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Adaptive NK cell response to human cytomegalovirus: Facts and open issues

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ABSTRACT

Human cytomegalovirus (HCMV) infection exerts broad effects on the immune system. These include the differentiation and persistent expansion of a mature NK cell subset which displays a characteristic phenotypic and functional profile hallmarked by expression of the HLA-E-specific CD94/NKG2C activating receptor. Based on our experience and recent advances in the field, we overview the adaptive features of the NKG2C+ NK cell response, discussing observations and open questions on: (a) the mechanisms and influence of viral and host factors; (b) the existence of other NKG2C- NK cell subsets sharing adaptive features; (c) the development and role of adaptive NKG2C+ NK cells in the response to HCMV in hematopoietic and solid organ transplant patients; (d) their relation with other viral infections, mainly HIV-1; and (e) current perspectives for their use in adoptive immunotherapy of cancer.

1. Introduction

Human Natural Killer (NK) cells mediate cytotoxicity and pro-inflammatory cytokine production in response to infected and tumor cells. NK cell differentiation, proliferation and effector functions are regulated by cytokines, under the control of an array of inhibitory and activating/co-stimulatory receptors (NKR). Human peripheral blood NK cells comprise a variety of subsets which differ by their maturation stage and the combinatorial expression of some NKR. Moreover, upon activation NK cells may undergo clonal expansion and late differentiation events, further diversifying their phenotypic/functional profile [1–4].

Among NKR, Killer cell immunoglobulin-like receptors (KIR) and CD94/NKG2 lectin-like heterodimers specific for HLA class I (HLA-I) molecules have a fundamental role regulating the response to pathological cells and self-tolerance. KIRs are encoded by a family of polymorphic genes located on chromosome 19q13.4, and a number of haplotypes containing different gene sets have been identified [5]. Most inhibitory KIR (iKIR) specifically recognize structural motifs shared by groups of HLA class Ia (i.e., HLA-A, -B, -C) allotypes. Upon interaction with their HLA ligands iKIR recruit SHP tyrosine phosphatases through cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM), repressing NK cell activation. Moreover, under physiological conditions, iKIR specific for self HLA class I molecules promote functional NK cell maturation through a process termed “education” or “licensing”, still incompletely understood at the molecular level [6,7]. Activating KIRs (aKIR) are coupled to protein tyrosine kinase (PTK) pathways through an ITAM-bearing adapter (DAP12/KARAP). Some aKIR share the same HLA ligands with structurally homologous iKIR, whereas the specificity for others remains ill-defined [8].

A second set of NKR specific for HLA-I molecules are C-type lectin-like heterodimers constituted by the covalent association of CD94 with members of the NKG2 family, encoded on human chromosome 12p12-p13. CD94/NKG2A functions as an inhibitory receptor recruiting SHP-1 through NKG2A ITIMs whilst CD94/NKG2C is coupled to DAP12 and delivers activating signals [9]. Both NKR interact with the HLA-E non-classical (class Ib) molecule, which presents conserved leader

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sequence peptides from most other HLA-I molecules, including allotypes not recognized by iKIRs [10–12]. The structural basis of CD94/NKG2A interaction with an HLA-E/peptide complex was characterized [13]. CD94/NKG2A and iKIRs complement each other to prevent the response of mature NK cells against normal autologous cells, as originally predicted by the "missing self" hypothesis [7,14]. In a paradigm of convergent evolution, the biological role of human KIRs is fulfilled in mice by Ly49lectin-like receptors, whereas CD94/NKG2 NKR are conserved in both species [15,16].

Without known exception, CD94/NKG2C and aKIR bind to HLA-I ligands with lower affinities than their inhibitory homologs [17–19] and it has been hypothesized that they evolved for responding to microbial pathogens. This view was reinforced by the evidence that Ly49H interacts with the m157 murine CMV (MCMV) protein and triggers NK cell functions, contributing to defense against the viral infection [20–22]. The specific clonal expansion of long-lived Ly49H+ NK cells and their ability to mediate a secondary response to MCMV infection are reminiscent of lymphocyte adaptive responses, leading to consider them "memory" NK cells [22,23]. Conversely, m157 engagement of inhibitory Ly49I/C receptors constitutes an immune evasion mechanism for MCMV [24,25]. No functional Ly49 homologs exist in humans and similar direct recognition of pathogen-derived molecules has not been shown for KIRs nor CD94/NKG2, but peptides bound to HLA-I molecules may influence the affinity of some of these NKR with functional implications. This becomes particularly relevant for the interaction of CD94/NKG2 with HLA-E, extensively addressed in the next sections.

2. Adaptive NKG2C+ NK cell response to HCMV infection

Human cytomegalovirus (HCMV) causes a prevalent lifelong infection in all human populations. In healthy individuals, the course is generally mild/asymptomatic and the virus remains latent, undergoing occasional reactivation [26,27]. Yet, HCMV may cause severe disorders in congenital infection and in immunocompromised patients [28–30]. Among a number of viral evasion mechanisms which impair T and NK cell functions, several HCMV molecules (i.e. US2, 3, 6, 10, 11) inhibit HLA class I expression at different levels, interfering with specific T cell mediated recognition of viral antigens [31]. Other HCMV molecules hamper T and NK cell activation either reducing the expression in infected cells of ligands for activating/costimulatory receptors (e.g. NKG2D, CD2, DNAM-1) or engaging inhibitory receptors (e.g. ILT2, CD94/NKG2A). In particular, the UL40 HCMV protein contains a peptide which mimics HLA-I leader sequences binding to HLA-E [31,32]. Differently from HLA-E-bound endogenous peptides, UL40 nonamers can be presented by infected cells in a TAP-independent manner, thus being refractory to inhibition by US6 which blocks the peptide transporter. In that way, engagement of CD94/NKG2A by HLA-E/UL40 peptide complexes in infected cells interferes with the response of the NKG2A+ NK cell subset to HLA-I downregulation. Given the fact that NKG2A expression is inducible in other NK cell subsets by IL-12 [33], UL40 might exert a broader immune subversion in an inflammatory context.

HCMV infection has been related with different alterations in the immune system [34]. We originally reported that healthy HCMV-seropositive (HCMV+) individuals displayed to a variable degree persistent expansions of NK cells expressing high surface levels of CD94/NKG2C associated with changes in the distribution of other NKR [35]. Such reconfiguration of the NK cell compartment was later described in response to HCMV infection in a primary T cell immunodeficiency [36], in infants experiencing congenital or postnatal HCMV infection [37,38], as well as in hematopoietic stem cell (HSC) and renal transplant recipients [39–41] (see Section 5). NKG2C+ NK cell expansions observed in other viral infections (e.g., HIV-1, Hantavirus, Chikungunya, EBV) were in every case associated to HCMV co-infection [42–47], consistent with the specificity for this pathogen (see Section 7). By analogy with the response of Ly49H+ NK cells to MCMV, the terms "adaptive" or "memory-like" were originally adopted to designate NKG2C+ NK cells with a characteristic differentiated phenotype [48] (see Section 3).

Though murine CD94/NKG2A/C NKR interact with the non-classical MHC class I molecule Qa-1b bound to H-2 leader peptide nonamers [15,16], no relation with MCMV infection has been reported, hampering the development of animal experimental models. Progress in the characterization of the role played by CD94/NKG2 receptors in the response of non-human primates to CMV may circumvent this limitation [49].

3. Phenotype, function and differentiation of adaptive NKG2C+ NK cells

As compared to NKG2C+ NK cells detected in HCMV- individuals, the common adaptive phenotype associated to HCMV infection is characterized by the following features: (a) absence of NKG2A and increased surface levels of the receptor (NKG2C$^{bright/high}$); (b) co-expression of the CD57 differentiation marker and inhibitory NKR specific for HLA-I molecules (i.e. iKIR, ILT2); (c) reduced surface levels of activating NKp30 and NKp46 NCR, Siglec7 and CD161; (d) normal/increased expression of activating/costimulatory receptors (i.e. CD16, NKG2D, CD2); (e) epigenetic downregulation of signaling molecules (e.g., FcεRIγ chain, SYK) and transcription factors (e.g. PLZF1), and enhancement of IFN-γ production [35,50–55]. Of note, CD16 remains coupled to CD3ζ in FcεRIγ(-) adaptive NK cells, efficiently triggering antibody-dependent effector functions and proliferation in response to HCMV-infected and other target cells [54,56–58]. Though this profile is conventionally considered to define adaptive NKG2C+ NK cells, some degree of phenotypic heterogeneity is perceived. This becomes particularly evident following their development in HCMV-infected HSCT recipients (see Section 5) and in vitro generation, pointing out to a stepwise differentiation process and/or to in vivo selection events as discussed next.

A thorough analysis of NKG2C+ adaptive NK cells assessing gene expression and chromatin accessibility at the single cell level has been recently reported [59]. An epigenetic signature common to adaptive NKG2C+ NK cells was detected, whereas other features were differentially displayed by NKG2C+ subpopulations in the same individual. These latter observations in combination with analysis of mitochondrial DNA mutations, as barcodes for clonality tracing, further supported a clonal pattern of differentiation, expansion and persistence of adaptive NKG2C+ cells in response to HCMV. The different factors that determine the selection of NKG2C+ "naïve" clones to undergo this process is uncertain, but co-expression of iKIRs specific for self HLA-I are likely involved, as indirectly supported by their known oligoclonal distribution in adaptive NKG2C+ NK cells [51] and their "educational" function [6,7].

The mechanisms underlying adaptive NKG2C+ cells development remained unclear for some time. Early studies showed that co-culture with HCMV-infected fibroblasts elicited an increase of NKG2C+ NK cells in PBMC from HCMV+ donors [60,61] that was inhibited by an anti-CD94 mAb. These observations suggested that specific recognition by CD94/NKG2C of a viral ligand promoted their expansion in response to IL-2 or IL-15, and other studies pointed out an involvement of IL-12 and HLA-E [61]. Assessment in these assays of the response to infection by HCMV deletion mutants in a BACmid, which lacks genes downregulating HLA class I expression (i.e. US2, US3 and US6), failed to define a CD94/NKG2C viral ligand. Moreover, an involvement of the receptor in triggering NK cell effector functions against HCMV-infected cells was not formally demonstrated by blocking experiments with specific mAbs [48,60,62,63]. Experimental observations in a cell reporter assay, in which CD94/NKG2C expression was segregated from other NKR [64], suggested a low affinity/avidity for its putative viral ligand in infected cells.

The question as to whether CD94/NKG2C interaction with HLA-E/UL40 peptide complex in HCMV-infected cells promoted an adaptive

NK cell response was re-addressed by Romagnani et al. [65]. Their experimental approach was based on the generation of HCMV recombinants displaying three different UL40 sequences in a BACmid in which US2, US3, US6 genes had been restored, allowing HLA-I down-regulation in infected cells. The response of adaptive NKG2C+ NK cells against endothelial cells infected with the UL40 variants was assessed. NK cell effector functions were clearly elicited by HCMV displaying the UL40 VMAPRTVFL peptide. This nonamer, known to endow HLA-E with the highest affinity for CD94/NKG2 receptors [66,18,19], is naturally present in the HLA-G leader sequence and in UL40 from a small proportion (~1 %) of HCMV strains/clinical isolates [65]. By contrast, a modest response was detected to cells infected with the virus displaying the UL40 VMAPRTLIL sequence, corresponding to one of the canonical HLA-I leader peptides more frequent in HCMV (~40 %). Altogether these observations supported that engagement of CD94/NKG2C by HLA-E/UL40 peptide complexes expressed by infected cells may trigger NK cell effector functions and, on the other hand, were consistent with the previous difficulties to formally demonstrate in vitro an involvement of the CD94/NKG2C receptor in the response against cells infected by common wild-type HCMV strains [60,62,64]. Induction of NKG2C+ NK proliferation in response to infected cells was not reported. However, stimulation of peripheral blood mononuclear cells (PBMC) from HCMV-seronegative donors with the TAP-deficient murine RMA-S cell line transfected with HLA-E and CD48 (RMAS/HLA-E), loaded with UL40/HLA-leader peptides, in the presence of proinflammatory cytokines (i.e. IL-12, IL-18, IL-15) promoted the development of NKG2C+ NK cells with an adaptive profile.

Phenotypic differences with samples treated only with cytokines illustrated the importance of CD94/NKG2C engagement to selectively drive the instructive differentiation process. NK cells with low surface levels of NKG2C ($NKG2C^{dim/low}$), partially co-expressing NKG2A, are present in blood samples from HCMV- individuals, likely representing naïve progenitors of adaptive NK cells capable of differentiating in response to HCMV infection. Considering the dominant inhibitory function of CD94/NKG2A, HLA-E/UL40 peptide complexes presumably trigger the response of the NKG2C+ NKG2A- NK cell subset, as supported by epigenetic changes detected early (12 h) after stimulation with peptide-loaded RMA-S/HLA-E and IL-15 [59]. However, following their prolonged expansion in the presence of IL-15, IL-12 and IL-18, substantial proportions of NKG2C+ populations co-expressed NKG2A [65], at variance with the conventional phenotype of adaptive NK cells. This reveals differences with their physiological generation in response to HCMV infection, at least partially attributable to the effects of in vitro stimulation with IL-12 and IL-18 [33,59]. From a practical standpoint, CD94/NKG2A co-expression by in vitro generated adaptive NK cells is a relevant issue as it might dampen their response and potential for cancer immunotherapy (see Section 8).

In summary, according to in vitro studies, minimal requirements for adaptive NK cell development include: (a) a timely interaction of NKG2C+ precursors with HCMV-infected cells, in which HLA class Ia molecules have been downregulated and HLA-E/UL40 peptide complexes are displayed; (b) an inflammatory context providing suitable cytokines. Consistent with observations in HSCT (see Section 5), bone marrow is an optimal location to catalyze this process considering the presence of hematopoietic progenitors together with HCMV tropism for hematopoietic lineages (e.g. myelomonocytic cells) and stromal cells. Development of suitable experimental models to further dissect the cellular/molecular interactions involved in adaptive NKG2C+ differentiation in response to HCMV is warranted.

The notion that circulating $CD56^{bright}$NKG2A+KIR- NK cells generated from CD34+ precursors undergo further development to $CD56^{dim}$NKG2A-KIR+NK cells has been supported by observations in different settings, particularly during immune reconstitution following HSCT [67]. According to this linear model of peripheral NK cell development, it has been postulated that adaptive NKG2C+$CD56^{dim}$KIR+ NK cells derive from NKG2A+$CD56^{bright}$KIR- NK cells through intermediate stages [68], yet precise information on the expression of NKG2C along this process is limited. Given the complexity of hematopoietic differentiation [69,70], the existence of alternative differentiation pathways deserves further attention (see Section 7).

As development of adaptive NKG2C+ NK cells depends on the specific receptor interaction with the HLA-E/UL40 peptide complex, they should be distinguished for the sake of precision from cytokine-induced memory-like (CIML) NK cells. These are generated stimulating polyclonal NK cell populations with IL-12, IL-15 and IL-18, to be used for immunotherapy of hematopoietic malignancies [71]. On the other hand, the term "memory" is occasionally extended to describe NKG2C- NK cell populations sharing some phenotypic features with adaptive NKG2C+ cells. Yet, it is uncertain which stimuli promote their differentiation and, particularly, whether other specific activating NKR/ligand interactions fulfill the role of CD94/NKG2C (see Section 5). Studies combining transcriptional, epigenetic and mass cytometry analyses might contribute to clarify these issues.

4. Dynamics of the adaptive NKG2C+ NK cell response to HCMV: Influence of viral and host genetic factors

The magnitude of steady-state adaptive NKG2C+ NK cell expansions is quite variable in different HCMV+ healthy individuals and remains rather stable along time, yet current knowledge on their turnover (i.e., early differentiation, proliferation, survival and loss) is limited. Studies on GATA2-deficiency supported the long-term survival of NK cells displaying differentiation features shared with adaptive NKG2C+ NK cells [72] and their increased lifespan is attributable to expression of anti-apoptotic molecules (e.g., Bcl-2) [54].

Observations in immunocompromised individuals suggest that the magnitude of adaptive NKG2C+ NK cell expansions in healthy subjects may, in general, be inversely related to T cell-mediated viral replication control, depending on virus/host genetics and other circumstantial factors (e.g., age at primary infection) [48]. Secondarily, antibody-dependent activation triggered by HCMV and other pathogens may contribute to amplify the adaptive NKG2C+ NK response.

Genetic factors governing CD94/NKG2C interaction with the HLA-E/UL40 peptide complex have received special attention. Miyashita et al. identified in healthy blood donors a deletion of the *NKG2C* gene (formally designated as *KLRC2*). This was confirmed in other populations with some frequency variability related to their ethnic/geographic origin [73–76]. Thus, the receptor is dispensable for immune defense against HCMV infection but its contribution is not ruled out, as supported by observations in $NKG2C^{del/del}$ children [75] and in transplant recipients (see Section 6). Steady state numbers of adaptive NKG2C+ NK cells were reported to be lower in $NKG2C^{wt/del}$ than $NKG2C^{wt/wt}$ HCMV+ individuals [37,50,75]. Moreover, *NKG2C* copy number influenced CD94/NKG2C surface expression levels and the functional response to receptor engagement [50]. The increased CD94/NKG2C expression levels, characteristic of differentiated adaptive NK cells, may enhance the avidity for the relative low surface density of HLA-E/peptide complexes. Recently allelic polymorphisms of the *NKG2C* gene have been reported [77] and further studies are warranted to assess their putative relation with the magnitude of the adaptive NKG2C+ NK cell response. Differences between surface expression levels of HLA-E*01:01 and HLA-E*01:03 allotypes might also subtly modulate the avidity of recognition by CD94/NKG2 receptors [78].

On the viral side, differences in the UL40 peptide sequence deserve special attention. UL40 nonamers from the majority (~70%) of HCMV strains/clinical isolates replicate three canonical HLA class Ia leader peptides (i.e. VMAPRTLIL, -LLL, -LVL). In contrast, low individual frequencies of the UL40/HLA-G (VMAPRTLFL) peptide (~1 %) and other sequences with changes at different positions have been observed [19,65]. As originally shown for endogenous HLA-I leader peptides, UL40 nonamers have been reported to influence HLA-E binding and/or recognition by CD94/NKG2 receptors, affecting the response of both

NKG2A+ and NKG2C+ NK cells [19]. From an evolutionary standpoint, it is plausible that UL40 peptides which efficiently inhibit NKG2A+ NK cells but minimize the adaptive NKG2C+ response might provide an advantage to the virus. This view would be consistent with the predominance of UL40 peptides which accurately replicate the endogenous HLA sequences as well as with the low frequency of the HLA-G/UL40 VMAPRTLFL peptide, which confers to HLA-E the highest affinity for both CD94/NKG2 receptors promoting the strongest adaptive NK cell response [18,65,66]. In this regard, an analysis of UL40 in HCMV isolates from NKG2C$^{del/del}$ subjects might be informative, though challenging given the relatively low frequency of that genotype and limitations to detect virus replication in healthy individuals.

5. Development of adaptive NKG2C+ NK cells in HSCT

HCMV infection/reactivation in HSCT was shown to be a key driver of NK cell differentiation inducing a rapid and progressive expansion of adaptive NKG2C+ NK cells [40,41]. Notably, HCMV infection/reactivation, but not other common viral infections (e.g., EBV, ADV) exerted similar effects in both autologous [79] and allogenic HSCT, including umbilical cord blood transplantation (UCBT) [40] and HLA-haploidentical HSCT, either T-cell depleted [80] or T-cell replete (e.g. post-transplant cyclophosphamide HSCT) [81,82].

However, the frequency of HCMV-driven adaptive NKG2C^{+} NK cells developing in HSCT patients is variable and can be influenced by multiple factors, as described above in HCMV^{+} healthy individuals; in particular, reduced intensity conditioning favors adaptive NK cells expansion as compared to myeloablative regimens [83,84]. Furthermore, adaptive NK cells developing in HSCT have demonstrated long-term persistence without detectable HCMV replication [40,41,80,85]. Of note, when adaptive NKG2C^{+} NK cells contained in the graft were transferred to recipients, as in αβT/B-depleted haplo-HSCT, they could also persist for months in the absence of HCMV viremia [86]. Nevertheless, together with the prolonged lifespan of adaptive NKG2C+ NK cells, it cannot be excluded that their sustained levels might also result from proliferation and/or de novo generation in response to subclinical HCMV replication in immunocompromised HSCT recipients.

In patients receiving UCBT from donors homozygous for the *NKG2C* gene deletion, a rapid expansion of mature NKG2C- NK cells was observed in response to HCMV infection, pointing out a broader NKG2C-independent influence on the NK cell compartment [87]. A role for activating KIRs (aKIR) has been proposed in HCMV recognition and in promoting NK cell maturation [51,87,88]. In line with this concept, KIR haplotypes of HSC donors encoding aKIR have been associated with a reduced risk of HCMV infection [89,90]. Yet, the influence of HCMV in the NK cell compartment has been also observed in rare individuals lacking both NKG2C and aKIRs, consistent with the contribution of additional mechanisms to late NK cell differentiation, possibly involving cytokines as well as CD16 and CD2 signaling [52,91].

6. Adaptive NKG2C+ NK cells and HCMV infection control: observations in transplant recipients

Several studies suggested a role in HCMV control for NKG2C+ NK cells emerging after HSCT [92–94]. Interestingly, a recent study reported that donor *NKG2C* homozygosity (i.e. full gene dosage) can contribute to HCMV clearance after haplo-HSCT. In particular, an earlier, larger and functionally stronger expansion of adaptive NKG2C^{+} NK cells, which correlated to a more rapid HCMV infection resolution, was observed in patients receiving HSCT from *NKG2C*$^{wt/wt}$ as compared to *NKG2C*$^{wt/del}$ donors [95].

In immunosuppressed HCMV+ kidney transplant recipients (KTR), the risk of post-transplant viremia, its clinical impact and control ultimately depends on the pre-transplant fitness of the immune system. Actually, frequencies of pp65 and IE-1 HCMV antigen-specific T cells have been related with a reduced risk of infection [96]. Similarly, the incidence of post-transplant DNAemia was inversely associated with pre-transplant proportions of adaptive NKG2C+ NK cells (NKG2A-, CD57+, ILT2+, FcεRIγ-) [97], without apparent relation with NKG2C- NK cell subsets sharing these differentiation markers. The association was limited to symptomatic but not asymptomatic infection, suggesting that adaptive NKG2C+ NK cells may contribute to contain HCMV infection progression, rather than controlling early viral replication. Of note, the effect did not appear attributable to the proportions of IE-1 and pp65 HCMV antigen-specific T lymphocytes nor to the TcRγδ Vδ2- T cell subset, reported to increase in response to HCMV infection [98]. Whether the putative antiviral effect of adaptive CD94/NKG2C NK cells involves recognition of the HLA-E/UL40 peptide complex and/or antibody-dependent effector functions against HCMV-infected cells is uncertain.

NKG2C+ NK cell expansions were also described to develop in KTR following post-transplant HCMV reactivation/reinfection [39,99–100]. Yet, individual differences in their magnitude and kinetics were observed in DNAemia(+) KTR. As reported in healthy individuals, increased circulating adaptive NKG2C+ NK cells generally persisted > 24 months; of note, a marked decline was observed in some cases suggesting an altered turnover which deserves attention. As discussed (Sections 3–4), the variable magnitude of adaptive NKG2C+ NK cell responses in KTR may be determined by host/viral genetic factors governing the effectiveness of viral replication control (e.g. T cell-mediated response) and the CD94/NKG2 interaction with HLA-E/UL40 peptide complexes. No relation with *NKG2C* copy number was substantiated [99] and further studies to assess the role of the UL40 nonamer are warranted. Considering that adaptive NKG2C+ NK cells were associated with a reduced incidence of symptomatic infection, it is plausible that their post-transplant development might contribute to restore HCMV control in KTR. Nevertheless, concomitant reactive expansions of CD8 + and TcRγδ T cell subsets detected in DNAemia (+) KTR did not allow to discern the relative role of adaptive NK cells in that setting [99,100].

Frequencies of NKG2C+ NK cells in bronchoalveolar lavage inversely correlated with HCMV blood titers in lung allograft recipients [101]. A relation of the HLA-E/UL40 axis with HCMV viremia has been reported in lung transplant recipients but no information on adaptive NKG2C+ cells was provided [102]. Further studies are required to systematically explore in different clinical scenarios the influence of UL40 sequences on the adaptive NKG2C+ NK cell response.

7. Adaptive NKG2C+ NK cells in the response to other viral infections: the HIV paradigm

The involvement of NK cells in the response to HIV-1 is supported by observations linking specific KIR/HLA combinations with HIV-1 outcome [103,104], and by the selection of HIV-1 sequence polymorphisms that enhance binding of inhibitory KIRs on NK cells to infected CD4+ T cells, thus reducing the activity of NK cells [105]. A decreased NK cell activity has been reported at all stages of HIV-1 infection and is particularly relevant in individuals with opportunistic infections or with subsequent progression to Kaposi's sarcoma or to AIDS [106,107]. Several mechanisms have been shown to contribute to decreased NK cell function during HIV infection [108–110].

NKG2C+ NK cell expansions reported in HIV-1 infected patients [42,43,111] and other viral infections (i.e. hantavirus, chikungunya, chronic HBV and HCV hepatitis, EBV) [44–47] were systematically linked to HCMV seropositivity/co-infection, consistent with their specificity for this pathogen. For HIV-1 the association may be attributed to a decreased control of HCMV replication and, more generally, to proliferation of adaptive NKG2C+ NK cells triggered by antibody-dependent activation in response to the different pathogens.

A bone marrow resident CD34+DNAM-1^{bright}CXCR4+ population was identified in peripheral blood of HIV-infected patients as well as in other chronic infections and inflammatory disorders [112]. Following a

two-week culture of purified CD34+DNAM-1^{bright}CXCR4- cells with IL-15, IL-7 SCF and FLT3-L mixed T/NK cell progenies were detected. These NK cell populations displayed a mature phenotypic/functional profile different from that of cells generated from umbilical cord blood and bone marrow CD34+ progenitors, thus pointing to an unconventional NK cell differentiation pathway. Cultures of CD34+DNAM-1^{bright}CXCR4+ cells in the presence of irradiated PBMC and.221-AEH cells, obtained by transfecting the 721.221 HLA-I-negative cell line with HLA-E together with the HLA-A2 leader sequence to allow its surface expression [11], promoted the generation of NKG2C+ NK cells with an adaptive phenotype [113]. These observations are reminiscent of the response of NK cells from HCMV- subjects stimulated with the RMA-S/HLA-E+ cell line loaded with UL40 peptides [65], suggesting that NKG2C+ cells present among the CD34+DNAM-1^{bright}CXCR4+ progeny may further differentiate/expand upon interaction with .221-AEH cells.

CD56- CD16+ NK cell populations are rare in healthy individuals but may represent up to 20–40% of NK cells in HIV+ patients [114]. In HSCT recipients a fraction of CD56- CD16+ cells are observed early after HCMV reactivation followed by a rapid maturation to adaptive NKG2C+ NK cells [40]. Stimulation with.221-AEH cells of a CD34- CD16+ CD56- Perf- CD7- CD94- CXCR4+ subset promoted the growth of NKG2C+ NK cells with adaptive features [113]. Further studies are required to precisely characterize these putative unconventional differentiation pathways, with special attention to NKG2C expression, key for the instructive step of adaptive NK cell development in response to HCMV.

Higher frequencies of circulating adaptive NKG2C+CD57+ NK cells in early treated HIV-1 infected patients have been associated with a more rapid control of viremia upon initiation of antiretroviral treatment [115] and a more favorable balance of immunological and virological parameters [116]. On the other hand, the impact of *NKG2C* deletion on HIV infection has remained unclear due to difficulties in replicating data in different settings. A recent analysis on a large group of patients and controls failed to find and association with HIV-1 susceptibility or clinical course [117]. In contrast, *NKG2C* deletion [118] and the proportions of circulating adaptive NKG2C+ NK cells [119] have been proposed to influence the course of SARS-CoV-2 infection.

8. Adaptive NKG2C+ NK cells in the response to tumors: Implications for immunotherapy

Although adaptive NK cells are generated in response to a viral infection, they have been associated with a protective effect against leukemia recurrence in HSCT. Indeed, a significant reduction in the leukemia relapse rates was observed in transplanted patients expanding adaptive NKG2C+ NK cells [82–84]. A direct role for CD94/NKG2C-HLA-E interactions in killing leukemic cells is plausible only for AML but not for ALL that usually express low levels of HLA-E [120,121]. On the other hand, the anti-leukemic properties of adaptive NK cells could also rely on HCMV-induced expansion of alloreactive KIR+ NKG2A- NK cells in KIR-mismatched donor/recipient pairs in haplo-HSCT [86]. Furthermore , based on the effectiveness of adaptive NK cells to mediate ADCC [54,56–58] major benefits could be achieved in HSCT patients displaying significant numbers of this subset by the use of immune engagers potentiating the response against leukemic cells [122].

NK cell activity and intra-tumoral NK-cell numbers have been associated to a reduced risk of cancer incidence and improved prognosis in different solid tumors [123–126]. NK cell cytotoxicity as well as secretion of pro-inflammatory cytokines and immune-cell recruiting chemokines promote the development of an efficient anti-tumor immunity [127,128]. In addition, several reports support the contribution of NK cell-mediated ADCC to the therapeutic efficacy of tumor antigen-specific antibodies and checkpoint blockers [128–131]. Tumor-infiltrating NK cells are mainly localized in the invasive margin and stromal-rich areas in solid tumors [129,132]. They show reduced surface expression of activating receptors (i.e., NKG2D, NKp30, 2B4, DNAM-1, CD16), as well as of terminally differentiation/cytotoxic markers, including granzyme B, perforin, KIR, ILT2 and CD57, concomitant to high proportions of CD94/NKG2A+ cells. The reduced levels of activating receptors and effector molecules together with the acquisition of tissue-residency molecules (CD9, CD103 and CD49a) is suggestive of an important imprint of TGFβ on tumor-infiltrating NK cells [133,134]. Though not formally addressed, these observations indirectly argue against the spontaneous presence of adaptive NKG2C+ NK cells in tumor immune infiltrates [126,132,134–136]. In the same line, single cell and bulk transcriptomic characterization of tumor infiltrating NK cells showed specialized gene expression programs (i.e. high levels of XCL1 and XCL2 chemokines) imprinted by the tumor microenvironment [137] and different from those described for adaptive NKG2C+ NK cells.

Few studies have directly assessed the putative relationship between adaptive NKG2C+ NK cell expansions in cancer patients, their presence in tumor immune infiltrates and clinical outcomes. The expression of NKG2C has been reported to be generally low in NK cells from non-small cell lung cancer (NSCLC), gastrointestinal stromal tumor (GIST) and breast cancer lesions [132,134,138]. In our experience, the proportions and absolute numbers of circulating NKG2C+ NK cells were comparable in patients regardless of the complete response to anti-HER2 antibodies [139]. Moreover, CD57+ NK cells were remarkably scarce in tumor infiltrates as compared to peripheral blood, consistent with paucity of adaptive NKG2C+ NK cells in solid tumors [139].

Nevertheless, the functional profile of adaptive NKG2C+ NK cells renders them of potential interest for cancer immunotherapy. In that context, features of special relevance include: (a) proficiency to mediate effector functions (cytotoxicity and cytokine production) upon antibody-dependent activation [54,56–58]; (b) oligoclonal iKIR expression without NKG2A; (c) prolonged lifespan. Moreover, adaptive NKG2C+ NK cells appear to be resistant to contact-dependent myeloid derived suppressor cells (MDSC) and Treg suppression in vitro, potentially conferring them advantage in the tumor microenvironment [140,141].

The use of adoptive NK cell infusions has been mostly explored as adjuvant therapy in the context of allo-HSCT or following immunosuppressive chemotherapy in hematological malignancies, where KIR-based NK alloreactivity has been shown to influence on the risk of relapse [142]. Prior experience showed the good safety profile of allogenic NK cells infusions, exempt of graft-vs-host disease (GvHD) [143–145] and severe adverse reactions (e.g., cytokine release syndrome or neurotoxicity) when engineered for chimeric antigen receptor (CAR) expression [146].

Most NK cell products for adoptive cell transfer explored in the clinic are generated through protocols involving their prior expansion/activation in vitro (e.g., CIML and haploidentical NK cell donor selection). Polyclonal NK cell expansions contain variable percentages of alloreactive NK cells and NKG2A co-expression, factors that could influence their anti-tumor efficacy. Recently, a GMP-compliant protocol has been described for enriching and expanding, from selected healthy blood donors, adaptive NKG2C+ NK cells with homogenous expression of a single self-HLA specific KIR, which retain an adaptive transcriptional signature [147]. This first step towards an off-the-shelf adaptive NKG2C+ NK cell-based therapy should allow selecting alloreactive adaptive NK cell products with enhanced anti-leukemic and ADCC activity. The adaptive NKG2C+ NK cell product has been recently tested in three completed phase I clinical trials including intravenous infusion in refractory/relapsed AML patients (NCT03081780); intraperitoneal delivery in women with recurrent ovarian, fallopian tube or primary peritoneal cancer (NCT03213964) and its combination with monoclonal antibodies (trastuzumab or cetuximab) for the treatment of advanced solid tumors (HER2+ breast and gastric cancers or EGFR+ colorectal and head and neck tumors) (NCT03319459).

Finally, circulating adaptive NKG2C+ NK cells in cancer patients

have been described to display enhanced expression of PD-1 in some cancer contexts [148] and chronic stimulation through NKG2C was reported to induce an enhanced expression of LAG3, TIGIT and PD-1 associated to impaired IFNγ production by adaptive NK cells [149]. On that basis, the possible combination of adaptive NKG2C+ NK cell products with immune checkpoint blockers can be also envisaged as a strategy to promote their anti-tumoral activity.

9. Concluding remarks

HCMV infection specifically promotes the adaptive differentiation, expansion and survival of an oligoclonal mature NK cell subset with characteristic phenotypic/functional profile and epigenetic signature, hallmarked by high expression of the activating CD94/NKG2C NKR. Ex vivo experimental observations support that a specific driver of this process is the CD94/NKG2C interaction with HLA-E-bound peptides from the UL40 viral protein, that mimic and replace endogenous nonamers in HCMV-infected cells, where HLA class Ia expression has been downregulated. Despite progress in understanding at the molecular level this process, some issues deserve further attention including: (a) To explore whether additional host/viral factors, other than NKG2C, UL40 and HLA-E, contribute to the marked variability of the adaptive NK cell response in healthy HCMV+ individuals. (b) To characterize the epigenetic signature induced in NKG2C- NK cells, combining engagement of other activating receptors (e.g. aKIR, NCR, CD16) with proinflammatory cytokines. (c) To define the hematopoietic developmental pathways generating NKG2C+ NKG2A- progenitors responding to HCMV infected cells and, particularly, the variables involved in their clonal selection. (d) To develop experimental models in non-human primates reproducing the CD94/NKG2C and MHC-E interactions in the response to CMV.

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