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NK Cell Regulation of CD4 T Cell-Mediated Graft-versus-Host Disease

Magali Noval Rivas,* Marc Hazzan,* Kathleen Weatherly,* Florence Gaudray,* Isabelle Salmon,* and Michel Y. Braun*

CD3-negative NK cells are granular lymphocytes capable of producing inflammatory cytokines and killing malignant, infected, or stressed cells. We have recently observed a new role for NK cells in the control of the proliferation of CD4 T cells under persistent antigenic stimulation. Monoclonal anti-male CD4 T cells transferred into Rag2−/− male recipients did not expand or were rapidly eliminated. Remarkably, T cells transferred into NK cell-deficient Rag2−/− Il-2Ryc−/− male hosts expanded extensively and mediated tissue lesions usually observed in chronic graft-versus-host disease (GVHD). T cell failure to proliferate and to induce chronic GVHD was the result of NK cell activity, because depletion of the recipient’s NK1.1+ cells by Ab treatment induced T cell expansion and chronic GVHD. T cells under chronic Ag stimulation upregulated ligands of the activating receptor NKG2D, and regulatory activity of NK cells was inhibited by the injection of Abs directed to NKG2D. On the contrary, blocking NKG2A inhibitory receptors did not increase NK cell regulatory activity. Finally, we show that NK regulation of T cell expansion did not involve perforin-mediated lytic activity of NK cells, but depended on T cell surface expression of a functional Fas molecule. These results highlight the potential role played by NK cells in controlling the Ag-specific CD4+ T cells responsible for chronic GVHD. The Journal of Immunology, 2010, 184: 6790–6798.

Natural killer cells belong to the innate immune system. They are radio-resistant, large, granular lymphocytes with potent perforin- and IFN-γ-mediated effector functions, and they participate in the early defense against pathogens (1, 2). NK cell activity is regulated by a series of stimulatory or inhibitory membrane receptors that can bind different classes of cell surface ligands expressed by normal or infected cells (1, 3, 4). NK cell-activating receptors include cytokine receptors, integrins, and receptors that detect the presence of infectious nonself ligands (e. g., via Ly49H in the mouse) or stress-induced self ligands (e. g., via NKG2D). The best characterized mouse NK cell inhibitory receptors are those that recognize self histocompatibility molecules expressed at the surface of target cells (CD94-NKG2A, inhibitory Ly49 receptors, NKR-P1B, NKR-PD, KLRG1). The balance between the signals provided by activating and inhibitory receptors will determine whether NK cells are activated.

After chemotherapy or hematopoietic stem cell transplantation, NK cells are the first lymphoid cells to recover (5, 6). Surprisingly, such postgrafting regeneration of NK cells does not cause clinical graft-versus-host disease (GVHD); this has led to the conclusion that normal nonhematopoietic tissues lack ligands able to activate NK cell lysis. This conclusion is supported by the so-called hybrid resistance phenomenon observed in the F1 hybrid murine transplantation model, in which immunocompetent F1 offspring of MHC-disparate parents is tolerant to organ grafts from either parent, but reject parental bone marrow cells (7). These observations led to the idea that, by targeting hematopoietic cells, NK cell activity could play an important role in the regulation of immune responses. The concept of an NK-mediated regulatory function is also supported by the observation that a higher number of bone marrow NK cells has been associated with a decreased incidence of chronic GVHD after HLA identical sibling bone marrow transplants in human (8). Recent findings indicate that this regulatory function can be indirect, through the interplay and molecular crosstalk with dendritic cells (DCs) (9, 10). On the one hand, DCs can prime, further the activation of, augment the expansion of, and enhance the activities of NK cells through the production of cytokines such as IL-2, IL-12, IL-15, IFN-α/β, and TNFα. On the other hand, mostly through the production of IFN-γ, NK cells can enhance the differentiation, survival, and function of DCs, especially their expression of costimulatory molecules and production of IL-12 and TNFα, skewing the T cell-stimulatory capacity of DCs toward Th1-type responses. NK cell-mediated immature DC elimination has also been observed in the mouse (11). By killing immature DCs, NK cells might thus regulate DC homeostasis and thereby the capacity to stimulate T cells.

The regulatory function of NK cells on adaptive immune responses appears also to be mediated through direct lysis of activated T cells (12, 13). This pathway has been postulated to play an important role in the generation of memory T cell repertoire. Several recent observations suggest that certain subpopulations of NK cells promote allograft tolerance via a cytolyis-dependent regulatory pathway (14–16). Ab-mediated depletion of certain subpopulations of NK cells or impaired NK cell cytotoxicity indeed prevented the development of transplantation tolerance or accelerated the rejection of cardiac allografts. When faced with mismatched allogeneic targets, NK cells sense the missing expression of self-HLA class I alleles and mediate alloreactions. Studies
in mice show that, in these conditions, direct killing of allogeneic DCs by bone marrow-derived NK cells could represent the main mechanism by which NK cells prevent the development of T cell-mediated alloreactivity (17, 18). Cytokine secretion might also play a role in the regulatory function of NK cells. Deniz et al. (19) identified a small population of human NK cells capable of suppressing Ag-specific T cell proliferation and secretion of IL-13 and IFN-γ, particularly through the secretion of IL-10.

Donor T cells are considered to be the main cause of GVHD in patients receiving bone marrow transplantation. The control of their activity represents a key target for hematologists and immunologists in their search for therapies against GVHD. After hematopoietic stem cell transplantation, donor NK cells may represent up to 80% of peripheral blood leukocytes for the first month after treatment (5, 6). However, little is known about the effects of NK cells on donor T cells after bone marrow transplantation. In this study, we show that NK cells can regulate chronic GVHD by limiting recipient minor histocompatibility Ag (mHA)-driven proliferation of donor CD4+ T cells. These results support the idea that NK cells have potent regulatory capacities on donor CD4+ T cells and show that NK-mediated resistance to GVHD after bone marrow transplantation can be independent of MHC allo-disparities between donor and recipient.

Materials and Methods

Animals

Female RAG2-/- Marilyn mice, transgenic for a TCR (TCRAV1.1, TCRBV6) specific for the male H-Y peptide NAGFSNNSRANSRSS presented by I-Aα, have been previously described (20). RAG2-/- mice, Thy1.1 congenic C57BL/6 (B6) mice and Fas[b/b] B6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). B, T, and NK cell-deficient lymphopenic mice were H-2k or H-2b, RAG2-/- male mice lacking NK cells. Male-specific TCR-BV6-transgenic T cells 28 d after T cell adoptive transfer was assessed by flow cytometry. TCRBV6-transgenic T cells or TCR-transgenic male-specific TCRBV6 CD4+ T cells were injected i.p. (1 × 10⁶ cells per recipient) into male and female Rag2-/- and Rag2-/-/γc-/- recipients.

Flow cytometry

The following anti-mouse mAbs were purchased from BD Biosciences (Erembodegem-Aalst, Belgium): PB-conjugated anti-CD4, FITC- or PE-conjugated anti-TCRBV6, PE- or FITC-conjugated anti-CD49b/Pan NK cells, PB-conjugated anti-CD3, PerCP-conjugated anti-CD90.1, biotin-conjugated anti-Qa-1, PE-conjugated anti-2B4 and PE-conjugated anti-CD48. Biotin-conjugated anti-mouse NGK2A, PE-conjugated anti-Mult1 and APC-conjugated anti-NKG2D were purchased from eBioscience (San Diego, CA). APC-conjugated anti-H60 and APC-conjugated anti-Rae-1 were purchased from R&D Systems Europe (Oxon, U.K.). Streptavidin-PE was from BD Biosciences. Immunostained cell samples were analyzed on a CyAn ADP LX 9 Color with Summit v4.3 software (Dako, Glostrup, Denmark).

RT-PCR

The presence of mRNA coding for cell surface ligands of receptors known for their activating or inhibitory function on NK cells was assessed by real-time RT-PCR in CD4 T cells. Naive (n = 5) and chronically stimulated (n = 5; day 14) anti-male TCR-transgenic CD4+ T cells were purified by positive selection (DynaM鼠CD4 Isolation Kit; Invitrogen Dynal, Oslo, Norway). Cell purity was >97%. Total RNA was isolated using Qiagen RNaseasy Mini Kit (Qiagen Benelux, Venlo, Netherlands). cDNA was synthesized by using a cDNA Quantitect kit (Qiagen) and mixed with LightCycler FastStart DNA Master Plus® SYBR Green I (Roche Diagnostics Belgium, Vilvoorde, Belgium). Reactions were performed on a LightCycler 2.0 (Roche Diagnostics Belgium). The sequences of primers were: CD1d-Fw 5’-ATCTTGGCAGAGGGCTTCTAG-3’, CD1d-Rv 5’-CTGGGGCCATGGGAGAT-3’, CD1d-5F 5’-TTGCCGTGCCTGTGGGGGAT-3’, CD1d-14R 5’-GCGACGACGGGATCTGGAGGATT-3’, CD1d-48R 5’-GAGCTTCTTTTGGGAAAAGG-3’, CRACC-Fw 5’-AGTTTCTCTAAACCCCCGTCTT-3’, CRACC-Rv 5’-TACCATCTTGGGAGATGTGC-3’, H60-5F 5’-TGCTGATTCTGAGCCTTTT-3’, H60-Rv 5’-TCGCTGATTCTGAGCCTTTT-3’.
The expression of target genes was normalized by using 18S mRNA as an endogenous control to correct for differences in the amount of total RNA added to each reaction. We used a relative quantification method (ΔΔCt method) to calculate the gene expression values. Genes for which no amplified product could be detected were given an arbitrary Ct of 40 cycles, the maximum number of cycles performed by the thermocycler.

**Histology**

Histologic analysis was performed on tissue sections obtained from different organs and stained with H&E after paraffin embedding. Chronic GVHD scores were assigned by a trained pathologist who was unaware of the treatment group, using ×40 magnification of formalin-fixed histologic tissue sections.

**Statistical analysis**

For cell counts, an unpaired *t* test was used to determine the degree of significance.

**Results**

Male-specific CD4+ T cells undergo extensive Ag-driven proliferation in lymphopenic male recipients lacking NK cells

The principal aim of the study was to develop an in vivo model in which the capacity of NK cells to regulate donor CD4+ T cells could be demonstrated. We adoptively transferred Rag2−/− anti-male TcR-transgenic CD4+ T cells into lymphopenic male recipients lacking T and B cells (Rag2−/−/−/−) or lacking T, B, and NK cells (Rag2−/−γc−/−). The percentage and absolute number of male-specific CD4+ T cells increased extensively in the spleen of

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Male-specific CD4+ T cells transferred into NK-deficient lymphopenic male hosts infiltrate several organs and mediate chronic GVHD. **A,** At day 28 after adoptive transfer, the presence of male-specific TCRBV6-transgenic T cells was assessed by flow cytometry in the spleen, inguinal lymph nodes, lung, liver, and kidney of adoptively transferred recipients. **B,** Twenty-eight days after adoptive transfer of TcR-transgenic TCRBV6+ CD4 T cells or polyclonal Thy1.1+ CD4 T cells, lymphopenic male recipients lacking NK cells showed important periportal infiltration in the liver. H&E staining. Original magnification ×200. **C,** Relative number of hepatic vessels with periportal infiltration 28 d after transfer of TcR-transgenic TCRBV6+ CD4 T cells or polyclonal Thy1.1+ CD4 T cells in NK-deficient recipients that received control isotype matched Ab, anti-NK1.1 Ab, or anti-NKG2D Ab. *p < 0.01.
Rag2−/− γc−/− male recipients as early as 14 d after adoptive transfer (Fig. 1). Ag-dependent proliferation was not seen, however, at day 14 and was less pronounced after 21 and 28 d in NK cell-sufficient Rag-2−/− male recipients, suggesting that the presence of NK cells in these recipients prevented Ag-specific T cells to proliferate (Fig. 1). T cell expansion in NK cell-deficient animals was Ag-specific, because a weak homeostatic proliferation was observed (Fig. 1) when male-specific CD4+ T cells were transferred into Rag2−/− γc−/− female recipients, or in male recipients that did not express the correct MHC molecules (H-2b males) for Ag presentation. Flow cytometry analysis revealed that adoptively transferred male-specific CD4+ T cells were present in different organs of Rag2−/− γc−/− male recipients (Fig. 2A). Histologic analysis demonstrated that the proliferation of Ag-specific CD4+ T cells was associated with mild dermal leukocyte infiltration of the skin (data not shown) and extensive cellular periportal infiltration in the liver (Fig 2). Thus, proliferation of recipient mHA-specific CD4+ T cells in NK cell-deficient recipients led to the development of pathologic conditions similar to those observed in patients suffering from chronic GVHD. These lesions were much less pronounced or absent in NK cell-sufficient recipients, suggesting that the presence of NK cells inhibited the proliferation of mHA-specific CD4+ T cells and prevented the development of chronic GVHD.

To validate the hypothesis that NK cells had developed the ability to control recipient mHA-driven proliferation of donor CD4+ T cells, Rag2−/− male recipients were treated with NK-depleting Ab. Treatment with α-NK1.1 mAb depleted NK cells from Rag2−/− male recipients and both the percentage and the number of CD3− CD49b+ NK cells present after Ab treatment were similar to those found in Rag2−/− γc−/− male recipients (Supplemental Fig. 1A). Male-specific CD4+ T cells adoptively transferred into α-NK1.1 mAb-treated Rag2−/− male recipients underwent extensive proliferation (Fig. 1) and infiltrated the recipient’s organs (Fig. 2A). Histologically, signs of chronic GVHD similar to those observed in Rag2−/− γc−/− recipients were noticed in NK-depleted RAG−/− recipients (Fig. 2B) and important inflammation of periportal vessels was evident in the liver (Fig. 2C). On the contrary, injection of control isotype mAbs did not stimulate T cell proliferation or liver inflammation. Thus, depletion of NK cells by Ab treatment had the same effect on Ag-specific T cell expansion and development of chronic GVHD as genetic depletion of NK cells. To demonstrate that the control of donor CD4+ T cell proliferation by recipient’s NK cells was not due to the use of a transgenic monoclonal population of CD4+ T cells, we performed experiments with nontransgenic polyclonal CD4+ T cell populations. Normal CD4+ T cells from female Th1.1-congenic B6 mice were transferred into Thy1.2+ Rag2−/− male recipients or not with α-NK1.1 mAb. The absence of NK cells allowed the Ag-driven proliferation of adoptively transferred Thy 1.1 CD4+ T cells (Fig. 3). Moreover, recipient mice treated with α-NK1.1 mAb developed histologic lesions of chronic GVHD (Fig. 2B, 2C). These results supported the idea that NK cells have the potential to control mHA-driven expansion of CD4 T cells. Interestingly, when polyclonal B6 Thy 1.1 CD4+ T cells were adoptively transferred into female NK cell-depleted Rag2-deficient recipients, T cell expansion occurred to similar levels as those observed in male recipients. Thus, NK cells could control homeostatic, as well as Ag-driven, proliferation of polyclonal T cells.

Subpopulations of NK cells present in Rag2−/− male recipients

It could be argued that NK cells present in Rag2-deficient mice are different from NK cells present in normal wild type animals;
Mult-1 protein is known to be expressed at the cell surface (Fig. 5). However, Mult-1 protein expression was not detected by flow cytometry at the cell surface (Fig. 5).

CD4+ T cells under chronic antigenic stimulation modulate cell surface expression of ligands for activating NK receptors

Next, we investigated the possibility that CD4 T cells chronically stimulated by mHA could activate NK cell regulatory function by modifying expression of activating or inhibitory NK receptor ligands at their surface. We compared the expression of several mRNA encoding ligands of different receptors described in the literature to be expressed by T cells and known to modulate the activity of NK cells. As depicted in Fig. 5A, comparison between naïve TCR-transgenic T cells and chronically stimulated TCR-transgenic T cells isolated from NK cell-deficient recipients revealed that chronic Ag stimulation induced the upregulation of CD48, the ligand of receptor 2B4 (CD244) (21, 22). This finding was also observed at the level of protein expression (Fig. 5B). 2B4 has been originally considered as an NK cell activating receptor whose engagement was able to trigger non-MHC-dependent NK cytotoxicity (21). Recently, however, 2B4 was reported as an efficient inhibitory signaling receptor of NK cells (23).

The expression of two ligands of the activating receptor NKG2D, namely H60 and Mult-1, were also upregulated at the mRNA level in CD4 T cells under chronic antigenic stimulation (Fig. 5A). Mult-1 protein expression, however, was not detected by flow cytometry at the surface of naïve (Fig. 5B). Mult-1 protein is known to undergo ubiquitination dependent on lysines in its cytoplasmic tail and lysosomal degradation (24). Mult-1 ubiquitination is reduced in response to stress imparted by heat shock or UV irradiation (24). The lack of Mult-1 cell surface expression suggests that ubiquitination of the protein operates in chronically stimulated CD4 T cells. H60 exists under three forms expressed by different genes in the mouse—H60a, H60b, and H60c (25). In mice of the B6 genetic background, such as Marilyn mice, H60a expression cannot be detected, whereas it can be expressed by most cell types and tissues in other mouse strains (25, 26). There is no Ab available to analyze the expression of H60b or H60c. However, PCR primers used to detect H60 mRNA expression in our study were designed to amplify products from H60a or H60b. Thus, it is possible that Marilyn transgenic T cells, stimulated chronically by the male Ag, upregulate the NKG2D ligand H60b at their surface. The third type of ligands known to bind NKG2D, namely Rae-1 Ags (Rae-1 in B6-background mice), were not expressed in naïve T cells or in T cells under persistent antigenic stimulation, as evidenced by flow cytometry using a pan Rae-1 mAb (Fig. 5B).

Contrary to CD48 and NKG2D ligands, nonclassical MHC class I molecule CD1d, which presents lipid/glycolipid to type I NKT cells expressing invariant TCR, and CD1b appeared to be slightly downregulated in T cells that were chronically stimulated by male Ags (Fig. 5A). Expression of other MHC class I molecules, such as classical H-2K or nonclassical Qa-1, the ligands of Ly49 and NKG2A receptors, respectively, did not significantly vary between naïve and chronically activated T cells (Fig. 5). Among members of the recently defined family of SLAM-related activating receptors, Ly-9.2 and CRACC, which are also expressed by NK cells and act as self-ligands, were similarly expressed in naïve and chronically stimulated T cells (Fig. 5).
regulation of T cell activity by NK cells: 2B4 and NKG2D, because both were expressed by NK cells in Rag2−/− mice and their ligands were upregulated by chronically activated T cells. However, previous studies (27, 28) have shown that activating receptors 2B4 and NKG2D can also be expressed at the cell surface by T cells. Therefore, we analyzed by flow cytometry the expression of these receptors on naive or chronically activated T cells. As depicted in Fig. 5B, naive CD4 T cells did not express CD48 or NKG2D. However, although chronically activated T cells did not express NKG2D, CD48 was present at their surface (Fig. 5B). Thus, NKG2D was the only receptor in our system that could singularize regulatory NK cells. Together, these results prompted us to consider the possibility that the NK cell regulatory activity we observed in Rag2−/− mice was mediated by the specific engagement of NKG2D present at the surface of NK cells. To test this hypothesis, Rag2−/− male mice were reconstituted with donor male-specific CD4+ T cells. One group received blocking anti-NKG2D mAb for 21 d (29). The effect of anti-NKG2D treatment on male-specific CD4+ T proliferation was assessed by flow cytometry. As depicted in Fig. 6, the injection of blocking anti-NKG2D Abs induced T cell proliferation, and a high number of male-specific T cells were observed in the spleen of anti-NKG2D-treated recipients. T cell expansion was similar to that observed after injection of depleting anti-NK1.1 Abs. Histologic analysis demonstrated that the proliferation of Ag-specific CD4+ T cells was associated with signs of GVHD, and extensive cellular perportal infiltration in the liver was noticed after anti-NKG2D treatment (Fig. 2C). On the contrary and as expected, blocking the interaction between the NK inhibitory receptor NKG2A and its ligand Qa-1b did not appear to increase the capacity of NK cells to control the proliferation of Ag-specific T cells (Fig. 6). The number of NK cells present in recipients treated with blocking anti-NKG2D Abs was similar to that seen in animals receiving depleting anti-NK1.1 Abs (Fig. 6). This observation was due to the increase in the number of T cells present within the spleen rather than to Ab-mediated depletion of NK cells, because our preliminary experiments showed that anti-NKG2D Ab treatment did not eliminate NK cells in vivo (Supplemental Fig. 1). Thus, NKG2D engagement appeared to be essential for the control of mHA-driven T cell proliferation by NK cells.

**FIGURE 6.** NK regulatory activity is mediated by NKG2D. A, Number of TCR-transgenic CD4 T cells 28 d after adoptive transfer in Rag2−/− recipients untreated or treated with isotype control mAb, depleting anti-NK1.1 mAb, blocking anti-NKG2D mAb, or blocking anti-Qa-1 mAb, or in Rag2.perf−/− recipients. Relative number (B) and absolute number (C) of DX5+ CD3− NK cells in the spleen 28 d after adoptive transfer in Rag2−/− recipients untreated or treated with isotype control mAb, depleting anti-NK1.1 mAb, blocking anti-NKG2D mAb or blocking anti-Qa-1 mAb, or in Rag2.perf−/− recipients. *p < 0.01.
NK cell regulation of T cell proliferation does not involve perforin-dependent cytotoxicity, but depends on a functional Fas molecule expressed at the surface of T cells

NK cells are well known for their capacity to develop perforin-dependent cytotoxic activity. Some in vitro data support the idea that NK cells could regulate T cell activity by killing activated T cells (12, 13). We tested this possibility in our system by transferring male-specific CD4 T cells into perforin-deficient (Pfp−/−) Rag2−/− double-knockout recipients. Pfp−/− Rag2−/− mice and Pfpwt/wt Rag2−/− mice have a similar number of splenic NK cells (see Supplemental Material). Pfp−/− Rag2−/− male mice were injected with anti-male CD4+ T cells, and splenic T cell expansion was assessed 21 d after adoptive transfer. As seen in Fig. 6, there was no significant difference between the number of CD4+ T cells present in the spleen of Rag2−/− mice with normal NK cells and that in the spleen of Rag2−/− recipients with NK cells lacking perforin function. Thus, it was concluded that regulation of mHA-driven expansion of T cells mediated by NK cells in lymphopenic recipients did not involve perforin-dependent cytotoxic activity.

It is well established that T cells under chronic antigen stimulation are sensitive to Fas (CD95)-mediated apoptosis (30, 31). Alternatively, NK cells can develop FasL (CD178)-dependent cytotoxic activity against cell targets that express Fas at the cell surface (32, 33). Therefore, in our model, there was the possibility that NK cells regulated CD4 T cell expansion by a Fas/FasL interaction. To test this possibility, anti-male TcR-transgenic CD4 T cells expressing a nonfunctional Fas were generated by crossing (Marilyn X Fas−/− B6)F1 mice. As seen in Fig. 7, when adoptively transferred into Rag2-deficient male recipients, Fas−/− Marilyn T cells expanded 2-fold more than did Fas-sufficient TcR-transgenic T cells. This difference was not observed when T cells were transferred into recipients devoid of NK cells (Fig. 7A). These observations attested the capacity of NK cells to regulate the Ag-dependent proliferation of T cells through a Fas-dependent mechanism.

We have previously shown that the activity of T cells under chronic Ag exposure is controlled by the negative regulator PD-1 (34). Interestingly, we also observed that NK cells constitutively express high levels of PD-1 ligand at their surface (Supplemental Fig. 2). Thus, it was possible that NK cells could control T cells by engaging PD-1 on T cells. We investigated whether PD-1/PD-L1 interaction played a role in NK cell regulation of T cell activity in our model by injection of blocking anti–PD-L1 Abs. As seen in Fig. 7B, blockade of PD-1/PD-L1 interaction did not abolish the capacity of NK cells to inhibit the expansion of anti-male T cells in Rag2−/− male recipients. As observed previously (34), however, injection of anti–PD-L1 Abs into NK-deficient male recipients allowed the dramatic expansion of anti-male CD4 T cells (Fig. 7B). This expansion was accompanied by the development of an acute wasting disease characterized by massive leukocyte infiltration and tissue destruction in several organs such as the liver, kidney, skin, and gut (34). Thus, the Ag-driven expansion and function of CD4 T cells in lymphopenic Rag2−/− recipients is controlled by at least two independent mechanisms: the engagement of PD-1 at the surface of T cells and the regulatory activity of NK cells. We show in this study that this regulatory activity depends on the expression of functional Fas molecules at the surface of T cells.

Discussion

Our study demonstrates the importance of NK cell regulatory function on the development of chronic GVHD in lymphopenic recipients reconstituted with recipient’s mHA-specific CD4 T cells. NK cell regulation of GVHD resulted from the capacity of NK cells to control the proliferation of CD4+ T cells submitted to persistent antigenic stimulation. This conclusion has important implications in the clinical setting of bone marrow transplantation. Bone marrow graft infusion in patients is usually followed by a rapid proliferation of NK cells that appear to proceed directly from the hematopoietic stem cells transferred (5, 6). In view of the results obtained in this study, one could predict that T cell expansion in transplanted patients is controlled by the activity of NK cells. By extension, our observations also offer the challenging perspective of using protocols aimed at activating NK cells as a novel strategy to limit the expansion of competent T cell populations that cause chronic GVHD.

In a clinical study of patients transplanted with HLA identical bone marrow, Larghero et al. (8) correlated the risk of chronic GvHD with a lower bone marrow NK cell dose. Although the study supported the notion that NK cells could regulate chronic GVHD, it did not provide any clues on how NK regulation could proceed. It has become clear that NK cells interact with various components of the immune system, and therefore have the potential to function as regulatory cells. Whereas NK cells can assist in DC maturation and T cell polarization, increasing evidence attests the capacity of NK cells to regulate the proliferation of CD4 T cells in lymphopenic recipients. Activated mouse and human T lymphocytes, however, have been shown in vitro to become susceptible to autologous NK lysis via an NKG2D/
NKG2DL interaction (12, 13). In humans, NK lytic activity toward autologous T cells was shown to be dependent on calcium, suggesting that T cell killing was mediated by the granule exocytosis pathway (13). These results contrast our own observation that NK regulation is perforin independent. One explanation for these divergent results might reside in the fact that in the human study NK cells were generated in vitro and activated with rIL-2, a cytokine that has been shown to participate in the selection of the perforin-dependent cytotoxic pathway in NK cells (35).

NK cells can also express FasL at their surface (36) and TCR-mediated activation of T cells is known to induce cell surface expression of the death receptor Fas (37, 38). FasL expression on NK cells is regulated by IFN-γ (39), a cytokine specifically produced by CD4 T cells under chronic antigenic stimulation in our model (34). Thus, NK cell regulatory activity on T cell expansion could be, in our model, the result of Fas engagement at the surface of expanding CD4 T cells. This hypothesis is also supported by the previous observation that Fas mediates the deletion of T lymphocytes undergoing homeostatic proliferation in Rag2−/− recipients (40). The observation that NK cells appeared unable to control the expansion of Fas-deficient T cells in our model provides the best evidence so far on the role played by Fas/FasL-mediated cytotoxicity in NK cell-mediated regulatory activity.

Finally, the regulatory cytokine IL-10 has been shown recently to be produced by a subset of human NK cells for the control of T cell activity (19). Therefore, NK cells could mediate their regulatory function on T cells in our system by the production of IL-10. However, we have not been able to detect IL-10 production by NK cells isolated from mice with T cells undergoing Ag-driven expansion.

NK cells can detect stressed, infected, or transformed cells through the existence of cell surface activating and inhibitory receptors, the engagement of which regulates NK cell activities. Based on the elimination of activated T cells by NK cells, Lu et al. (41) recently identified NKG2A inhibitory receptor and its ligand, the nonclassical class I MHC molecule Qa-1, as possible targets for immunotherapy. In our system, we could not correlate a lack of Qa-1 expression with T cells undergoing Ag-driven proliferation. Moreover, blocking NKG2A/Qa-1 interaction did not enhance the capacity of NK cells to inhibit the proliferation of donor mHA-specific CD4 T cells. Thus, the NKG2A/Qa-1 molecular pathway does not appear to be involved in NK-mediated regulation of T cells under chronic antigenic stimulation. Our data clearly support a major role for activating receptor NKG2D in this process. First, NKG2D ligands were expressed by T cells under chronic antigenic stimulation. Second, blocking in vivo the interaction between NKG2D and its ligands abrogated the regulatory activity of NK cells. Interestingly, though effective at early time point after T cell injection, the regulatory activity of NK cells appeared to lose efficiency with time, and expansion of Ag-specific T cells was clearly visible 28 d after T cell adoptive transfer. This observation suggests that T cells might adapt to the pressure exerted by NK cell activity and with time become insensitive to regulation. In support of this concept are the observations made mostly in humans by several groups that the release of soluble NKG2D ligands by activated CD4 T cells inhibits the activity of NKG2D-expressing CD8 T cells, presumably by blocking the interaction between NKG2D on CD8 T cells and its ligands expressed at the surface of target cells (42). Activated CD4 T cells have also been described for their apparent capacity to retain NKG2D ligands, mostly inside their cytoplasm, suggesting that low surface expression of NKG2D ligands could be a safeguard mechanism to protect them from NK cell activity (43). Whether secretion or sequestration of NKG2D ligands is the mechanism used by chronically stimulated CD4 T cells to escape NK regulatory activity, and promote late T cell expansion in our system, is currently under investigation.

Although our study clearly demonstrates in vivo the regulatory capacity of NK cells on mHA-specific T cells, it is unclear whether this observation has any relevance to clinical bone marrow transplantation. Several observations, however, support NK regulatory function in bone marrow transplant recipients. As mentioned before, Largethor et al. (8) associate the presence of NK cells with a reduced incidence of chronic GVHD in patients transplanted with HLA identical sibling bone marrow. In another study analyzing the kinetics of leukocyte subsets in patients after transplantation of allogeneic stem cells, NK cells were identified as negative predictors of chronic GVHD (44). In allogeneic conditions, NK cell alloreactivity derives from a mismatch between donor NK cells bearing inhibitory killer cell Ig-like receptors (KIR) for self-HLA class I molecules and their HLA class I ligands (KIR ligands) on recipient cells. When faced with mismatched allogeneic targets, these NK cells sense the missing expression of self-HLA class I alleles and mediate alloreactions. As mentioned earlier, studies in mice show that, in these conditions, direct killing of allogeneic DC by bone marrow-derived NK cells could represent the main mechanism by which NK cells prevent the development of T cell-mediated GVHD (17, 18). Our results clearly demonstrate that, in situations in which recognition of donor–recipient histocompatibility mismatches does not involve sensing of missing self by inhibitory killer cell receptors, the development of NK cell-mediated regulatory activity targeting T cells and involving NK activating receptor NKG2D could represent an effective way to control chronic GVHD.

A third human study investigated the association of NK cell recovery with clinical outcomes after unmanipulated haploidentical blood and marrow transplantation (45). Human NK cells can be divided into two subpopulations according to their expression of the molecule CD56: CD56dim NK cells are good producers of cytokines and develop a poor granule-dependent cytotoxic activity upon activation, whereas CD56bright NK cells produce little cytokines and are naturally cytotoxic. Statistical analysis revealed that the patients with more CD56bright NK cells in the recovery stage had a higher survival rate and the patients with a higher ratio of T/NK had a higher chance of getting GVHD. These results suggest that regulatory function on T cell activity might not represent a common feature shared by all NK cells.

Our results have revealed an unexpected role played by NK cells in both the control of T cell proliferation and resistance to GVHD in MHC-compatible hosts. They suggest the use of immunotherapies aimed at the activation of NK cells as a way to protect against leukemia relapse and to prevent the development of chronic GVHD after bone marrow transplantation in patients with cancer.

Disclosures
The authors have no financial conflicts of interest.

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