAR, it features an IL-15/IL-15R fusion protein that enhances *in vivo* persistence and expresses the cleavage-resistant CD16a 158V, which enhances ADCC (Goodridge et al. 2020). Furthermore, a biallelic CD38 knockout allows for treatment with CD38 antibodies, avoiding fratricide induced by upregulated CD38 expression in NK cells (Goodridge et al. 2020).

Three completed Phase I clinical trials of iPSC-derived NK cells (NCT03841110, NCT04023071, NCT04614636) show no iNK-related toxicities or adverse effects. Three phase I clinical trials on anti-CD19 CAR-iNK cells are currently ongoing (NCT04023071, NCT04245722, NCT04555811). A phase I clinical trial testing the anti-CD19-iNK cell product FT596 has shown no dose-limiting toxicities, only two cases of CRS as well as objective responses in eight out of eleven efficacy-evaluable patients, as of June 2021 (Bachanova et al. 2021).

7.4 iPSC-derived CAR-macrophages (CAR-iMΦ)

Considering the restricted expansion potential of macrophages and their inherent resistance to genetic alterations, the differentiation of genetically modified iPSC into macrophages (iMΦ) presents a more viable route for manufacturing CAR-macrophages. iPSC-to-macrophage differentiation protocols generally encompass four fundamental steps and can be divided in 3D and 2D culture systems, summarized in the review of Lyadova et al. (Lyadova et al. 2021).

Senju et al. developed the first CAR-iMΦ, by introducing an anti-CD20 CAR into iPSC and differentiating these into macrophages (Senju et al. 2011). Following this innovative approach, a further group developed anti-Mesothelin CAR-iMΦ which possessed typical macrophage marker gene expression and functions (phagocytosis and cytokine secretion), as well as an M1 polarization upon incubation with tumor cells (Zhang et al. 2023a). *In vivo*, the anti-Mesothelin CAR-iMΦ persisted for 20 days and demonstrated antigen-specific antitumor effects.

8 Summary

In conclusion, the exploration of alternate cell sources for CAR-based therapies presents a promising avenue to address the challenges and limitations faced by CAR-T cell therapy in cancer treatment. Despite some impressive clinical outcomes of CAR-T cell therapy, its efficacy is hampered by restricted tumor infiltration, inadequate persistence, antigen escape, systemic toxicities, and manufacturing complexities. Macrophages, NK cells, iNKT cells, γδT cells and neutrophils inherently possess cytotoxic mechanisms that can be harnessed to combat malignancies, and the integration of CAR technology enhances their specificity and potency. iPSC provide a renewable cell source that could streamline manufacturing and turn CAR-therapies into “off-the-shelf” ready to use products. Continued research and innovation are required to navigate these potential solutions towards effective and safe clinical applications, ultimately providing new hope for patients with cancer.

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