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TriKEs and BiKEs join CARs on the cancer immunotherapy highway

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ABSTRACT

Unprecedented clinical success has recently been achieved in cancer immunotherapy using cytotoxic T cells armed with activating tumor-specific Chimeric Antigen Receptors (CARs). Natural killer (NK) cells, potent cytotoxic effectors, also hold potential to be effectively harnessed for immunotherapy. The anti-tumor efficacy of NK cell therapies has been limited by a lack of antigen specificity and the poor persistence of NK cells *in vivo*. To address these limitations, Vallera and colleagues developed novel *Tri*specific *K*iller cell *E*ngagers (TriKEs), reported in the Feb. 2016 issue of *Clinical Cancer Research*.[1] The novel TriKE immunomodulator evolved from the *Bi*specific *K*iller cell *E*ngager (BiKE), a precursor developed by the same team. BiKEs comprise 2 antibody fragments, a first recognizing a tumor antigen and a second directed against CD16 on NK cells, which together trigger antibody-dependent cell-mediated cytotoxicity. IL-15 was further integrated to create TriKEs in order to drive NK cell expansion. Compared to BiKEs, TriKEs elicit far superior NK cytotoxicity and NK cell persistence in a xenograft tumor model *in vivo*, and are proposed to be effective adjuncts to existing NK transfer protocols. Importantly, TriKEs provide a versatile and cost-effective platform onto which novel targeting ligands can be incorporated and hold the potential to stimulate endogenous NK cells in order to circumvent the need for cell transfers altogether, heralding a new generation of immunotherapeutics.

NK cell killing of tumors

Natural Killer (NK) cells are known to play a role in cancer immune surveillance.[2] They are potent cytotoxic effectors that were first identified in the 1970s as being able to lyse tumors without prior antigen sensitization.[3,4] NK cells can kill via several mechanisms: by delivering lytic granules containing perforin and granzymes (degranulation),[5] by secreting effector cytokines [6] and by engaging death-inducing receptors through surface ligands such as FasL and TRAIL to induce target cell apoptosis.[7] NK cells also express CD16 (or FcγRIII), an activating receptor for the conserved Fc portion of IgG. When IgG-coated target cells bind to CD16, target cell killing is triggered via antibody-dependent cell-mediated cytotoxicity (ADCC).[8] NK cell-mediated ADCC is potent and can occur without the need for co-activation from other receptors (Fig. 1A, B).

Unlike T cells that rearrange gene segments to generate antigen-specific receptors, NK cells recognize their target ligands using an array of germ-line encoded receptors. These include both activating and inhibitory receptors, from which signals are integrated to determine NK cell function (reviewed in[9]). Activating receptors recognize a number of stress ligands that may be overexpressed by tumors.[10] Tumors may also downregulate MHC molecules recognized by the NK inhibitory receptors. It is believed that NK cells are therefore activated to kill tumors due to overriding activating signals and/or lack of inhibitory signals due to 'missing-self'[11] (Fig. 1A, B). This mode of target cell recognition lacks specificity however, and a key challenge in harnessing NK cells for cancer immunotherapy has been to develop strategies that enhance their specificity for tumors. Another challenge of NK cell-based therapies is to maintain NK cell numbers and function *in vivo*.[12-14] Therefore, many genetic and pharmacological approaches are currently being pursued to re-direct NK cells, augment their function and sustain their *in vivo* expansion and persistence (reviewed in[9,15]).

BiKEs and TriKEs effectively drive NK cell anti-tumor effects

The TriKE reagent builds upon previous versions of engineered bispecific molecules designed to crosslink tumors and NK cells, termed *Bi*specific *K*iller cell *E*ngagers (BiKEs) (Fig. 1B). An example of a BiKE created by Vallera and colleagues is one that comprises a single-chain variable fragment (scFv) domain specific for CD16 on NK cells and a second scFv specific for the acute myeloid leukemia (AML) antigen CD33 (known as 1633 BiKE).[16] *In vitro*, 1633 BiKEs could overcome inhibitory killer-cell immunoglobulin-like receptor (KIR) signaling to stimulate NK cells to kill AML blasts.[16] Stimulation of NK cells with 1633 BiKEs *in vitro* also restored NK cell functions that had been inhibited in patients with myelodysplastic syndrome (MDS).[17]

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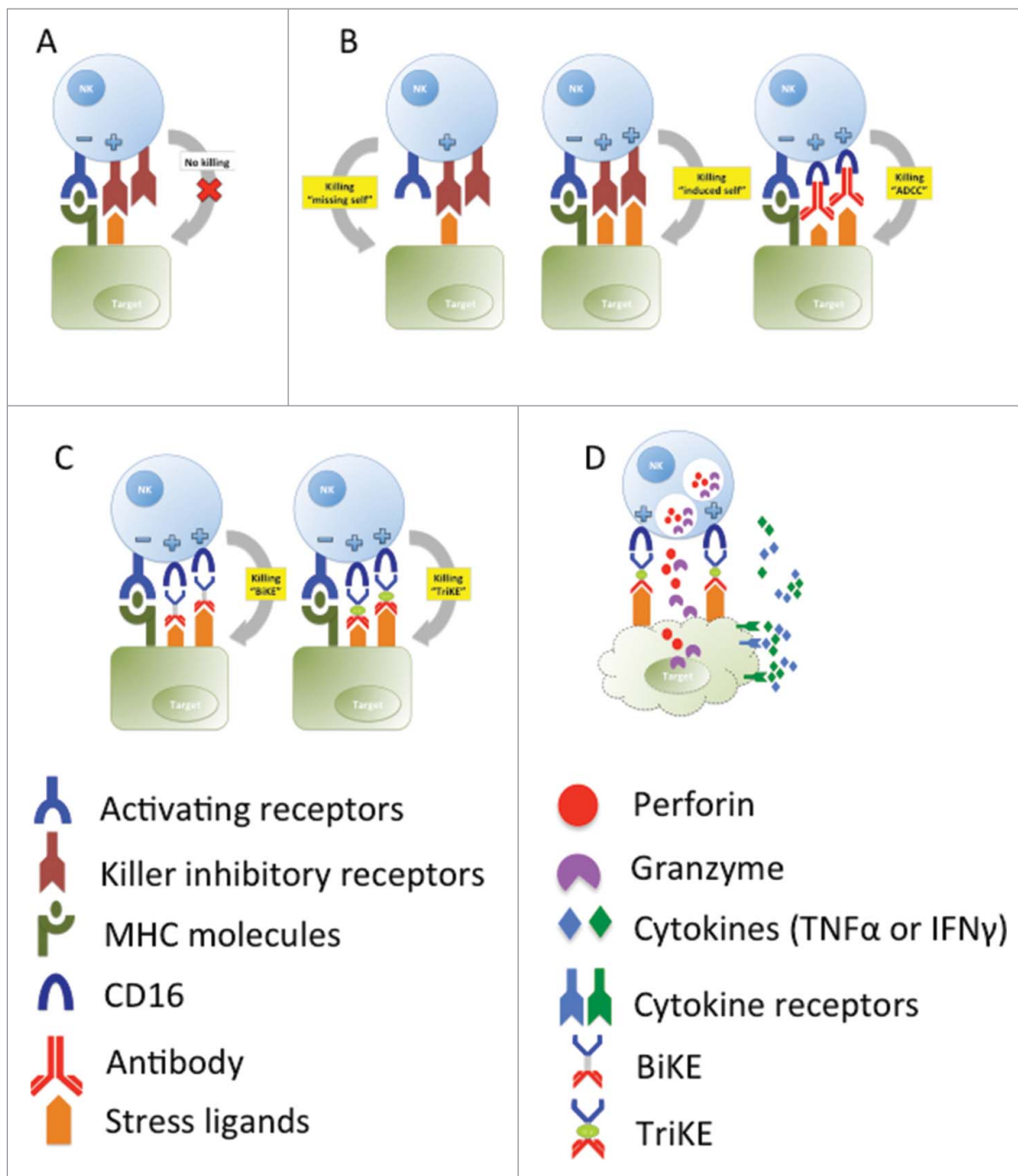
Figure 1. Natural killer cell-mediated killing of target cells. (A) No killing of healthy cells due to a balance of activating and inhibitory signals. (B) Killing of targets due to down-regulation of MHC molecules ("missing self"); killing due to overexpression of stress ligands ("induced self") that can override inhibitory signals; killing due to the recognition of antibodies bound to target cells by CD16 on NK cells ("antibody-dependent cell-mediated cytotoxicity" ADCC). (C) BiKEs and TriKEs facilitate NK cell interaction with tumor targets via CD16, (D) triggering degranulation and cytokine production via the ADCC pathway.

In the current study, Vallera and colleagues integrated IL-15 into the existing BiKE to create the novel 161533 TriKE[1] (Fig. 1C). The TriKE therefore performs 3 key functions: (a) to direct NK cells to tumors by facilitating formation of intracellular synapses; (b) to bind CD16 on NK cells to trigger ADCC; and (c) to drive *in vivo* NK cell expansion. IL-15 was selected (instead of IL-2) to promote NK cell activation, expansion and survival in order to avoid problems associated with the use of IL-2: the risk of systemic vascular leak [18] and the concurrent activation of CD25+ T regulatory cells that could inhibit NK cell function.[12,19]

When compared to its predecessor BiKE, the TriKE elicited superior NK cell killing of CD33+ myeloma cell lines and primary AML blasts, by enhancing NK cell degranulation, cytokine production (Fig. 1D), proliferation and survival *in vitro*.[1] It was also superior to the BiKE in restoring NK proliferation and killing functions in samples obtained from patients who had recently undergone haematopoietic stem cell transplantation (HSCT)[1]. The significance of this capacity is discussed in the subsequent section. *In vivo* efficacy of the TriKE was further demonstrated in a xenograft model where human NK cells were adoptively transferred

into mice to eradicate engrafted human CD33+ myeloma cells [1] (Fig. 2A). Tumor load was reduced significantly 3 weeks after NK cell infusion when the transferred NK cells were stimulated with TriKE but not with BiKE, and at this time point, significant increases in NK cells in the blood after TriKE stimulation was recorded. Together, this
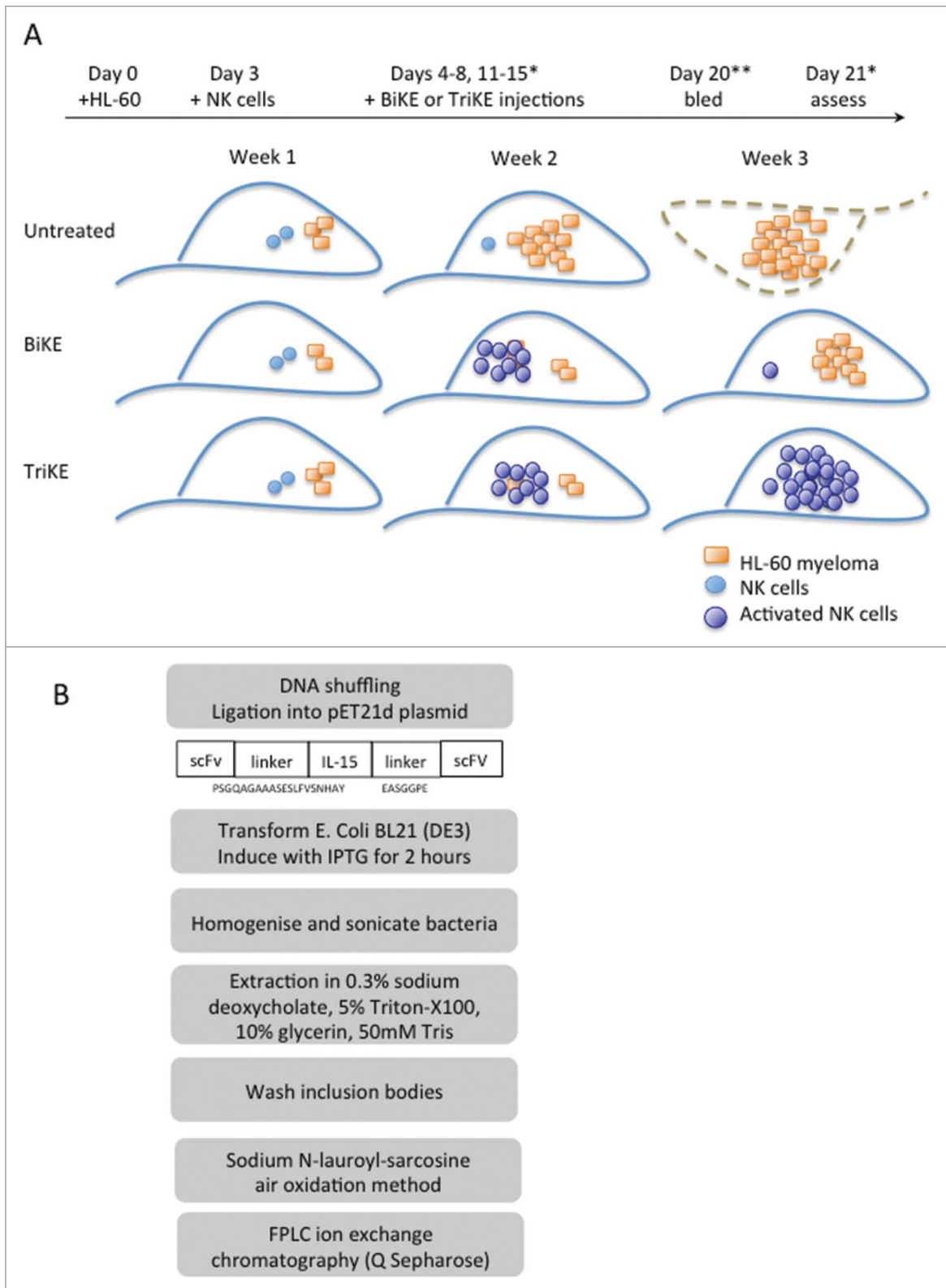

Figure 2. Production of TriKEs and functional testing in a xenograft model.[1] (A) TriKEs are expressed as recombinant proteins in bacteria before refolding and purification. Peptide linkers flanking IL-15 are indicated. (B) TriKEs and BiKEs promote NK cell-mediated killing of engrafted HL-60. TriKEs further support NK cell expansion and persistence that is associated with tumor clearance. A luciferase-expressing HL-60 myeloma cell line (7.5×10^5) was delivered intravenously 3 d before adoptive transfer of 1×10^6 CD3/CD19-depleted NK cells that were stimulated overnight with IL-15. TriKEs or BiKES were injected subcutaneously (50 μg each injection) for 10 d. *Tumor load was assessed by chemiluminesence after luciferin administration on days 14 and 21. **NK cells in peripheral blood were enumerated.

study showed that TriKE elicited superior anti-tumor responses from NK cells and supported their *in vivo* persistence.

TriKEs complement NK transfer therapies for haematological malignancies

Allogeneic HSCT is a current standard treatment for acute myeloid leukemia (AML) and for myelodysplastic syndrome (MDS) but is compromised by treatment-associated mortality and high relapse rates.[20,21] It is thought that defective NK function early after HSCT may play a role in these relapses.[22] Therefore, infusion of fully functional NK cells is an option for consolidation therapy while patients are in remission.[19] Since TriKEs were found to restore NK function in samples from recipients that had undergone HSCT *in vitro*, it is expected that TriKEs may also find clinical application when administered *in vivo* to activate reconstituting NK cells early after HSCT. It was noted in the current study (1) that TriKEs activated NK cells but did not induce T cell proliferation within the same post-HSCT samples. The mechanism for this differential activation is unknown but a strategy that avoids expanding T cells (that could mediate graft-versus-host responses) while being able to potentiate the graft-vs.-leukemia responses of NK cells warrants further investigation.

Apart from HSCT, the infusion of haploidentical NK cells (without aiming for donor haematopoesis) has also been trialled for treating AML or ALL. Pilot studies have produced good outcomes with increased safety profiles when improved NK purification protocols and reduced IL-2 doses have been used.[13,14,23] The use of IL-2 is still not ideal, however, and by selecting an IL-15-containing TriKE to support NK cell expansion, Vallera and colleagues hope to avoid IL-2-mediated toxicities and to avoid the expansion of regulatory T cells.[24] It was also hypothesized that incorporating IL-15 within a TriKE would reduce the risk of systemic toxicity as this restricts IL-15 availability to local NK-target cell synapses (1 and personal communication, D. Vallera). Further, IL-15 may be more relevant than IL-2 in this clinical setting, as it was noticed in 2 clinical trials that a transient surge in IL-15 production correlated with NK reconstitution,[13,14] suggesting that the presence of IL-15-stimulated NK cells may be associated with leukemic remission.

TriKEs are versatile and amenable to further optimization

The TriKE is a versatile platform amenable to incorporation of unique combinations of targeting ligands by straightforward molecular cloning approaches (Fig. 2B) and,[25] with IL-15 integrated, flanked by linkers, in between the dual targeting scFv domains. The TriKE is then readily produced as a recombinant protein in bacteria and purified by chromatography. Relevant scFv domains against clinically tested tumor-associated antigens, including those from solid tumors such as EpCAM [25] can be expressed within a TriKE. It is also possible to target 2 tumor antigens simultaneously, as described in a tri-specific molecule comprising scFv against CD19 and CD22 as well as against CD16.[26]

As TriKEs can be generated with different specificities, they provide the option to re-target NK cells in accordance with the emergence of tumor-associated antigens during tumor escape and immunoediting, which can impede the long-term success of cancer therapies. A remaining question pertinent to repeated TriKE stimulation, however, is whether TriKE-expanded NK cells sustain CD16 expression and whether their progeny will express unoccupied CD16 available for further stimulation (personal communication, D. Vallera). To overcome the problem of CD16 shedding upon NK cell activation, Vallera and colleagues had previously investigated if concurrent administration of an ADAM17 inhibitor with TriKES to prevent CD16 shedding might be useful.[16]

Apart from altering targeting specificities, intrinsic TriKE function might be enhanced by simple engineering of scFv variants with higher affinity for CD16. For instance, modifying the Fc fragment of humanised antibodies to increase CD16 binding enhances ADCC[27,28] a similar strategy could work in the context of TriKEs.

While a CD16-specific TriKE (and BiKE) primarily takes advantage of ADCC, additional NK cell receptors might also be suitable for TriKE-mediated targeting. A particularly promising candidate is the activating receptor Natural Killer Group 2D (NKG2D), which has important roles in tumor surveillance. The NKG2D pathway is the target of several tumor escape mechanisms,[29] including shedding of NKG2D ligands by tumors, and/or de-sensitization and down-regulation of NKG2D due to chronic stimulation. Of particular interest, in 2015, a soluble murine-UL16-binding protein-like transcript (MULT)-1 ligand was identified. This soluble MULT1 was found to, contrary to expectations, increase NK cell activity by binding to NKG2D.[30] It is thought that soluble MULT1 stabilises NKG2D on the cell surface instead of inducing its downregulation, in part due to its higher affinity for NKG2D compared to previously described ligands. This mode of engagement could overcome inhibitory signals delivered via a second NKG2D ligand, retinoic acid early inducible (RAE)-1, resulting in maintenance of NK functions. A targeting modality based on soluble MULT1 could be tested within the context of a TriKE.

The TriKE platform not only provides options to target different receptors, or novel tumor ligands, but also provides a way to explore new targeting strategies based upon newly discovered molecules reasonably rapidly.

Could TriKEs become a cost-effective off-the-shelf alternative to NK cell transfer?

Rapid improvements are being made in technologies for producing clinical-grade NK cells suitable for adoptive transfer. The sources of NK cells are diverse, including cell lines, peripheral and cord blood, or pluripotent stem cells (reviewed in [31]). Similarly, many protocols for *ex vivo* primary NK cell expansion using cytokine cocktails and/or feeder cells are also being developed for clinical use.[32,33] TriKEs could be used to potentiate these NK expansion protocols by conferring antigen specificity, by boosting ADCC and by supporting NK cell expansion, pre- or post-infusion. For instance, in addition to *in vivo* applications, TriKEs may also be useful for stimulating

and expanding NK cells *in vitro* in the presence of appropriate ligands (email discussion with D. Vallera).

At this stage, however, it is unclear if TriKEs could be used to bypass the need of NK cell transfer altogether. TriKE activity has thus far only been tested on transferred human NK cells in a xenograft model. While TriKES could drive dramatic levels of NK cell expansion (up to 350 fold compared to BiKE) for up to 3 weeks,[1] it is not known if *in vivo* administration of TriKEs alone will be sufficient to stimulate expansion of endogenous NK populations and their activity to therapeutically relevant levels (personal communication, D. Vallera). It will be interesting to see the outcomes of further preclinical testing of TriKEs: for instance, to determine whether they can expand and redirect a physiologically relevant quantity and diversity of human NK cells in mice with humanised immune systems. The development of humanised models able to support full human NK cell reconstitution and suitable for testing NK cell functions, including ADCC, would further these investigations.[34]

Safety and efficacy considerations

Clinical trials for haematological malignancies using patient T cells armed with activating, tumor-specific chimeric antigen receptors (CARs) have achieved unprecedented remission rates (recently reviewed in [35]). CAR T cells targeting CD19 have brought 70 to 90% of patients with refractory or relapsed ALL into remission.[36,37] Despite these spectacular successes, there have been reports of treatment-related deaths and severe side effects associated with cytokine-release syndrome after infusion of CAR T-cells.[38-40] By virtue of IL-15's potential to stimulate and expand both T cells and NK cells, TriKEs could present similar risks of activating systemic immune responses by triggering of cytokine cascades.[1] Indeed, the recent report of unexpected and severe acute graft-versus-host responses in several patients who had been given infusions of NK cells (pre-activated with IL-15 and TNFSF9), cautions that depending on their mode of activation, NK cells could initiate damaging immune responses.[33] It was encouraging that Vallera and colleagues showed more than 10 doses of $20\mu g$/day of IL-15-incorporating-TriKE administered to xenografted SCID immunodeficient mice for 2 weeks did not cause observable detrimental effects despite successful activation and expansion of NK cells. However, longer-term safety evaluations in additional model systems are required to monitor for TriKE-mediated immune toxicities, or their separate biological effects on tumors. One such system could be tumor-engrafted immunocompetent mice, since TriKE reacts with the mouse IL-15 receptor (personal communication, D. Vallera).

Another important risk after cancer immunotherapy is the untoward killing of healthy cells, as tumor antigens can be shared with healthy cells. Indeed, persisting CAR T cells specific for CD19 or CD38 have been reported to cause long-term B cell depletion or bone marrow suppression in patients, respectively. While the shorter lifespan of NK cells are among several properties that led researchers to consider them "better CAR drivers" (discussed in [41]), leading to genetic modifications of NK cell lines (NK-92 in particular) and primary NK cells with CARs to target different tumors,[42,43] TriKEs provide a simpler alternative for re-targeting NK cells *in vivo* without

genetic modification. However, further characterisations of the proliferative and functional responses of NK cells under various TriKE dosing regimens are required. These studies would provide insight into the biological effect of TriKEs on NK cells, in regard to their activation and maturation and conversely, exhaustion; their activities upon cessation of TriKE administration; and their potential to develop into long-lived "memory" populations.[44]

The road ahead for cancer immunotherapy

Cancer immunotherapy has been hailed as the biggest breakthrough in modern cancer treatment. While the field is currently dominated by CAR T cell therapies and antibody therapies that aim to reverse immunosuppression of T cells, NK cell-based approaches are beginning to move to the forefront. TriKES and BiKES, with their potential to enable tumor-specific targeting, to boost NK functions and to drive cellular expansion hold promise as versatile, cost-effective and widely accessible off-the-shelf solutions, when compared to more complex and costly genetic modifications, *ex vivo* expansion protocols or more personalised therapies. TriKES and BiKES can be used in conjunction with existing therapies and also have the potential to be used *in lieu* of NK cell transfer. We are optimistic that positive outcomes from further preclinical evaluations of the safety and efficacy profiles of TriKES and BiKES will support progress toward clinical testing, and herald potent novel avenues for immunotherapeutic treatments for cancers.

Disclosure of potential conflicts of interest

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