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Cytomegalovirus: an unlikely ally in the fight against blood cancers?

A. B. Bigley*† , F. L. Baker*† and R. J. Simpson*†‡§

*Department of Nutritional Sciences, The University of Arizona, Tucson, AZ, †Department of Health and Human Performance, University of Houston, Houston, TX, ‡Department of Pediatrics, The University of Arizona, Tucson, AZ, and §Department of Immunobiology, The University of Arizona, Tucson, AZ, USA

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Correspondence: A. B. Bigley, Research Assistant Professor, Department of Nutritional Sciences, The University of Arizona, Tucson, AZ 85721, USA.
E-mail: abbigley@email.arizona.edu

Summary

Cytomegalovirus (CMV) infection is a potentially fatal complication in patients receiving haematopoietic stem cell transplantation (HSCT), but recent evidence indicates that CMV has strong anti-leukaemia effects due in part to shifts in the composition of natural killer (NK) cell subsets. NK cells are the primary mediators of the anti-leukaemia effect of allogeneic HSCT, and infusion of allogeneic NK cells has shown promise as a means of inducing remission and preventing relapse of several different haematological malignancies. The effectiveness of these treatments is limited, however, when tumours express human leucocyte antigen (HLA)-E, a ligand for the inhibitory receptor NKG2A, which is expressed by the vast majority of post-transplant reconstituted and *ex-vivo* expanded NK cells. It is possible to enhance NK cell cytotoxicity against HLA-E^{pos} malignancies by increasing the proportion of NK cells expressing NKG2C (the activating receptor for HLA-E) and lacking the corresponding inhibitory receptor NKG2A. The proportion of NKG2C^{pos}/NKG2A^{neg} NK cells is typically low in healthy adults, but it can be increased by CMV infection or *ex-vivo* expansion of NK cells using HLA-E-transfected feeder cells and interleukin (IL)-15. In this review, we will discuss the role of CMV-driven NKG2C^{pos}/NKG2A^{neg} NK cell expansion on anti-tumour cytotoxicity and disease progression in the context of haematological malignancies, and explore the possibility of harnessing NKG2C^{pos}/NKG2A^{neg} NK cells for cancer immunotherapy.

Keywords: immunotherapy, leukemia, myeloma, NK-cells, NKG2C

CMV and cancer: a double-edged sword?

Cytomegalovirus (CMV) is a prevalent β-herpesvirus that infects 50–80% of all adults in the United States [1]. CMV is typically asymptomatic in immunocompetent hosts; however, it has been linked to increased risk of cancer-related and post-transplant mortality in haematopoietic stem cell transplantation (HSCT) and organ transplant recipients, respectively [2,3]. Moreover, research into immunological function and causes of death in elderly people have led to the conclusion that CMV is an immunological burden within the T cell compartment that impacts negatively upon both immune status and overall health [4]. These findings caused some to speculate that it may be beneficial to eradicate CMV or vaccinate against it as a matter of interest to public health [5]; however, recent evidence suggests that CMV may also play a beneficial role in 'arming' natural killer (NK) cells to destroy cancerous blood cells [6,7]. This is due, in part, to the fact that NK cells have evolved countermeasures that allow them to control viral replication by up-regulating activating receptors and down-regulating inhibitory receptors for human leucocyte antigen (HLA) expressed on the surface of virally infected cells [8–11]. For example, both acute and latent CMV infections trigger substantial expansions of NKG2C^{pos}/NKG2A^{neg} NK cells [9,12–14], which enables the elimination and containment of CMV-infected cells that up-regulate HLA-E as a means of evading detection by NKG2A^{pos} NK cells [10]. HLA-E can signal through either the inhibitory receptor NKG2A or the activating receptor NKG2C; however, the inhibitory receptor is dominant, thus only NKG2C^{pos}/NKG2A^{neg} NK cells are able

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to effectively kill HLA-E^{pos} target cells [15–17]. These NKG2C^{pos}/NKG2A^{neg} NK cells generate recall responses to CMV that are evocative of immunological memory [13], which has led some to refer to them as 'adaptive' or 'memory-like' NK cells [18,19]. These memory-like properties of NK cells were first demonstrated in mice when it was shown that Ly49H^{pos} NK cells (Ly49H is the functional counterpart to human NKG2C) were expanded preferentially in mice subjected to murine CMV infection and that these CMV-induced Ly49H^{pos} NK cells were long-lived and able to mount recall responses to subsequent CMV exposure [20]. Recently, it has been shown that there are two distinct types of long-lived NK cells in mice, one being antigen-specific (memory) and the other being cytokine-driven [21]. Specifically, Ly49H^{pos} memory NK cells show enhanced effector functions and anti-tumour cytotoxicity (similar to our findings with NKG2C^{pos} NK cells in humans) [8], while cytokine-activated long-lived NK cells (Ly49H^{neg}) have greater responsiveness to interleukin (IL)-15 and persist longer in a CMV-free milieu [21]. Evidence has also been provided for immunological memory in human NK cells, as CMV has been shown to induce epigenetic and phenotypical changes to NK cells that are stable over many years [22,23]. Supporting further the notion of 'memory' NK cells in humans, NKG2C^{pos} NK cells protect against acute CMV infection in solid organ transplant recipients [20], and NKG2C^{pos} NK cells from CMV^{pos} donors expand preferentially in response to CMV reactivation following allo-HSCT, but NKG2C^{pos} NK cells from CMV^{neg} donors do not [24]. Moreover, expansion of NKG2C^{pos} FcRγ-deficient NK cells can be driven by CMV-specific antibodies, which suggests an additional adaptive component to this 'memory' response [19].

Up-regulation of NKG2C on 'adaptive' or 'memory-like' NK cells responding to CMV has been linked to decreased expression of the transcription factor promyelocytic leukaemia zinc finger (PLZF) and downstream hypomethylation (up-regulation) of transcripts encoding NKG2C isoforms [18]. These 'adaptive' NKG2C^{pos} NK cells have enhanced target-specific cytotoxicity (antibody-dependent and -independent) and production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α [8,25], but markedly reduced effector responses to the innate cytokines IL-12 and IL-18 [18]. Furthermore, hypermethylation (down-regulation) of promoters for the transmembrane intracellular signalling proteins [Fc receptor common gamma chain (FcεRγ)], spleen tyrosine kinase (SYK) and EWS/FLI1-activated transcript 2 (EAT-2) has also been observed in NKG2C^{pos} NK cells taken from CMV^{pos} donors [18,19] with many purported downstream effects, including enhanced antibody-dependent cellular cytotoxicity (ADCC) [25], impaired innate production of immunoregulatory cytokines and reduced elimination of activated T cells (i.e. heightened viral-specific T cell responses) [18]. Furthermore, CMV-specific antibody-dependent expansion of FcRγ-deficient NK cells [19] can augment further the proportion of NKG2C^{pos} NK cells, as lack of FcεRγ correlates strongly with NKG2C expression 18.

While an increased proportion of NKG2C^{pos} NK cells has been reported to be protective against CMV reactivation [24], no direct link has been established between the NKG2C receptor and increased NK cell activation in response to CMV as NKG2C^{pos} NK cells have poor direct effector responses toward CMV-infected cells [25–27]. However, more recent studies have reported that NKG2C^{pos} NK cells have enhanced ADCC markedly against CMV-infected cells [28,29], due probably to down-regulation of FcRγ [25]. Thus, it may be that ADCC is required for NKG2C^{pos} NK cell-mediated killing of CMV-infected cells.

As with CMV infection, tumour escape has been linked to up-regulation of HLA-E on transformed cells [10,30], which has led researchers to explore the role of CMV and NKG2C^{pos} NK cells on cancer prognosis and treatment response. Interestingly, it has been reported in multiple prospective cohort studies that reactivation of CMV post-transplant is associated with a remarkable reduction in the risk of 100-day relapse in acute myelogenous leukaemia (AML) [31–34] and chronic myelogenous leukaemia (CML) [35] patients receiving allogeneic HSCT. The mechanism underpinning this beneficial effect of CMV reactivation on leukaemic relapse is currently unknown; however, we and others have hypothesized that the effect may be mediated by CMV-induced shifts in the composition of NK cell subsets [33,36]. Our work suggests that this unknown mediating factor may be NKG2C^{pos}/NKG2A^{neg} NK cells. Specifically, we have shown that the CMV-driven accumulation of NKG2C^{pos}/NKG2A^{neg} NK cells is associated with a strong anti-leukaemia and anti-myeloma effect that is commensurate with the magnitude of tumour cell HLA-E expression [8,37]. Using an HLA-E-transfected 721.221 lymphoma cell line (221.AEH), we were able to show that the beneficial effect of CMV on anti-tumour cytotoxicity was HLA-E-dependent, as CMV-infected individuals showed greater NK cell activity against lymphoma cells constitutively expressing HLA-E (221.AEH), but not HLA-E void 721.221 cells [8]. This effect was also shown to be NKG2C-dependent, as antibody blockade of the NKG2C receptor eliminated the CMV effect on NK cell activity against 221.AEH cells, and it was also shown that it was only the NKG2C^{pos} NK cells that degranulated and produced IFN-γ in response to 221.AEH cells [8]. Furthermore, the increased cytotoxic activity of NK cells seen in people with CMV was replicated in CMV-seronegative individuals by expanding NKG2C^{pos}/NKG2A^{neg} NK cells preferentially using 221.AEH feeder cells and IL-15 *in vitro* [8]. Liu *et al.* [38] recently corroborated our findings by showing that *ex-vivo* expansion

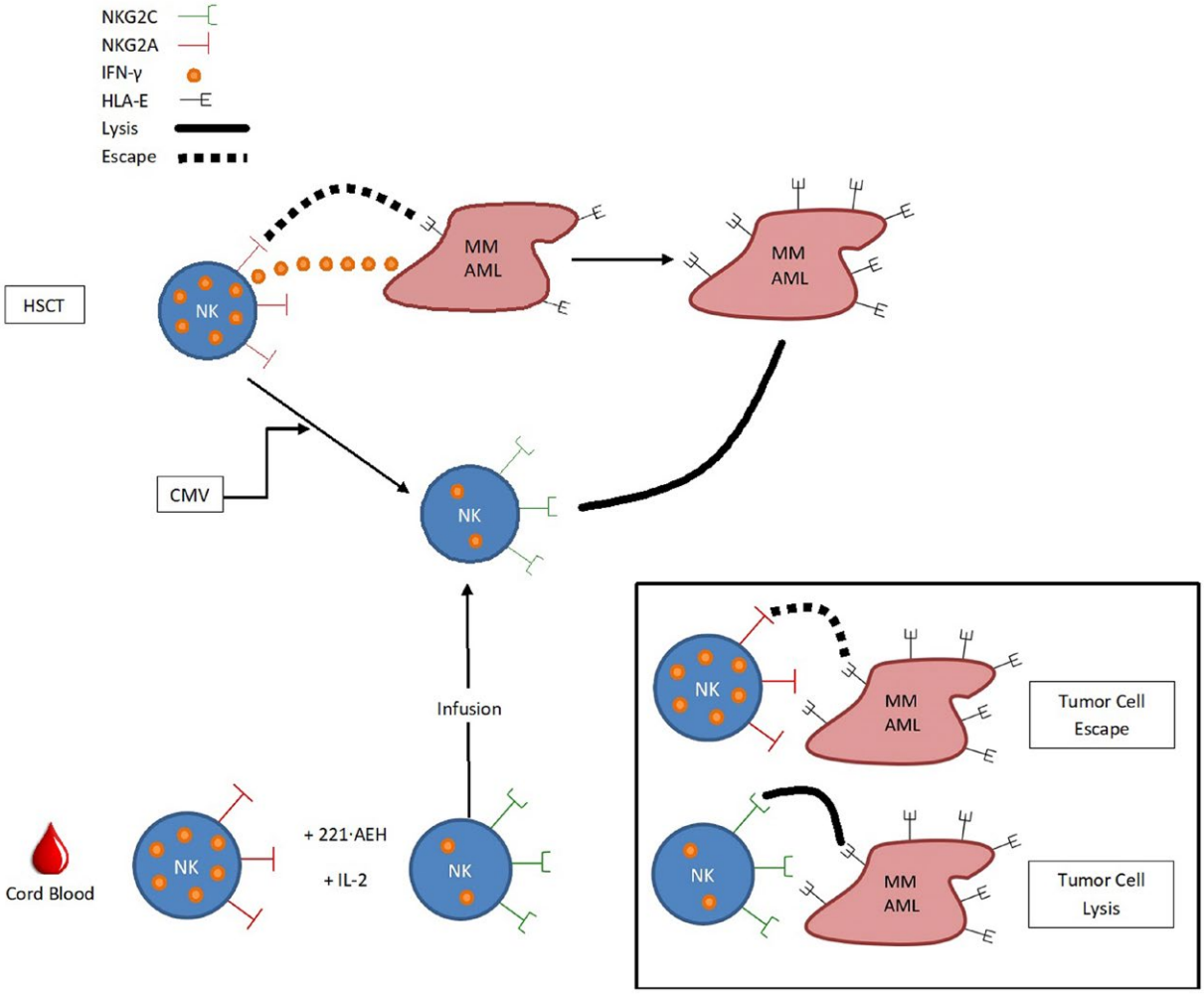

Fig. 1. Cytomegalovirus (CMV)-driven accumulation or *ex-vivo* expansion of NKG2C^{pos}/NKG2A^{neg} natural killer (NK) cells enhances NK cell cytotoxicity against human leucocyte antigen (HLA)-E^{pos} tumour cells. HLA-E signals through the activating receptor NKG2C and the inhibitory receptor NKG2A, with signalling through NKG2A being dominant, thus only NKG2C^{pos}/NKG2A^{neg} NK cells are able to effectively lyse HLA-E^{pos} target cells. NK cells are the first lymphocyte subset to recover following an allogeneic haematopoietic stem cell transplantation (HSCT), but the cells that are first to reconstitute are ~80% NKG2A^{pos}, which makes them unable to kill acute myeloid leukaemia (AML), multiple myeloma (MM) and other haematological malignancies characterized by high HLA-E expression. Furthermore, these 'naive' NKG2A^{pos} NK cells produce copious amounts of interferon (IFN)-γ, which further up-regulate HLA-E expression by haematological tumour cells. CMV infection/reactivation increases the proportion of NKG2C^{pos}/NKG2A^{neg} NK cells, enhances cytotoxicity against HLA-E^{pos} tumour cells and reduces the risk of leukaemic relapse markedly. While CMV seropositivity and reactivation have been correlated positively with decreased risk of leukaemic relapse, this benefit is outweighed by increased non-relapse mortality due to complications of CMV infection. To mimic the beneficial anti-tumour effect of CMV without the negative consequences of CMV infection, we developed a protocol to mass produce NKG2C^{pos}/NKG2A^{neg} NK cells from peripheral or cord blood samples and enhance cytotoxicity against HLA-E^{pos} malignancies. Infusion of these *ex-vivo* expanded NKG2C^{pos} NKcells may improve immunotherapy for the treatment of HLA-E^{pos} malignancies.

of NKG2C^{pos} NK cells using 221.AEH feeder cells enhances NK cell cytotoxicity against paediatric acute lymphoblastic leukaemia (ALL) blasts. Additional support for the important role of HLA-E comes from Sarkar *et al.* [39], who reported that primary myeloma cells express heterogeneously high levels of HLA-E and that HLA-E inhibits stoichiometrically the anti-myeloma cytotoxicity of NKG2A^{pos} NK cells. Collectively, these data suggest that CMV infection might prime NK cells to recognize and destroy malignant HLA-E^{pos} cells through the accumulation of highly functional NKG2C^{pos} NK cells (see Fig. 1).

Recent clinical data also support this hypothesis [32,40], as NKG2C^{pos} NK cells expand during CMV reactivation in allogeneic HSCT recipients [24] and leukaemic blasts, in turn, have a high expression of HLA-E [41]. In one major investigation, Cichocki *et al.* [31] showed prospectively in 674 allogeneic HSCT recipients that the expansion of highly differentiated (CD56^{dim}/CD57^{pos}) NKG2C^{pos} NK

cells is associated with a decreased incidence of leukaemic relapse and increased disease-free survival up to 1 year post-transplant. This study reported a broadly beneficial effect of CMV reactivation on relapse risk for haematological malignancies, as the study included patients with AML (n = 313), ALL (n = 187), myelodysplastic syndrome (MDS) (n = 85), non-Hodgkin lymphoma (NHL) (n = 42), CML (n = 28), Hodgkin's disease (n = 14) and multiple myeloma (MM) (n = 5) [31]. Interestingly, in a smaller prospective study of 106 allogeneic HSCT patients afflicted with a variety of haematological malignancies (including 37 patients with AML, 21 with MDS and 12 with CLL), Bjorklund *et al.* [42] reported a protective effect of the naive NK cell repertoire (defined as $NKG2C^{neg}/NKG2A^{pos}/CD57^{neg}$) on leukaemic relapse. This study directly contradicts the findings of Cichocki *et al.* [31], as it reports a deleterious effect of increased NK cell differentiation ($NKG2C^{pos}/CD56^{dim}/CD57^{pos}$) on leukaemic relapse, disease-free survival and overall survival 9–12 months after allogeneic HSCT [42]. It is important to note when resolving the discrepancies between the two studies that the patients and donors were completely different. For example, the donors for the Bjorklund *et al.* [42] study were all adults, while the donor source for the Cichocki *et al.* [31] study was primarily cord blood [31,42]. In addition, all the patients in the Bjorklund *et al.* [42] study received myoablative (MA) conditioning, while the beneficial effect of CMV reported in the Cichocki *et al.* [31] paper was observed only in patients who received reduced intensity conditioning (RIC) 31,42. Thus, the beneficial effect of CMV-driven expansion of $NKG2C^{pos}$ NK cells on treatment response in the Cichocki *et al.* [31] study may be at least partially attributable to residual host immunity (although 90% of NK cells were donor-derived at 6 months) [31]. Furthermore, monocyte concentrations were found to correlate strongly with $NKG2C^{pos}$ NK cell expansion and these were higher in patients receiving RIC than in those conditioned with MA; thus, it could be that residual host monocytes are required for the beneficial effect of CMV and they may explain the difference between results obtained with RIC and MA conditioning [31]. Conversely, the beneficial effect of a naive donor NK cell repertoire [42] may be attributable to lower expression of inhibitory killer-cell immunoglobulin-like receptor (KIR) for host HLA on the surface of immature $NKG2A^{pos}$ donor NK cells [42]. Adding further complexity to the issue is the report by Manjappa *et al.* [43], that the beneficial effect of CMV reactivation on leukaemic relapse in allogeneic HSCT recipients is observed only in those receiving MA conditioning and not those receiving RIC (again contradicting Cichocki *et al.* [31]). Use of common anti-graft-*versus*-host disease (GVHD) prophylactic drugs such as anti-thymocyte globulin has also been shown to blunt the anti-leukaemia effect of CMV reactivation in allogeneic HSCT recipients [43]. Moreover, Achour *et al.* [44] have reported that the incidence of head/neck and colorectal tumours is correlated positively with the expansion of $NKG2C^{pos}$ NK cells in CMV-infected liver transplant patients, which suggests that the effect of CMV and $NKG2C^{pos}$ NK cells is actually negative with some categories of cancer [45]. Thus, the field is currently beset with equivocal findings and future studies should seek to resolve the ambiguities and also determine if the benefits of CMV-induced NK cell reconstitution on prognosis/relapse risk extend to HLA-E-expressing malignancies beyond leukaemia (e.g. MM) [39].

It is important to note that while donor CMV seropositivity [46] and CMV reactivation [32,33] have been correlated positively with a decreased risk of leukaemic relapse in allogeneic HSCT recipients, this salubrious effect does not outweigh the risk of increased non-relapse mortality that CMV carries [34,46]. Our work and that of others suggests that the benefit of CMV on the incidence of leukaemic relapse may be attributable to an elevated frequency of $NKG2C^{pos}$ NK cells that are able to target HLA-E-expressing leukaemic blasts effectively [8,31,41]. Thus, it follows that infusion of *ex-vivo* expanded $NKG2C^{pos}$ NK cells may allow us to simulate the beneficial effect of CMV on incidence of leukaemic relapse, while simultaneously reducing the risk of CMV reactivation [24,47] and consequently reducing the risk of non-relapse mortality in HSCT recipients. It is also plausible that the potential immunotherapeutic benefits of $NKG2C^{pos}$ NK cells could be extended to other HLA-E-expressing cancers besides leukaemia [30,39].

Harnessing the power of CMV: $NKG2C^{pos}$ NK cells and immunotherapy

The idea of harnessing the power of NK cells for the treatment of cancer can be traced back to the landmark study by Ruggeri *et al.* [48], who showed that AML patients receiving an allogeneic HLA-mismatched HSCT with KIR–ligand incompatibility had extremely favourable outcomes relative to those receiving transplants without it. Specifically, it was shown that KIR–ligand mismatch completely protected against haematopoietic graft rejection, acute GVHD and leukaemic relapse at 5 years (0 *versus* 75% in KIR-matched donors), and that this effect was entirely attributable to anti-recipient and anti-leukaemia NK cell clones that arose post-transplant [49]. The promising results of Ruggeri *et al.* [48] led to the idea that adoptive transfer of NK cells may serve as an effective means of controlling AML in the absence of HSCT.

In one study, 19 patients with AML were infused with a single NK cell-enriched product derived from a haploidentical-related donor [50]. Of the four patients receiving NK cells from KIR-mismatched donors, three achieved a complete remission while only two of 15 patients receiving NK cells from KIR-matched donors achieved remission. Other studies have shown beneficial effects of KIR–ligand mismatch on HSCT and cord blood transplantation in AML patients. For example, KIR–ligand mismatch was associated with a decreased incidence of relapse and improved survival in AML patients receiving an unrelated cord blood transplant [51], and the absence of one or more KIR ligands for donor inhibitory KIR was associated with improved survival and a decreased incidence of relapse in AML and MDS patients receiving T cell-depleted (NK-rich) HSCT from unrelated donors [52]. While most studies reported a beneficial effect of KIR–ligand mismatch on treatment outcome (increased remission or decreased relapse) in leukaemia patients receiving either allogeneic NK cells directly or some form of allogeneic HSCT [50–56], some others reported no benefit [57–60], and one even reported an increased incidence of relapse [61]. Furthermore, a larger follow-up study by the aforementioned Miller group demonstrating the potential to enhance allogeneic NK cell immunotherapy through selective depletion of regulatory T cells (T_{regs}) showed no correlation between KIR–ligand mismatch and remission rates in AML patients [62]. Overall, however, the generally successful record of allogeneic, KIR-mismatched NK cells in the prevention of relapse in leukaemia patients has led to attempts to utilize the technique to treat other haematological malignancies, such as MM [63–65].

High-dose chemotherapy in combination with autologous HSCT is the standard treatment for MM [66,67] and allogeneic HSCT is relatively rare [68], thus there are not the multitude of studies examining the potential anti-myeloma activity of allogeneic NK cells that we see with leukaemia patients who are treated commonly with varying forms of allogeneic HSCT [69]. However, given the fact that most MM patients fail to achieve a complete remission post-transplant [70], new strategies are needed to improve treatment outcomes for MM, and adoptive transfer of allogeneic KIR-mismatched NK cells is one avenue that has been explored with some clinical benefit being reported. For example, it has been shown that KIR–ligand mismatch in T cell-depleted (NK cell-enriched) allogeneic HSCT protects against relapse in MM patients [71] and infusion of haploidentical, KIR-mismatched NK cells led to complete or near-complete remission in 50% of patients with relapsed MM [65]. While KIR–ligand mismatch has improved outcomes in allogeneic transplantation for MM, infusion of an adequate dose of alloreactive NK cells is difficult [65], which has led to the development of new techniques to achieve large clinical-grade NK cell expansion [64,72]. Existing protocols generate exponentially large NK cell expansions [64,72], but alloreactivity of donor NK cells is still highly variable [49,65,73] and expression of NKG2A is far greater than NKG2C [64], which limits the capacity of NK cells to kill myeloma cells with high HLA-E expression [65,73]. This can be extremely important in the context of immunotherapy, as HLA-E expression is up-regulated on MM cells as the disease progresses [74] and HLA-E can eliminate the beneficial effects of KIR–ligand mismatch by engaging NKG2A on NK cells [39]. Moreover, $NKG2A^{pos}$ NK cells typically make up more than 80% of NK cells expanded from peripheral blood or stem cells [64,75,76] as well as reconstituted NK cells during the early post-transplant period of allo-HSCT [41]. This inhibitory effect of HLA-E on $NKG2A^{pos}$ NK cells may explain why infusion of KIR–ligand mismatched NK cells in relapsed myeloma patients produced a complete remission in only two of 10 patients [65]. New protocols should exploit the immunoevasive strategies of MM to generate NK cells with high cytotoxicity against primary myeloma, especially those $HLA\text{-}E^{bright}$ cells which are particularly resistant to existing protocols that generate large numbers of $NKG2A^{pos}$ NK cells [64,72]. Such an approach would probably also be beneficial in the context of leukaemia, because AML and other leukaemic blasts are characterized by high HLA-E expression [30,77] due, in part, to up-regulation of HLA-E by IFN-γ-producing $NKG2A^{pos}$ NK cells, which make up the great majority of early post-allogeneic HSCT NK cells [41]. One such approach would be to mimic the effect of CMV on NK cells, as CMV has been shown to induce a marked expansion of $NKG2C^{pos}/NKG2A^{neg}$ NK cells [12] which are, in turn, able to lyse HLA-E expressing tumour cell lines that $NKG2A^{pos}$ NK cells cannot [8,78].

To this end, our laboratory has shown that *ex-vivo* expansion of $NKG2C^{pos}/NKG2A^{neg}$ NK cells derived from fresh peripheral blood mononuclear cells (PBMCs) can be achieved using 221.AEH feeder cells (HLA-E transfected) and IL-15, with a resultant increase in NK cell activity against several distinct $HLA\text{-}E^{pos}$ targets [8]. Current expansion protocols rely on the proliferation-inducing cytokines IL-2/-15 and transgenic feeder cell lines that constitutively express transmembrane pro-growth cytokines such as IL-15 and IL-21, all of which enhance expression of NKG2A relative to NKG2C on stimulated NK cells [64,79]. By co-culturing NK cells with the transgenic, $HLA\text{-}E^{bright}$ 221.AEH cell line, we mimic the effect of CMV and counter cytokine-driven up-regulation of NKG2A by preferentially activating and expanding $NKG2C^{pos}/NKG2A^{neg}$ NK cells [8]. Our approach can enhance the proportion of $NKG2C^{pos}/NKG2A^{neg}$ NK cells from < 5%

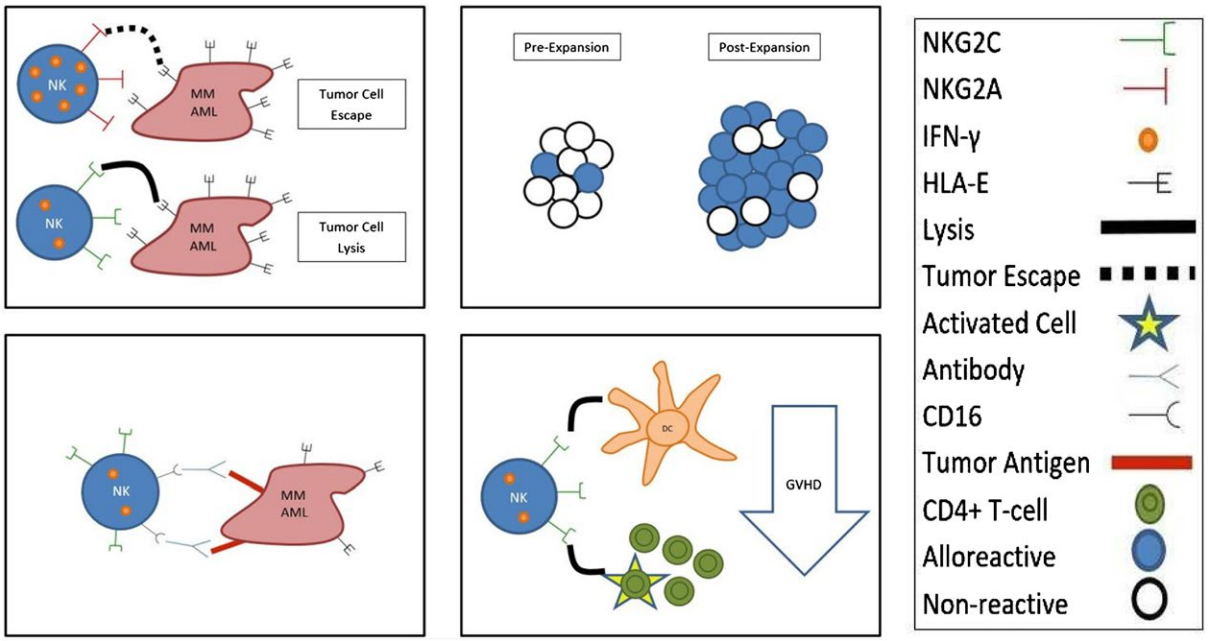

Fig. 2. NKG2C^{pos}/NKG2A^{neg} natural killer (NK) cells have a broad range of benefits that can be harnessed effectively for cancer immunotherapy. (1) NKG2C^{pos}/NKG2A^{neg} NK cells are the only NK cells capable of efficiently lysing HLA-E^{pos} tumour cells. (2) The *ex-vivo* expansion of NKG2C^{pos}/NKG2A^{neg} NK cells skews the killer cell immunoglobulin-like receptor (KIR) repertoire towards self-KIR, thus enhancing alloreactivity of allogeneic NK cells against malignancies from human leucocyte antigen (HLA)-mismatched recipients. (3) NKG2C^{pos} NK cells are potent mediators of antibody-dependent cell-mediated cytotoxicity (ADCC), thus adoptive transfer of NKG2C^{pos} NK cells is expected to synergize well with monoclonal antibody-based treatments. (4) NKG2C^{pos}/NKG2A^{neg} NK cells have strong anti-graft-*versus*-host disease (GVHD) effects due in part to their ability to lyse residual host dendritic cells and activated CD4^{pos} T cells. Thus, infusion of *ex-vivo* expanded NKG2C^{pos}/NKG2A^{neg} NK cells could reduce incidence of both acute and chronic GVHD in the context of allogeneic haematopoietic stem cell transplantation (HSCT). Overall, adoptive transfer of NKG2C^{pos}/NKG2A^{neg} NK cells shows great promise as a tool for the treatment of a variety of haematological malignancies characterized by high HLA-E expression, and the approach can be combined synergistically with other established techniques, including KIR/ligand mismatch and monoclonal antibody infusions.

to greater than 50% when compared to conventional expansion techniques [8,64]. These NKG2C^{pos}/NKG2A^{neg} NK cells are able to recognize and eliminate CMV-infected cells [12,28,29] and haematological malignancies that up-regulate HLA-E [8] as a means of immunoevasion. This enhanced anti-tumour and anti-viral cytotoxicity comes without damaging healthy tissue, as NKG2C^{pos} NK cells express self-KIR [22] and healthy cells have lower expression of HLA-E and lack the co-stimulatory molecules required for full activation [80,81]. Furthermore, the cytotoxic effects of NKG2C^{pos} NK cells against HLA-E^{pos} malignancies do not require ADCC, which is necessary for NKG2C^{pos} NK cell-mediated effector functions against CMV-infected cells [8,28,29]. It remains to be seen, however, if our approach for selectively expanding NKG2C^{pos}/NKG2A^{neg} NK cells can be combined with other clinical expansion protocols that generate higher NK cell yields (e.g. those using irradiated transgenic K562 cells) or if the final NK cell product will be effective *in vivo* or kill primary tumour cells as well as cell lines. If successful, the clinical grade *ex-vivo* expansion of NKG2C^{pos}/NKG2A^{neg} NK cells may enhance the efficacy of NK cell-based immunotherapy by enabling the production of highly cytotoxic 'off-the-shelf' NK cell lines for the treatment of MM, AML and other malignancies characterized by high HLA-E expression (see Fig. 1).

While the idea of harnessing the anti-tumour effects of CMV for NK cell immunotherapy is compelling, it is important to note that the effects of CMV on NK cell function are not universally beneficial. For example, Fielding *et al.* [82] report that members of the CMV US12 family of genes are able to down-regulate expression of ligands for NK cell-activating receptors on the surface of CMV-infected targets. Specifically, CMV infection induces expression of US18 and US20 which synergistically suppress cell surface expression of B7-H6 [82], a major ligand for the activating receptor NKp30 [83]. Additionally, CMV-induced gpUL16 is able to sequester ligands for the activating receptor NKG2D [84]. It is of note, however, that these immunoevasive strategies centre on protecting CMV-infected cells from being recognized by NK cells rather than inhibiting NK cell

function directly. Thus, NKG2C-mediated killing of tumour targets by *ex-vivo* expanded NKG2C^{pos}/NKG2A^{neg} NK cells would probably not be impaired by these CMV evasion strategies. Separate from our findings linking NKG2C with anti-tumour activity, other activating receptors, such as NKG2D, NKp30 and activating KIR, have also been shown to drive cytotoxicity against haematological malignancies [49,85]. Besides increasing the proportion of NKG2C^{pos} NK cells, co-culture of peripheral blood mononuclear cells with 221.AEH feeder cells can lead to an increased proportion of NK cells expressing activating KIR (similarly to CMV infection) [22]. Thus, it is plausible that other activating receptors besides NKG2C will play a role in the anti-tumour effect of the NKG2C^{pos} NK cell lines.

In addition to enhanced killing of HLA-E^{pos} malignancies, *ex-vivo* expansion of NKG2C^{pos} NK cells has other benefits that could be harnessed for use in cancer immunotherapy. First, expansion of NKG2C^{pos} NK cells leads to skewing of the KIR repertoire towards self-KIR [22] thus enhancing alloreactivity of NK cells against malignancies from HLA-mismatched recipients (i.e. C2/C2 donors and C1/C1 recipients). For example, *ex -vivo* expansion of NKG2C^{pos} NK cells has been shown to enhance cytotoxicity against mismatched primary paediatric ALL blasts, an HLA-E deficient malignancy, by enhancing the proportion of alloreactive NK cells [38]. Secondly, NKG2C^{pos} NK cells are far more potent mediators of ADCC than their NKG2C^{neg} counterparts [86] with ADCC being one of the primary mechanisms whereby NKG2C^{pos} NK cells mediate effector responses *in vivo* [29]. Thirdly, NKG2C^{pos} NK cells have been linked to the prevention of GVHD, as a low ratio of NKG2C to NKG2A on NK cells is associated with a marked increase in the risk of severe acute and chronic GVHD in HLA-mismatched allogeneic HSCT recipients [87]. Lastly, efficacy of NK cell immunotherapy protocols has been limited by poor persistence and loss of effector functions in transferred NK cells; however, NKG2C^{pos} NK cells have been reported to have 'memory-like' persistence *in vivo* [19,22]. Collectively, these findings show that the benefits of *ex-vivo* expansion of NKG2C^{pos} NK cells for cancer immunity go far beyond direct interaction of NKG2C with the HLA-E receptor and that this approach can be combined effectively with other immunotherapeutic tools such as KIR–ligand mismatch and monoclonal antibody treatments (see Fig. 2).

Conclusions

CMV infection is a potentially fatal complication in patients receiving HSCT for the treatment of haematological malignancies, but recent evidence indicates that it may also have inadvertent salubrious effects. Specifically, CMV reactivation is associated with a marked reduction in the risk of relapse in leukaemia patients after allogeneic HSCT [32,33]. This anti-leukaemia effect has been shown to correlate strongly with the expansion of NKG2C^{pos} NK cells [31], which are able to lyse leukaemic blasts that typically express constitutively high levels of HLA-E [41]. NK cells are a critical component of the anti-leukaemia effect of allogeneic HSCT, and infusion of allogeneic NK cells has shown promise as a means of inducing remission in leukaemia and myeloma patients [50,65] and preventing leukaemic relapse [88]. Efficacy of these treatments is limited, however, by the difficulty of acquiring adequate numbers of alloreactive NK cells [65] and high expression of NKG2A relative to NKG2C in expanded NK cells [64]. These limitations are particularly salient when dealing with haematological malignancies characterized by high expression of HLA-E, as HLA-E^{pos} tumour cells can only be lysed by NKG2C^{pos}/NKG2A^{neg} NK cells [39]. The proportion of NKG2C^{pos}/NKG2A^{neg} NK cells is typically low in healthy adults, but is elevated markedly in individuals infected with CMV [13,22]. We have shown in healthy donors that even latent CMV infection is associated with increased NK cell cytotoxic activity against a variety of haematological malignancies (leukaemia, MM and lymphoma), and that this effect is proportionate to the magnitude of target cell HLA-E expression and is NKG2C-dependent [8,37]. Furthermore, we demonstrated that this CMV effect could be mimicked in NK cells taken from CMV-seronegative individuals by expanding NKG2C^{pos}/NKG2A^{neg} NK cells preferentially *ex vivo* using HLA-E transfected feeder cells and IL-15 [8]. From all the above, it is clear that NKG2C^{pos}/NKG2A^{neg} NK cells represent an intriguing immunotherapeutic target for the treatment of a variety of cancers characterized by high HLA-E expression. Furthermore, NKG2C^{pos}/NKG2A^{neg} NK cells can be combined with other immunotherapy procedures, such as KIR–ligand mismatch and monoclonal antibody treatments to potentiate their effects. Future studies should explore the role of NKG2C^{pos} NK cells in the progression of other non-leukaemic cancers and determine if NKG2C^{pos}/NKG2A^{neg} NK cells expanded *ex vivo* or taken directly from CMV-infected individuals are able to kill primary tumour cells as effectively as cell lines [6,8].

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Disclosure

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