Adoptively transferred natural killer cells maintain long-term antitumor activity by epigenetic imprinting and CD4⁺ T cell help

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**ABSTRACT**

Natural killer (NK) cell infusions can induce remissions in subsets of patients with different types of cancer. The optimal strategies for NK cell activation prior to infusion are still under debate. There is recent evidence that NK cells can acquire long-term functional competence by preactivation with the cytokines IL-12/15/18. The mechanisms supporting the maintenance of long-term NK cell antitumor activity are incompletely understood. Here, we show that NK cells preactivated in vitro with IL-12/15/18, but not with IL-15 alone, maintained high antitumor activity even 1 mo after transfer into lymphopenic RAG-2⁻/⁻γc⁻/⁻ mice. The NK cell intrinsic ability to produce IFNγ coincided with demethylation of the conserved non-coding sequence (CNS) 1 in the Ifng locus, previously shown to enhance transcription of ifng in a xenograft melanoma mouse model. Human IL-12/15/18-preactivated NK cells rejected tumors more efficiently. In RAG-2⁻/⁻γc⁻/⁻ mice, co-transfer of CD4⁺ T cells further improved the long-term competence of NK cells for IFNγ production that was dependent on IL-2. CD4⁺ T cell activation during homeostatic proliferation required macrophages and further promoted the long-term NK cell antitumor activity. Thus, NK cells can "remember" a previous exposure to cytokines by epigenetic imprinting resulting in a remarkable stability of the IFNγ-producing phenotype after adoptive transfer. In addition, our results support combination of cytokine-preactivated NK cells with CD4⁺ T cell activation upon lymphopenic conditioning to achieve long-term NK cell effector function for cancer immunotherapy.

**Introduction**

Adoptive Immunotherapy based on natural killer (NK) cells has shown clinical benefits in patients suffering from different kinds of leukemia. For instance, a study with 154 acute myeloid leukemia (AML) patients demonstrated that 33% of the patients remained tumor-free and survived for at least 5 y after killer cell immunoglobulin-like receptors (KIR)-mismatched T cell-depleted haploidentical transplantation. Moreover, a complete hematologic remission was observed in 5 of 19 AML patients upon infusion of IL-2-treated KIR-mismatched NK cells after chemotherapy, which is promising for elderly high-risk patients who are not candidates for stem-cell transplantation. NK cell transfers also showed clinical responses in subsets of patients suffering from other types of hematological neoplasias such as chronic myeloid leukemia (CML) and lymphoma. However, adoptive transfer of autologous IL-2 expanded NK cells in patients suffering from solid tumors such as melanoma or renal cell carcinoma did not result in clinical benefits. Since the functional activity of NK cells recovered from cancer patients is frequently impaired, novel protocols to ensure long-term antitumor activity of NK cells are instrumental to improve current therapies.

In this context, Cooper et al. showed that mouse NK cells preactivated in vitro for 13–15 h with the cytokine combination IL-12, IL-15, and IL-18 produced increased levels of IFNγ upon restimulation compared to NK cells precultured with IL-15 alone after adoptive transfer into RAG-1⁻/⁻ mice. Moreover, our previous study revealed that adoptive transfer of IL-12/15/18-pretreated NK cells into tumor-bearing, irradiated mice resulted in high numbers of NK cells with potent effector function in adoptive hosts and greatly reduced tumor growth, whereas IL-2 or IL-15-pretreated NK cells were inefficient. Intriguingly, the brief exposure of NK cells to the cytokines IL-12/15/18 resulted in the ability for IFNγ production observed up to 3 mo after transfer that was maintained after homeostatic proliferation. Human IL-12/15/18-pretreated NK cells showed similar properties when cultured in vitro with IL-2 or IL-15 and in NOD-SCID IL-2γ⁻/⁻ (NSG) mice after adoptive transfer. Thus, upon activation with cytokines, long-term functional NK cell antitumor activity can be achieved upon lymphopenic conditioning.

**Abbreviations:** CNS, conserved non-coding sequences; NK, natural killer; PBMC, peripheral blood mononuclear cells

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term competence for NK effector function, such as IFNγ production, was generated resembling characteristics of memory cells.13 The epigenetic configuration of the Ifng locus determines accessibility for transcription of Ifng by transcription factors.14 Naive T cells display a closed configuration with high CpG methylation of the Ifng locus. In Th1 cells that produce high levels of IFNγ, an open configuration with CpG demethylation of the promoter and the conserved non-coding sequences (CNS) 1 region in the Ifng locus has been shown to be crucial to enhance transcription of Ifng.15-18 Naive NK cells already show an open configuration of the Ifng promoter.19,20 Recently, Romagnani et al.20 have shown that human naive NK cells, unlike Th1 cells, display a close configuration of the CNS1 at the IFNG locus, despite their prompt ability to produce IFNγ. Moreover, CpG demethylation of the CNS1 that facilitated IFNγ production was demonstrated to be a selective hallmark of human NKG2C+ “memory-like” NK cells expanded in Human Cytomegalovirus (HCMV) seropositive individuals.20 So far, molecular mechanisms underlying the long-term stability of a polarized NK phenotype in vivo have not been addressed.

It is well established that CD4+ T cell help is instrumental for primary and memory CD8+ T cell responses.21 In addition, evidence emerged that NK cell-mediated immune responses also benefit from CD4+ T cell help.9,22-28 In certain infectious disease and tumor models, the cross-talk between CD4+ T cells and NK cells was shown to improve NK cell responses mostly involving the cytokine IL-2.9,22-28 Regulatory T cells were reported to restrain IL-2 dependent CD4+ T cell help for NK cell proliferation and activity.29,31 Our previous study demonstrated that adoptive transfer of IL-12/15/18-pretreated NK cells into irradiated tumor-bearing mice resulted in antitumor activity that required the presence of host CD4+ T cells and IL-2.9 However, the mechanisms and prerequisites of the CD4+ T cell and NK cell cross-talk are still incompletely understood.

IFNγ is a critical cytokine involved in cancer immunosurveillance.32,33 Accordingly, we observed that adoptive transfer of IL-12/15/18-pretreated IFNγ-deficient NK cells failed to control tumor growth.9 In the present study, we aimed at unraveling the mechanisms driving the long-term ability of high IFNγ production and antitumor activity by IL-12/15/18-pretreated NK cells in an adoptive transfer setting.

Results

Preactivation of mouse NK cells with IL-12/15/18 results in subsequent epigenetic imprinting of the CNS1 in the Ifng locus

To investigate cell intrinsic characteristics of cytokine-pretreated NK cells, we transferred IL-12/15/18- or IL-15-pretreated syngeneic NK cells into lymphopenic RAG-2−/−/γc−/− mice and determined IFNγ production before, 11, and 28 d after transfer (Fig. 1A). After the preactivation before adoptive transfer, IFNγ was produced by more than 90% NK cells activated with IL-12/15/18 but not with IL-15 (Fig. 1B) or by naive NK cells (Fig. S1A). IL-12/15/18-pretreated NK cells killed different tumor targets more efficiently than IL-15-pretreated NK cells (Fig. S1B). Four days after transfer, IL-12/15/18-pretreated NK cells rapidly proliferated and produced high amounts of IFNγ upon restimulation with RMA-S lymphoma cells (Fig. 1C), whereas IL-15-pretreated NK cells proliferated much less and displayed lower IFNγ production. Of note, no IFNγ production by adoptively transferred preactivated NK cells was observed without restimulation (data not shown). Eleven days after transfer IL-12/15/18-pretreated NK cells still produced much higher levels of IFNγ (~20% IFNγ-producing cells) (Fig. 1D) and Granzyme B (data not shown) in response to RMA-S cells, compared with IL-15-pretreated NK cells with less than 5% IFNγ-producing cells (Fig. 1D) or naive NK cells (Fig. S1A). On day 28 after transfer, IL-12/15/18-pretreated NK cells still showed a significantly increased ability of IFNγ production in response to different tumor cell lines such as RMA-S, RMA-RAE1γ, and YAC-1, whereas IL-15-pretreated NK cells almost completely lacked IFNγ production (Fig. 1D and E).

Neither Ifng mRNA nor expression of T-bet protein, a transcription factor controlling IFNγ production,34 differed in NK cells re-isolated 11 d after adoptive transfer regardless whether these cells were preactivated with IL-15 or with IL-12/15/18 (data not shown). Next, we determined the methylation status within defined regions of the Ifng locus. The Ifng promoter was largely demethylated in naive splenic NK cells to a similar extent as in vitro generated Th1 cells (Fig. S2A). Next, we analyzed methylation status of CNS1 in the Ifng locus, which is an important enhancer of Ifng gene transcription in Th1 cells.15,16 We depicted four selected CpG residues that are conserved in human and mouse.18 Naive mouse NK cells displayed methylated CNS1, similar to naive T cells and in vitro generated Th2 cells (Fig. 1F). The high levels of methylation remained unchanged after overnight (16 h) culture with either IL-15 or IL-12/15/18 (Fig. 1F). Strikingly, 11 d after transfer, the CNS1 locus of IL-12/15/18-pretreated NK cells was greatly demethylated similar to Th1 cells (Fig. 1F). In contrast, no demethylation was observed with IL-15-pretreated NK cells after adoptive transfer. Of note, in vitro activation of mouse NK cells with IL-12/15/18 for 5 d also resulted in substantial demethylation of the CNS1 region (Fig. S2B) as previously reported for human NK cells.20 Thus, our data suggest that the brief in vitro exposure of NK cells to IL-12/15/18 initiated the program of demethylation in the Ifng locus occurring after transfer. Together, the cell intrinsic ability of IL-12/15/18-pretreated NK cells to produce high levels of IFNγ after transfer coincides with a demethylated CNS1 region of the Ifng gene.

Preactivation of human NK cells with IL-12/15/18 generates potent IFNγ producing and cytolytic NK cells with antitumor activity in a xenograft melanoma mouse model

To translate our findings to human cells, we transferred human IL-15- and IL-12/15/18-pretreated NK cells into NSG mice (Fig. 2A). Six days after transfer, IL-12/15/18-pretreated human NK cells produced elevated levels of IFNγ compared to IL-15-pretreated NK cells upon restimulation with the leukemia cell line K562 as well as IL-12/15 (Fig. 2A), consistent with a previous report.15 Human IL-12/15/18-pretreated NK cells showed...
Figure 1. Preactivation of mouse NK cells with IL-12/15/18 results in epigenetic imprinting of the CNS1 in the Ifng locus. (A) NK cells were purified from spleens and pre-activated with IL-12/15/18 or IL-15 alone overnight for 16 h. RAG-2−/−γc−/− mice (n = 3) were injected i.v. with 109 preactivated NK cells. Splenocytes were analyzed for ex vivo function of the transferred NK cells at different time points. (B) IFNγ production from preactivated NK cells was determined by intracellular staining. Anti-IFNγ, solid line; isotype control, filled histogram. (C) Preactivated NK cells were labeled with CFSE and transferred into RAG-2−/−γc−/− mice. Four days later, splenocytes were restimulated with RMA-S cells and IFNγ production from transferred NK cells was analyzed. Numbers indicate % cells in each quadrant. One representative dot-plot (n = 3) from each group is shown (gated on CD3−/NK1.1+). Data are representative of two independent experiments. (D, E) Eleven or 28 d after NK cell transfer, splenocytes from RAG-2−/−γc−/− mice were restimulated with RMA-S (D), RMA-RAE1γ or YAC-1 (E) cells. IFNγ production from transferred NK cells was analyzed. One representative dot-plot (n = 3) from each group is shown in D (gated on CD3−/NK1.1+, left panels). Numbers indicate % IFNγ-producing NK cells. The mean % IFNγ-producing NK cells from individual mice are shown in graphs (mean ± SD). *p < 0.05; **p < 0.01 using Student’s t-test. Data are representative of three independent experiments. (F) Preactivated NK cells before and after transfer were FACs-sorted (with >99% purity) and CpG methylation in the Ifng CNS1 region was analyzed by bisulfate pyrosequencing. Four CpGs from −5789 to −5690 bp in the CNS1 region (relative to Ifng gene) were analyzed. The mean % of methylation from individual mice (n = 3) at the four CpG sites in naive and preactivated NK cells before and after transfer, as well as in naive CD4, Th1, and Th2 cells is depicted in a heatmap (upper panel) and a graph (mean ± SEM) (lower panel).

higher cytotoxicity against different tumor cell lines (Fig. S3A). Similar to mouse NK cells, IL-12/15/18-pretreated human NK cells proliferated more rapidly and persisted at higher cell numbers in different organs compared to IL-12/15/18-sensitized human NK cells (Fig. S3B and C). To further investigate if those NK cells also possess antitumor activity after adoptive transfer, we injected melanoma cells SK-Mel-28-luc and IL-12/15/18-sensitized human NK cells into NSG mice (Fig. 2B). IL-12/15/18-pretreated human NK cells controlled tumor growth in the lungs more efficiently than IL-15-pretreated NK cells in vivo as assessed by bioluminescence (Fig. 2B). These data reveal a potent antitumor activity of human IL-12/15/18-pretreated NK cells after adoptive transfer in a melanoma xenograft model.

**Co-transfer of CD4+ T cells further enhances IFNγ production by IL-12/15/18-pretreated NK cells dependent on IL-2**

CD4+ T cells were shown to be instrumental for sustained effector function of IL-12/15/18-pretreated NK cells in irradiated tumor-bearing mice.9 In order to investigate whether CD4+ T cells affected IFNγ production in a tumor-free experimental system in the absence of irradiation, we transferred mouse NK cells with or without splenic CD4+ T cells into RAG-2−/−γc−/− mice (Fig. 3A). When co-transferred with CD4+ T cells, both IL-15- and IL-12/15/18-pretreated NK cells produced 2–3 times more IFNγ upon restimulation 11 d after transfer (Fig. 3B, upper panels). In contrast, naive NK cells produced only very low levels (<5%) of IFNγ after transfer even in the presence of CD4+ T cells (Fig. S4A). Importantly, on day 11 co-transfer of CD4+ T cells induced so called IFNγhigh producers among NK cells that express highly elevated levels of IFNγ upon restimulation with RMA-S cells (Fig. 3B, upper panels, red dots and red bars) or RMA-Rae1γ and YAC-1 cells (data not shown). Most remarkably, when CD4+ T cells were co-transferred the IFNγhigh producers within the IL-12/15/18-pretreated NK cells were still observed after 1 mo (Fig. 3B, lower panels, red dots and red bars). At this time point, IL-15-pretreated NK cells had lost the competence to produce IFNγ in response to RMA-S cells even in the presence of CD4+ T cells (Fig. 3B, lower panels). Co-transfer of CD8+ T cells did not improve NK cell function (Fig. S4B).
Of note, the increased competence for IFNγ production by NK cells upon co-transfer of CD4+ T cells did not coincide with further demethylation of the CNS1 region of the Ifng gene (Fig. S5). Moreover, similar levels of the T-bet protein and Ifng mRNA were observed in NK cells (data not shown). Our data demonstrate that co-transfer of CD4+ T cells into lymphopenic mice further enhances the ability of IFNγ production by IL-12/15/18-pretreated NK cells upon restimulation even four weeks after transfer.

Next, we characterized CD4+ T cells before and after transfer in RAG-2−/−γc−/− mice. Freshly isolated splenic CD4+ T cells displayed a CD44low, CD69neg, and CD122low phenotype. After adoptive transfer, these cells acquired an activated phenotype characterized by high expression of CD44, CD69, and CD122, which coincided with their rapid proliferation (Fig. S6A and B). Of note, increased levels of activation markers such as CD69 on transferred CD4+ T cells correlated with enhanced IFNγ production by NK cells upon restimulation (Fig. S6C). It has been shown that IL-2 is crucial for CD4+ T cell-mediated help for NK cell activation.9,22-27

To address the role of IL-2 in the NK/CD4+ T cell cross-talk in our system, we neutralized IL-2 in vivo. Upon IL-2 neutralization, the enhanced ability of IFNγ production by IL-12/15/18-pretreated NK cells, observed upon co-transfer of CD4+ T cells, was abrogated (Fig. 3C). In particular, the IFNγhigh producers among total IFNγ producers were lacking when IL-2 was neutralized (Fig. 3C, red dots and red bars). Intriguingly, depletion of regulatory T cells (Tregs) prior to adoptive transfer of CD4+ T cells did not increase IFNγ production by IL-12/15/18-pretreated NK cells compared to bulk CD4+ T cells (Figs. 3D and S7). Collectively, our data indicate that CD4+ T cells that become activated during homeostatic proliferation in vivo further enhance the competence of IFNγ production by IL-12/15/18-pretreated NK cells in an IL-2 dependent manner.

Macrophages are instrumental for CD4+ T cell activation and sustained NK cell function

To determine the cell population critical for CD4+ T cell activation after transfer in lymphopenic mice, we addressed the
role of macrophages, the most prominent cell population in RAG-2<sup>−/−</sup>γ<sup>−/−</sup> mice. We detected a rapidly and a slowly proliferating CD4<sup>+</sup>C<sub>T</sub> cell population (Fig. 4A) as previously described.35,36 The CD4<sup>+</sup>C<sub>T</sub> cells that rapidly proliferated expressed increased levels of CD44 (Fig. 4A), which has been correlated with the capacity to produce IL-2.37 Depletion of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages by Clodronate liposomes (CLIP) significantly reduced the percentage (Fig. 4A) and the numbers (data not shown) of activated CD4<sup>+</sup>C<sub>T</sub> cells (CD44<sup>high</sup>CFSE<sup>low</sup>). Furthermore, in absence of macrophages, we observed reduced frequencies of CD69- and CD122-expressing CD4<sup>+</sup>C<sub>T</sub> cells, consistent with a less activated phenotype (Fig. 4B). When we transferred IL-12/15/18-pretreated NK cells alone, macrophage depletion did not significantly affect NK cell IFNγ production (Fig. 4C), suggesting the requirement of macrophage/CD4<sup>+</sup>C<sub>T</sub> cell crosstalk for supporting NK cell effector function. However, when we co-transferred CD4<sup>+</sup>C<sub>T</sub> cells and preactivated NK cells, the increased ability of IFNγ production by NK cells was abrogated when macrophages were depleted (Fig. 4C). Of note, low frequencies of the CD69-expressing CD4<sup>+</sup>C<sub>T</sub> cells after macrophage depletion correlated with decreased IFNγ production by NK cells (open circles, Fig. 4D). All together, these results show that the presence of macrophages supported activation of CD4<sup>+</sup>C<sub>T</sub> cells during homeostatic proliferation and thereby affected NK cell function.

**IFNγ production by human IL-12/15/18-pretreated NK cells is enhanced by co-transfer of PBMC containing human CD4<sup>+</sup> and CD14<sup>+</sup> cells in NSG mice**

Next, we investigated whether co-transfer of human CD4<sup>+</sup>T cells could enhance the function of human NK cells (Fig. 5A). However, co-transfer of anti-CD3/CD28 activated autologous human CD4<sup>+</sup>T cells did not increase the recovery of human NK cells after transfer (data not shown). Thus, we provided additional cell populations by co-transfer of autologous peripheral blood mononuclear cells (PBMC). Co-transfer of PBMC supported in vivo proliferation of IL-12/15/18-pretreated NK cells, but not of IL-15-pretreated NK cells, to a similar extent as in vivo injection of IL-2 (Fig. 5B). Depletion of either CD4<sup>+</sup> or CD14<sup>+</sup> cells from PBMC resulted in a reduction of in vivo NK cell proliferation (Fig. 5C). Accordingly, the lack of either
CD4+ or CD14+ cells led to lower frequencies of the transferred IL-12/15/18-pretreated NK cells (Fig. 5D). Most importantly, on day 6 after adoptive transfer the presence of CD4+ T cells and CD14+ monocytes/macrophages further increased the IFNγ production by transferred IL-12/15/18-pretreated NK cells in response to K562 ex vivo (Fig. 5E). In conclusion, our data show that CD4+ T cells and monocytes/macrophages support the ability of IFNγ production by human IL-12/15/18-pretreated NK cells after adoptive transfer.

**Discussion**

Preserving the antitumor activity of NK cells after adoptive transfer over long time remains one of the major challenges for NK cell-based immunotherapy of cancer. We and others previously showed that the in vitro preactivation of NK cells with IL-12/15/18 leads to a long-term functional competence such as the production of IFNγ after adoptive transfer in tumor-bearing mice.9,10 Our present study reveals two mechanisms underlying and supporting long-term in vivo persistence of the IFNγ-producing NK cell phenotype: (i) epigenetic remodeling of the Ifng locus; (ii) help by CD4+ T cells. Thus, we unravel both cell intrinsic and extrinsic mechanisms promoting long-term effector potential of adoptively transferred NK cells.

We observed that the ability of high IFNγ production by IL-12/15/18-pretreated NK cells was preserved during proliferation, coinciding with demethylation of the CNS1 region of the Ifng gene. In contrast, the CNS1 region of IL-15-pretreated NK cells remained highly methylated after adoptive transfer as in naive NK cells. The brief exposure for 16 h to IL-12/15/18 in vitro did not immediately lead to demethylation of the CNS1 region, but enabled subsequent in vivo demethylation of CNS1 after adoptive transfer. In our study, prolonged in vitro exposure of mouse NK cells to IL-12/15/18 for 5 d also resulted in
demethylation in this region that was also previously observed with human NK cells. Moreover, after culture of NK cells in vitro in IL-2/12/18 for 5 d, significant demethylation of CNS1 was observed, indicating that IL-2 could replace IL-15 in our experimental setup (our unpublished observations). Human NKG2Chigh NK cells that are expanded in HCMV seropositive individuals showed CNS1 demethylation that correlated with enhanced IFNγ production. Thus, it is likely that, also in our study, demethylation of CNS1 region facilitates IFNγ transcription in transferred IL-12/15/18-preactivated NK cells. During MCMV infection, the cytokines IL-12 and IL-18 were shown to be critically involved in expansion of Ly49H+ NK cells in response to the viral antigen m157 and regulation of effector function such as IFNγ production. In Th1 cells that produce high levels of IFNγ, an open configuration with CpG demethylation of the promoter and the CNS 1 region in the Ifng locus has been shown to be crucial to enhance transcription of Ifng. IL-12 is required to increase demethylation of CNS1 during development of IFNγ-producing memory CD4+ T cells. Although the underlying mechanism is not completely understood, it was shown that IL-12 activates STAT4 that could further limit recruitment of DNA methyltransferase leading to DNA demethylation. In addition, NK cells pre-activated with IL-12/15/18 showed increased STAT5 phosphorylation in response to low doses of IL-2. STAT5 was shown to bind to CNS1 and promote optimal IFNγ production. Altogether these data indicate that the binding of transcription factors (STAT4 and STAT5) activated downstream of the IL-12/15/18 receptors to regulatory regions of the Ifng locus does not only acutely induce Ifng transcriptional activity in NK cells but also might contribute to the subsequent epigenetic remodeling of the locus. CpG demethylation of Ifng locus is inheritable in memory T cells and their progeny. Our data indicate that the demethylation of CNS1 region might also be inherited by the NK cell progeny during homeostatic proliferation leading to the long-term competence of IFNγ production. IL-12 and IL-18 were indispensable for the CNS1 demethylation during in vitro culture of human NK cells. IL-15 is also important because NK cells did not survive without IL-15 in the 5 d in vitro IL-12/18 activation (our unpublished data).

In the study by Romagnani et al., not only cytokine genes but also many other activation pathways were epigenetically regulated in HCMV-expanded NK cells. Two recent reports further dissected signaling and functional characteristics of the epigenetically modified HCMV-associated NK cell subset. Accordingly, it is likely that also additional epigenetic changes occur in NK cells in our experimental system subsequent to IL-12/15/18 activation. Importantly, human IL-12/15/18-preactivated NK cells efficiently controlled tumor growth in the melanoma xenograft model in NSG mice. This antitumor effect could be a result of the potent cytotoxicity of IL-12/15/18 NK cells against tumor cells, the persistent high cell numbers and their capability to produce high levels of IFNγ.

Figure 5. The ability of IFNγ production by IL-12/15/18-pretreated human NK cells is enhanced in the presence of CD4+ T cells and CD14+ cells after transfer into NSG mice. (A) Human NK cells were pretreated with IL-15 or IL-12/15/18 for 16 h, labeled with CFSE and transferred together with autologous PBMC into NSG mice. In parallel, one group of mice transferred with IL-12/15/18-preactivated NK cells were injected with IL-2. (B) Six days later, in vivo proliferation of the transferred NK cells (gated on CD45+CD3-CD56-) from blood was analyzed. (C) CFSE-labeled preactivated NK cells were co-transferred with autologous PBMC, CD4-depleted PBMC (PBMC dCD4), or CD14-depleted PBMC (PBMC dCD14) into NSG mice (six recipient mice from each donor, 2 mice/group). Six days later, in vivo proliferation of the transferred NK cells (gated on CD45+CD3-CD56-) from blood was analyzed. (D) Numbers of the transferred NK cells in blood were depicted in the graph (mean ± SEM). (E) Blood cells were restimulated with K562 and IFNγ production from transferred NK cells was analyzed by intracellular staining. The mean % IFNγ-producing NK cells are shown in graphs (mean ± SEM). *p < 0.05; **p < 0.01 using Student’s t-test. Data are representative of three donors.
Pre-existing antigen-specific memory CD4⁺ T cells were shown to improve IFNγ production by human NK cells upon in vitro stimulation. Infection and tumor models in mice also showed that antigen-specific CD4⁺ T cells were required for the early IFNγ induction from NK cells. Our previous study revealed that CD4⁺ T cells were indispensable for maintaining potent effector function of adoptively transferred preactivated NK cells in total-body irradiated tumor-bearing mice. To address whether CD4⁺ T cell help to NK cells can occur in absence of specific tumor antigens, tumor-derived factors and irradiation, we co-transferred CD4⁺ T cells together with preactivated NK cells in lymphopenic RAG-2⁻/⁻γc⁻/⁻ mice. Indeed, when CD4⁺ T cells were co-transferred, NK cells showed significantly increased ability of IFNγ production. Importantly, in the presence of CD4⁺ T cells, IFNγ⁺ producers among transferred NK cells were induced. Even 1 mo after adoptive transfer, IL-12/15/18-pretreated NK cells, but not IL-15-pretreated NK cells, still were highly competent of IFNγ production when co-transferred with CD4⁺ T cells. Similar to a previous report, the CD4⁺ T cells homeostatically proliferated, got activated, and acquired a memory phenotype (CD44high) after transfer into RAG-2⁻/⁻γc⁻/⁻ mice in our system. Thus, CD4⁺ T cell activation during homeostatic proliferation independent of a specific tumor antigen or irradiation further improved the long-term ability of NK cells for IFNγ production.

We observed that neutralization of IL-2 in vivo abolished CD4⁺ T cell-help for improving IFNγ production by transferred NK cells. In particular, the IFNγ⁺ producers among the transferred NK cells were not detected when IL-2 was neutralized. Co-transfer of CD4⁺ T cells increased IFNγ production without altering expression levels of T-bet or preformed Ifng mRNA (data not shown) or further changing the methylation status of the CNS1 region in the Ifng locus, although we cannot exclude that methylation status in other regions or histone modifications in the Ifng locus were induced. In this context, it was shown that IL-2 induces histone acetylation of a distal IFNG site in primary human NK cells facilitating IFNγ transcription. It is possible that also in our system IL-2 might induce further histone modifications in vivo in the preactivated NK cells after transfer. We also tested whether co-stimulatory molecules affected the CD4⁺ T cell/NK cell cross-talk. Using CD28⁻/⁻, CD40L⁻/⁻ and OX40⁻/⁻ CD4⁺ T cells, we excluded a contribution of these co-stimulatory molecules in the CD4⁺ T cell-mediated help to NK cell functional competence in our experimental system (data not shown).

Tregs can suppress activity of effector T cells, NK cells, and other immune cells. The ratio of Treg/effector T cells often increases in tumor patients correlating with immunosuppression. However, in our experimental system, co-transfer of Treg-depleted CD4⁺ T cells did not result in higher amounts of IFNγ in NK cells, compared to co-transfer with bulk CD4⁺ T cells, indicating that IL-12/15/18-pretreated NK cells might be insensitive to the presence of CD4⁺ Foxp3⁺ T cells. It is likely that IL-12/15/18-pretreated NK cells compete with Treg for IL-2 consumption due to their pronounced upregulation of CD25. A previous study from Yu et al. showed that NK cells activated by IL-12/15/18 antagonize TGF-β signaling by down-regulating the TGF-β receptor, suggesting that these cells resist to suppression by Tregs involving TGF-β. Whether the IL-12/15/18-pretreated NK cells can be suppressed by antigen-specific activated Tregs in a tumor environment is currently unknown. In clinical studies, the presence of Tregs negatively correlated with NK cell function in leukemia patients. Thus, potential resistance of IL-12/15/18-pretreated NK cells to Treg-mediated suppression might have major clinical implications.

We observed that the presence of macrophages was instrumental for the long-term competence for IFNγ production by mouse and human NK cells. Accordingly, CD14⁺ cells were shown previously to support human T cells for memory conversion upon transfer. Homeostatic proliferation of CD4⁺ T cells was shown to require their interaction with MHC class II-expressing cells. It is tempting to speculate that MHC class II expressed by macrophages drives CD4⁺ T cell activation in lymphopenic mice. The fact that CD40L⁻/⁻ CD4⁺ T cells improved NK cell function to the similar level as wild-type CD4⁺ T cells in our system (data not shown) indicates a redundant role of CD40/CD40L interaction in macrophage-mediated CD4⁺ T cell activation. Previously, by using diphtheria toxin (DT)-induced DC-deficient CD11c⁺DOG mice, we observed that the presence of DC was not required for the rapid proliferation of transferred IL-12/15/18 preactivated NK cells suggesting that CD4⁺ T cell help for NK cells occurred independently of priming by DCs. Moreover, we did not detect any change of effector function of transferred IL-12/15/18 preactivated NK cells in the DC-depleted mice (our unpublished data). Although intravenous injection of clodronate liposomes every 2 d could deplete precursors of dendritic cells from blood and thus might affect dendritic cells numbers, we did not detect a reduction of CD11c⁺I-A/I-E⁺ population in spleen (data not shown) after intra-peritoneal injection of CLIP every third day. Depletion of CD14⁺ cells (monocytes/macrophages) from human PBMC impaired the function of the transferred NK cells, indicating the importance of this population in our co-transfer system. To our knowledge only a minor population of blood DCs expresses CD14 and to our mind it would be unlikely that these cells would account for the observed effects.

In our study, IL-12/15/18-preactivated NK cells underwent cell proliferation and epigenetic imprinting at the Ifng locus after adoptive transfer into lymphopenic mice. According to the publications from Cooper et al. and our own, it is sufficient to stimulate NK cells by IL-12/15/18 for 16 h to induce the “memory-like” phenotype (persistent function after adoptive transfer). Importantly, a clinical study at the Washington University School of Medicine uses a similar protocol to generate the NK cells for adoptive transfer into AML patients (NCT01898793). In addition, homeostatically proliferating CD4⁺ T cells without the need of antigen-specificity further improved NK cell function. Accordingly, we assume that in cancer patients, lymphopenic conditioning by chemotherapy or radiotherapy might drive CD4⁺ T cell activation and thus the maintenance of effector function by adoptively transferred NK cells. In our study, human IL-12/15/18-preactivated NK cells showed potent antitumor activity in a xenograft tumor model, indicating their potential in the pre-clinical settings. Additional activation of CD4⁺ T cells, for example, by activating antibodies or by vaccination might further improve long-term NK cell functional competence upon transfer in cancer patients.
Together, our results support the design of innovative combined therapies incorporating IL-12/15/18-preactivated NK cells and CD4+ T cells for the treatment of cancer patients.

**Materials and methods**

**Mice**

C57BL/6N 8-week old mice were purchased from Charles River. RAG-2-/-γc-/-, NSG, and Foxp3-DTR mice of C57BL/6 background were bred at the DKFZ animal facility. Mice were housed under specific pathogen-free conditions and in accordance with all standards of animal care. All animal experiments were approved by the “Regierungspräsidium Karlsruhe.”

**Antibodies and flow cytometry**

Anti-mouse CD3ε (145-2C11), NK1.1 (PK136), CD4+ (H129.19), CD44 (IM7), CD69 (H1.2F3), CD122 (TM-b1), IFNγ (XMGL1.2), T-bet (4B10), Foxp3 (FJK-16s), and anti-human CD45 (HI30), CD3 (HIT3a), CD56 (HCD56), CD4 (XMG1.2), T-bet (4B10), Foxp3 (FJK-16s), and anti-human RAG-2 (145-2C11), NK1.1 (PK136), CD4+ (H129.19), CD44 (IM7), CD69 (H1.2F3), CD122 (TM-b1), IFNγ (XMGL1.2), T-bet (4B10), Foxp3 (FJK-16s), and anti-human CD45 (HI30), CD3 (HIT3a), CD56 (HCD56), CD4+ (OKT4), CD45 (M5E2), IFNγ (4S.B3) were obtained from BD Biosciences, BioLegend, and ebioscience. Flow cytometric analyses were performed with a FACS Canto II (BD Biosciences) and data were analyzed using FlowJo software (TreeStar). For staining of surface molecules, dead cells were excluded by gating on 7-AAD (BD Biosciences) negative cells.

**Cell isolation from mouse spleen and in vitro activation**

NK cells were isolated by negative selection from spleens of C57BL/6N mice with the NK cell isolation kit II (Miltenyi Biotech) and treated with 10 ng/mL IL-12 (Peprotech), 10 ng/mL IL-15 (Peprotech), and 50 ng/mL IL-18 (MBL) or 10 ng/mL IL-15 alone for 16–18 h in NK cell culture medium: RPMI-1640 (Sigma) supplemented with 10% FCS, 1% L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol, 1% MEM non-essential amino acids, and 1 mM sodium pyruvate (all from Life Technologies or Sigma). The cells were stained and analyzed by flow cytometry or washed four times with PBS before adoptive transfer. The purity of the NK cells (CD3ε−NK1.1+) before adoptive transfer was >90%. CD4+ (CD4 MicroBeads, Miltenyi) and CD8+ T cells (CD8a MicroBeads, Miltenyi) were isolated from spleens (purity >95%). For long-term adoptive transfer (28 d), preactivated NK cells and freshly isolated CD4+ and CD8+ T cells were FACs-sorted with the purity >90%. For Treg depletion, Foxp3-DTR mice were i.p. injected with DT (Sigma, 15ng/g BW) twice with 24 h interval. One day later, CD4+ T cells were isolated with CD4 MicroBeads from the spleens.

**Adoptive cell transfer and in vivo depletion in RAG-2-/-γc-/- mice**

RAG-2-/-γc-/- mice (8–14 weeks old) were i.v. injected with 10⁶ NK cells preactivated for 16–18 h with IL-12/15/18 or IL-15 alone or together with 10⁶ freshly splenic CD4+ or CD8+ T cells. After 11 or 28 d, splenocytes were restimulated and analyzed. Anti-IL-2 (S4B6 and JES6-1A12, 500 μg, 1:1, BioXcell) antibodies were i.p. injected on the day of adoptive cell-transfer and every second day afterwards. To deplete macrophages, CLIP or the control PBS liposomes (PLIP) (ClodronateLiposomes.com) (400 μg) were i.p. injected 2 d before adoptive cell transfer and every 3 d afterwards.

**Proliferation assay**

*In vitro* preactivated NK cells or freshly isolated CD4+ T cells were labeled with 1.5 μM CFSE (Sigma) at room temperature for 15 min. After three washes with PBS, cells were transferred into RAG-2-/-γc-/- mice. Four days later, single cell suspensions from spleens were prepared, restimulated with RMA-S cells as described above, stained, and analyzed by flow cytometry.

**Ex vivo function of mouse NK cells after transfer**

The NK cell sensitive target lymphoma cell lines RMA-S, YAC-1, and RMA transduced with the NKG2D-ligand RAE1γ (RMA-RAE1γ) were cultured as previously described in RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies or Sigma) for one week before use. Splenocytes were isolated and restimulated with tumor cells at a 2:1 ratio for 22 h in NK cell culture medium as described above. GolgiStop (BD Biosciences) was added 4 h before the end of co-culture. Cells were stained for surface markers, fixed, and permeabilized (ebioscience) followed by intracellular staining of IFNγ.

**CpG methylation analysis**

Genomic DNA of FACSorted CD3ε−NK1.1+ and CD3ε+CD4+ cell populations (with >99% purity) was extracted using the QIAamp DNA Mini Kit (Qiagen). The regions of interest of the Ifng gene were selected according to previous reports. Bisulfite conversion and pyrosequencing were performed by Varionostic GmbH as previously described. Detailed information of primers is provided in Tables S1 and S2.

**Transfer of human NK cells into NSG mice, ex vivo function and bioluminescence**

Buffy coats, collected according to the principles of the Declaration of Helsinki, were provided by Deutsches Rotes Kreuz DRK-Blutspendedienst Baden-Württemberg-Hessen gGmbH (Mannheim, Germany). Written informed consent was obtained from all human subjects prior to blood donation and ethical approval 87/04 was granted by the Ethik Kommission II of the Medical Faculty Mannheim (Mannheim, Germany). PBMC were isolated by Ficoll separation (LSM 1077 Lymphocyte Separation Medium; PAA). NK cells were isolated by negative selection (Human NK cell Isolation Kit; Miltenyi Biotec) with a purity of CD3−CD56− NK cells >95%. The other <5% cells are mainly CD3−CD56+ cells. NK cells were preactivated in SCGM medium (CellGenix) containing 20% human serum (PAA), 100 μg/mL penicillin, 100 μg/mL streptomycin (Life Technologies) with 10 ng/mL IL-12 (Peprotech), 20 ng/mL IL-15 (R&D), and 100 ng/mL IL-18 (MBL) for 16 h. To assess in vivo proliferation and function, preactivated human NK cells
were labeled with 2 μM CFSE (Sigma) and 1–3 × 10^6 preactivated NK cells were i.v. injected alone or together with 10^7 autologous PBMC into NSG mice which were irradiated with 3.5 Gy at the day of injection. Mice were i.p. injected with 20,000 IU human recombinant IL-2 every day when NK cells were transferred alone. Human CD4 and CD14 MicroBeads (Miltenyi Biotec) were used to deplete CD4^+ and CD14^+ cells from PBMC before adoptive transfer. Six days later, in vivo proliferation and ex vivo function of the transferred NK cells (gated on CD45^+CD3^+CD56^+) were analyzed by flow cytometry. For ex vivo function, blood cells were restimulated with K562 cells in a 2:1 ratio or with the cytokines IL-12 (10 ng/mL) and IL-15 (50 ng/mL) for 6 h with the GolgiStop (BD Biosciences) added after 1 h of stimulation. Cells were stained for surface markers, fixed, and permeabilized (eBioscience) followed by intracellular staining of IFNγ. The number of CD45^+CD3^+CD56^+ NK was calculated accordingly. The supernatant from the co-culture was collected for IFNγ ELISA (BioLegend). The amount of IFNγ in the supernatant was adjusted per 50,000 NK cells.

For bioluminescence, NSG mice were injected i.v. with 7 × 10^5 melanoma cell line SK-Mel-28 transduced with firefly luciferase (SK-Mel-28-luc cells) and with preactivated NK cells. 20,000 IU IL-2 was injected i.p. every day. Mice were imaged at the indicated time points using the IVIS® imaging system-100 (Xenogen). D-luciferin was injected i.v. at a dose of 150 mg/kg 10 min prior to the acquisition of the bioluminescent signal intensity (BLI). The pseudocolor luminescent images were analyzed by the IVIS Living Image Software (Xenogen, version 2.50.1). Luminescence is defined as relative light units (RLU) [photons/s/cm^2/sr].

Statistics

The statistical significance of results from experimental groups in comparison to control groups was determined by the Student’s t-test.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

A.C. and J.N. designed the study; J.N., O.H. and M.M. did the experiments; Q.H. M.L. and C.R. provided the CpG methylation data; J.N. and O.H. collected and analyzed data; J.N. and A.C. wrote the manuscript.

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