Preventing NK Cell Activation by Donor Dendritic Cells Enhances Allospecific CD4 T Cell Priming and Promotes Th Type 2 Responses to Transplantation Antigens

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Although much progress has been made in understanding the role of NK cells in bone marrow transplantation, little is known about their function in CD4 T cell-mediated allograft rejection. We have previously shown that in the absence of CD8 T lymphocyte priming, the in vivo default development pathway of alloreactive CD4 T cells was strongly biased toward Th2 phenotype acquisition. In this study, we investigate the impact of NK cells on the activation and differentiation of alloreactive CD4 T cells in various donor/recipient combinations. Our data demonstrate that defective inhibition of host NK cells by donor APCs including dendritic cells (DCs) results in diminished allospecific Th cell responses associated with the development of effector Th cells producing IFN-γ rather than type 2 cytokines. Turning host NK cells off was sufficient to restore strong alloreactive CD4 T cell priming and Th2 cell development. Similar results were obtained by analyzing the effect of NK cell activation on CD4 T cell responses to skin allografts. However, despite the dramatic effect of NK cells on alloreactive Th1/Th2 cell development, the kinetics of skin graft rejection were not affected. Thus, Th2 differentiation is a major pathway of alloreactive CD4 T cell development during solid organ transplant rejection, as long as host NK and CD8 T cells are not activated. We propose the hypothesis that MHC class I-driven interactions between donor DCs and host NK cells or CD8 T cells might result in DC-carried signals controlling the dynamics of alloreactive CD4 T cell priming and polarization. The Journal of Immunology, 2002, 169: 2979–2987.

Natural killer cells are large granular lymphocytes capable of killing cells without prior immunization (1). The recognition of tumors or pathogen-infected cells by NK cells is controlled by the engagement of multiple cell surface inhibitory or stimulatory receptors that bind to MHC class I or non-MHC ligands. Such interactions result in a cascade of events that transduces either inhibitory or activatory signals, with their balance regulating NK cell activation. Inhibitory receptors encompass several families of cell surface molecules such as Ly-49 and CD94/NKG2 receptors in mouse that recognize self MHC class Ia molecules (2). The activatory receptors recognize MHC class I-like molecules and other undefined ligands and trigger lysis and cytokine production (2). NK cells can distinguish allogeneic from syngeneic cells, and the expression of self MHC class I molecules protects target cells from NK cell lysis by interacting with NK cell inhibitory receptors. According to the missing self hypothesis, lack of expression or down-regulation of class I molecules on target cells render them susceptible to NK cell lysis (3).

Although much progress has been made in understanding the role of NK cells in bone marrow transplantation, little is known about their function in CD4 T cell-mediated allogeneic transplant rejection (4–7). For instance, in fully allogeneic combinations, it is likely that in addition to CD4 and CD8 T lymphocytes, NK cells can be activated by donor-derived APCs. Among donor APCs, tissue-resident dendritic cells (DCs)3 are likely to migrate from the graft to secondary lymphoid organs where priming of alloreactive T cells can occur initiating transplant rejection (8, 9). How NK cells can interfere with donor-derived DC and whether these interactions can modulate allospecific T cell development in vivo is presently not known. We have recently analyzed the development of alloreactive CD4 T cells in the absence of CD8 T cell activation in vivo. We showed that while immunization of adult mice with semiallogeneic splenocytes induces the differentiation of donor-specific CD4 T cells toward the Th1 phenotype, responses strongly polarized toward the Th2 type occurred in CD8-deficient mice. This led us to conclude that in the absence of CD8 T cell priming and whatever the genetic background of the host, the default response of alloreactive CD4 T cells is a sustained production of Th2-type cytokines (10).

In this study, we investigate the role of NK cell activation on the development of alloreactive CD4 T cells in vivo in the absence of CD8 T cell activation. The experimental model we have chosen involved priming of the CD8-deficient parental C57BL/6 (B6) or BALB/c strains with either semiallogeneic (BALB/c × B6) F1 (CB6 F1) or fully allogeneic APCs. Semiallogeneic APCs that expressed both parental MHC products were used instead of fully allogeneic cells to avoid NK cell activation (10). Although immunization of CD8-deficient mice with semiallogeneic APCs induced strong alloreactive CD4 T cell priming and Th2 cell development, injection of allogeneic APCs resulted in reduced allospecific CD4 T cell responses and in the selective expansion of IFN-γ-producing cells. Ab-mediated NK cell depletion before immunization resulted in dramatic expansion of alloreactive Th cells secreting high

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3 Abbreviations used in this paper: DC, dendritic cell; β2m, β2-microglobulin; BM-DC, bone marrow-derived DC; LNC, lymph node cell; SC, spleen cell; WT, wild type.
levels of IL-4 and IL-10 similar to the responses observed in β2-microglobulin (β2m) and CD8 double knockout mice where NK cell activity is impaired (11–13). Interestingly, grafting CD8-deficient mice with semiallogeneic skin also induced marked allo-specific Th2 cell priming; whereas rejection of allogeneic grafts was associated with the selective development of Th1 cells. In this latter combination, turning NK cells off resulted in a skewing of the alloreactive CD4 T cell response to the Th2 pathway without affecting the kinetics of skin graft rejection. Taken together, our data demonstrate that recognition of donor APCs by host NK cells strongly affects the magnitude of allo-specific Th cell responses as well as their cytokine secretion profiles in various donor/recipient strain combinations.

Materials and Methods

Mice and immunization

BALB/c (H-2d), C57BL/6 (H-2b), and (BALB/c × C57BL/6)F1 (CB6F1) mice were purchased from Center d’Elevage R. Janvier (Le Genest St Isle, France). H-2b mice with disrupted β2m genes were back-crossed to BALB/c mice as previously described (14). β2m−/− mice on BALB/c or B6 background were used after 10 back-crosses. CD8−/− mice on the B6 background were initially obtained from the Center National de la Recherche Scientifique (Centre de Développement des Techniques Avancées, Orléans, France) and were used to generate double-deficient CD8−/− and β2m−/− mice. H-2b mice with disrupted CD8 genes were back-crossed eight times to BALB/c mice in our own animal facility. BALB/c mice from mice immunized with allogeneic SCs were cultured at 3 × 105 cells/ml in a bacteriological petri dish (SCs) or for 0.5 × 106 bone-derived DCs (BM-DCs) from normal or β2m-deficient BALB/c or B6 or CB6F1 mice. Six days after immunization, the draining popliteal and inguinal lymph nodes were removed and further processed as described below.

In vivo mAb treatment

For in vivo NK cell depletion in B6 mice, the anti-NK cells PK136 mouse IgM2 (BD PharMingen), 1% pyruvate, 1% nonessential amino acids, 1%L-glutamine and 50 μg/ml gentamicin (Sigma-Aldrich). Cultures were incubated for 3 days in a humidified atmosphere of 5% CO2 in air. Supernatants from replicate cultures were collected after 72 h and pooled for cytokine analysis. IFN-γ, IL-4, IL-5, and IL-10 were identified by two sites of sandwich ELISA as previously described (10). For T cell proliferation assays, cell cultures were pulsed 16 h with 1 μCi [3H]Tdr (40 Ci/mmol; Radiochemical Center, Amersham, U.K.) before harvesting on glass fiber filter. Incorporation of [3H]Tdr was measured by direct counting using an automated β-counter plate (Matrix 9600; Packard Instrument, Meriden, CT).

Flow cytometric analysis of intracellular cytokine synthesis

LNC were cultured with allogeneic-irradiated (2400 rad) SCs from B6 or BALB/c or CB6 F1 mice as indicated above. After 72 h of culture, cells were harvested, washed, and cultured for an additional 72 h in complete medium. After Ficoll separation, living cells were collected, resuspended at 1 × 107/ml, and stimulated with PMA (50 ng/ml; Sigma-Aldrich) plus ionomycin (0.5 μg/ml; Sigma-Aldrich) for 4 h. Two hours before cell harvest, brefeldin A (Sigma-Aldrich) was added at a concentration of 10 μg/ml. Cells were harvested, washed in the presence of brefeldin A, and stained using biotinylated anti-CD4 mAb (BD Pharmingen), followed by streptavidin-phycoerythrin (BD Pharmingen). Labeled cells were then fixed with 2% paraformaldehyde (Fluka Chemie, Buchs, Switzerland). Intracytoplasmic staining for IFN-γ, IL-4, IL-5, and IL-10 were performed as described previously (10). Data were collected on 20,000 CD4+ cells on an L-Coulter cytometer (Beckman Coulter France S.A., Roissy, France) and analyzed using the CellQuest software (BD Biosciences, Mountain View, CA).

Skin grafting

Skin grafts ~1 cm in diameter were prepared from tails of female CB6 F1, B6, or BALB/c mice and grafted onto the flank of recipient mice. Petroleum gauze was placed over the graft and stirring plaster was applied around the trunk. After 7 days, bandages were removed and the grafts monitored daily. Grafts were considered rejected when complete epithelial breakdown had occurred.

Statistical analysis

Results are expressed as the mean ± SD, and statistical differences between variables were evaluated by the Mann-Whitney U test.

Results

Effect of MHC class I deficiency of allogeneic APCs on allospecific CD4 T cell development in vivo

We have previously shown that priming of alloreactive CD4 T cells with semiallogeneic APCs results in the development of effector Th cells producing large amounts of type 2 cytokines in the absence of CD8 T cell activation (10). This is illustrated in Fig. 1, where immunization of β2m−/−BALB/c mice with semiallogeneic β2m−/−-irradiated splenocytes resulted in the generation of IAα-reactive CD4 T cells producing high levels of IL-4 and IL-10 in agreement with our previous work (17). This was confirmed by the analysis of intracellular cytokine synthesis in the same T cell populations after 6 days of in vitro stimulation with CB6 F1 APCs (Fig. 2C). Strong expansion of IL-4- or IL-10-producing cells was observed, and accounted for the majority of effector CD4 T cells in this combination. In contrast, the frequency of CD4 T cells that produced IFN-γ was <10%. Conversely, immunization of BALB/c mice with CB6 F1 APCs induced the development of IAα-reactive Th1 cells, producing mainly IFN-γ (38%) but little type 2 cytokines (Fig. 2A). We have previously shown that the difference in alloreactive CD4 T cell phenotype acquisition between these two combinations was due to the in vitro activation of MHC class I-specific CD8 T cells (10). Because MHC class I deficiency in hemopoietic cells has been shown to promote NK
production by H-2b-reactive CD4 T cells. The T cell proliferative response, as well as IFN-γ synthesis were also up-regulated in anti-asialo-GM-1-treated mice (Fig. 3). Thus, impaired MHC class I expression by donor APCs dramatically affects the magnitude and the phenotype of the allospecific CD4 T cell response due to the absence of negative regulation of host NK cells.

**NK cell-mediated regulation of allospecific CD4 T cell development due to lack of expression of self MHC class I molecules by allogeneic APCs**

NK cell-mediated bone marrow rejection can be caused by the complete lack of expression of MHC class I molecules, but also by the lack of self MHC class I molecules on donor cells (2, 18) albeit less efficiently (11, 12). To evaluate the effect of NK cell activation on alloreactive CD4 T cell development, BM-DC from either fully allogeneic B6 or semiallogeneic CB6 F1 mice were used to immunize BALB/c CD8-/- mice. BALB/c recipients (Fig. 4, A and B). Similar results were obtained using allogeneic B6 SC as APCs (data not shown). In contrast, although a proliferative response could be induced in CD8-deficient BALB/c SC primed with B6 BM-DCs (Fig. 4A), these B6-specific CD4 T cells secreted less IFN-γ in primary culture and no type 2 cytokines (Fig. 4B). To test whether this effect was due to NK cell activation as a consequence of the lack of expression of self MHC class I molecules, mice were pretreated with asialo-GM-1 antisera. The B6-specific proliferative response was slightly up-regulated in NK-depleted mice as compared with control serum-treated and untreated CD8-deficient BALB/c recipients (Fig. 4A). Interestingly, in vivo elimination of

**NK cell depletion restores Th2 development in BALB/c mice**

Immunization of BALB/c mice with MHC class I-deficient CB6 F1 APCs impairs Th2 cell development. WT (n = 3 per group) or β2m-/- (n = 4) BALB/c mice were immunized into the hind footpads with 50 × 10^6 irradiated SC from WT or β2m-/- CB6 F1 mice. Six days later, draining lymph nodes were harvested. Immune LNC were cultured (3 × 10^6 cells/well) in duplicate in the presence of irradiated (2400 rad) CB6 SC (3 × 10^6 cells/well) in HL-1 synthetic medium during 72 h. To measure proliferation, [3H]TdR (0.1 μCi/well) was added during the last 16 h of culture. Cytokine production in 72-h culture supernatant was measured by ELISA. Results are expressed as mean ± SD of three to four mice per group and are from one representative experiment of four performed (*, p < 0.05).

**FIGURE 1.** MHC class I-deficient APCs induce allospecific Th2 priming in β2m-deficient but not WT recipients. WT (n = 3 per group) or β2m-/- (n = 4) BALB/c mice were immunized into the hind footpads with 50 × 10^6 irradiated SC from WT or β2m-/- CB6 F1 mice. Six days later, draining lymph nodes were harvested. Immune LNC were cultured (3 × 10^6 cells/well) in duplicate in the presence of irradiated (2400 rad) CB6 SC (3 × 10^6 cells/well) in HL-1 synthetic medium during 72 h. To measure proliferation, [3H]TdR (0.1 μCi/well) was added during the last 16 h of culture. Cytokine production in 72-h culture supernatant was measured by ELISA. Results are expressed as mean ± SD of three to four mice per group and are from one representative experiment of four performed (*, p < 0.05).

**FIGURE 2.** Immunization of BALB/c mice with MHC class I-deficient semiallogeneic APCs impairs Th2 cell development. WT (n = 3 per group) or β2m-/- (n = 4) BALB/c mice were immunized into the hind footpads with 50 × 10^6 irradiated SC from WT or β2m-/- CB6 F1 mice. Six days later, draining lymph nodes were harvested. Immune LNC were cultured (3 × 10^6 cells/ml) in the presence of irradiated CB6 F1 SC (1.5 × 10^6 cells/ml). After 6 days of culture, LNC were stimulated using PMA and ionomycin for 4 h and stained intracellularly for IFN-γ and IL-4. Analysis was performed on CD4 T cells. Results expressed as mean of three to four individual mice per group ± SD are from one representative experiment of four performed.

**FIGURE 3.** NK cell depletion restores Th2 development in BALB/c mice primed with MHC class I-deficient semiallogeneic APCs. BALB/c mice were immunized into the hind footpads with 50 × 10^6 SC from β2m-/- CB6 F1 mice. Mice were injected i.v. either with asialo-GM-1 rabbit antisera at days −2, 0, and +2 of the experiment or left untreated. Six days later, LNC were stimulated (3 × 10^6 cells/well) with the indicated amounts of irradiated B6 SC. Proliferation and cytokine production were measured as in Fig. 1. Results expressed as mean of three individual mice per group ± SD, are from one representative experiment of three performed (*, p < 0.05).
NK cells led to the expansion of allospecific CD4 T cells producing high amounts of IL-4 and IL-10 in BALB/c CD8−/− mice immunized with fully allogeneic B6 APCs (Fig. 4B). IFN-γ synthesis was also up-regulated in mice treated with anti-asialo GM-1 Abs (Fig. 4B). Because both type 1 and 2 cytokines were increased by turning NK cells off, we analyzed the frequency of IL-4- and IFN-γ-producing cells. As shown in Fig. 4C, NK cell depletion resulted in a dramatic increase in effector/memory Th2 cell expansion producing IL-4 but no IFN-γ (40–60%). The remaining effector T cells were composed of Th1 (2–6% IFN-γ+) and Th0 (3–7% IL-4+/IFN-γ+) lymphocytes. This pattern of cytokine-producing cells was similar to the one observed in CD8-deficient BALB/c mice primed with CB6 F1 APCs (Fig. 4C). Although T cell proliferative responses when measured in the fully allogeneic combination were associated with IFN-γ synthesis (Fig. 4A and B), the yield of allospecific CD4 T cells after 6 days of primary culture was very low (<0.2 × 10⁶ cells for 10⁶ input cells) precluding further analysis (data not shown). However, in some experiments where a sufficient number of T cells could be obtained from this combination, the response was dominated by IFN-γ-producing cells with a frequency of 20% (data not shown).

**NK cell-mediated regulation of allohelper T cell activation and differentiation is not restricted to the BALB/c genetic background**

Experiments described so far were performed in BALB/c mice using anti-asialo GM-1 Abs for NK cell depletion, which can also damage macrophages (19) or T cells (20). Therefore, we tested whether our observations were also valid in B6 mice in which the method of NK cell depletion using the anti-NK1.1 mAb PK136 is well established (21). To examine the allogeneic CD4 T cell response in the absence of CD8 T cell activation, we used CD8-deficient B6 mice as recipients. We compared two combinations where mice were injected with BM-DCs from either semiallogeneic CB6 F1 or allogeneic BALB/c mice. Unlike BALB/c BM-

**FIGURE 4.** Strong allospecific CD4 T cell response associated with type 2 cytokine production in CD8-deficient mice primed with semi-identical but not with fully allogeneic DCs. CD8-deficient BALB/c mice were immunized into the hind footpads with 5 × 10⁶ BM-DCs from either semiallogeneic CB6 F1 or fully allogeneic B6 mice. Treated mice were injected i.v. either with asialo-GM-1 rabbit antiserum at day −2, 0, and +2 of the experiment or left untreated. Six days later, LNC were stimulated (3 × 10⁶ cells/well) with the indicated amounts (A) or (3 × 10⁶ cells/well) (B) of irradiated CB6 F1 SC. A, To measure proliferation, [³H]TdR was added during the last 16 h of culture. B, Cytokine production in 72 h culture supernatant was measured by ELISA. C, After 6 days of culture, LNC were stimulated using PMA and ionomycin and stained intracellularly for IFN-γ and IL-4. Analysis was performed on CD4 T cells. Results expressed as mean of three individual mice per group ± SD, are from one representative experiment of three performed (*, p > 0.05).

**FIGURE 5.** NK cell activation in CD8-deficient B6 mice immunized with fully allogeneic BM-DC leads to an impaired CD4 T cell response. CD8-deficient B6 mice were immunized into the hind footpads with 5 × 10⁶ BM-DCs from either semiallogeneic CB6 F1 or fully allogeneic BALB/c mice. A, Six days later, immune lymph nodes were harvested and recruited cells were counted. B, LNC were cultured (3 × 10⁶ cells/well) in the presence of the indicated amounts of irradiated CB6 F1 SC. Cytokine production in 72 h culture supernatant was measured by ELISA. C, After 6 days of culture, LNC were stimulated using PMA and ionomycin and stained intracellularly for IFN-γ and IL-4. Results expressed as mean of three individual mice per group ± SD, are from one representative experiment of three performed (*, p < 0.05).
Inhibition of host NK cells in mice immunized with fully allogeneic DCs restores strong CD4 T cell responses and type 2 cytokine production. CD8<sup>+</sup>/ or CD8<sup>-</sup>/β<sub>2m</sub><sup>-/-</sup> B6 mice were immunized into the hind footpads with 5 x 10<sup>4</sup> BM-DC from fully allogeneic BALB/c mice. Treated mice were i.v. injected with anti-NK PK136 mAb at days -2, 0, and +2 of the experiment. Six days after immunization, immune LNC were cultured (3 x 10<sup>5</sup> cells/well) in the presence of the indicated amounts of irradiated BALB/c splenic cells (SC). A, Cytokine production in 72-h culture supernatant was measured by ELISA. Proliferation after 72 h of culture in the presence of irradiated BALB/c SC (1 x 10<sup>5</sup> cells/well) was determined by [H]Tdr incorporation. B, After 6 days of culture, LNC were stimulated using PMA and ionomycin and stained intracellularly for IFN-γ and IL-4. Analysis was performed on CD4<sup>+</sup> T cells. Results expressed as means of three individual mice per group ± SD are from one representative experiment of three performed.

DCs, CB6 F<sub>1</sub> BM-DCs express MHC class I molecules of H<sup>2</sup><sub>b</sub> haplotype; and therefore, are able to inhibit NK cell activation. As shown in Fig. 5, immunization of CD8-deficient B6 mice with allogeneic BALB/c BM-DCs resulted in a low lymphocyte recruitment in the draining lymph nodes (Fig. 5A). Upon allogenic stimulation with APCs expressing H-<sup>2</sup><sub>a</sub> class II molecules, LNC failed to proliferate (data not shown) and did not produce cytokines (Fig. 5B). In contrast, immunization of CD8-deficient B6 mice with semiallogeneic CB6 F<sub>1</sub> APCs resulted in a strong expansion of H-<sup>2</sup><sub>b</sub>-specific CD4 T cells that produce, in addition to IFN-γ, high levels of type 2 cytokines (Fig. 5, B and C), in agreement with our previous observations (10).

Using the PK16 mAb, we next assessed whether NK cell depletion in B6 CD8<sup>-/-</sup> mice before their immunization with fully allogeneic APCs could restore efficient CD4 T lymphocyte priming. Furthermore, because β<sub>2m</sub>-deficient mice have been shown to have an impaired NK activity due to the lack of expression of MHC class I molecules (11, 12), CD4 T cell priming in response to fully allogeneic APCs was also tested in CD8<sup>-/-</sup> and β<sub>2m</sub>-double knockout B6 mice. As shown in Fig. 6A in agreement with data in Fig. 5, CD8-deficient B6 mice injected with BALB/c BM-DCs failed to develop a significant allospecific CD4 T cell response. Lack of T cell priming was also observed in B6 CD8<sup>-/-</sup> mice treated with an irrelevant isotype-matched control mAb (data not shown). In striking contrast, in NK-cell-depleted B6 CD8<sup>-/-</sup> mice, a strong T cell proliferation and cytokine production following in vitro allogenic restimulation was observed. Similar CD4 T cell responses were recorded in NK-depleted B6 CD8<sup>-/-</sup> mice and in β<sub>2m</sub>-deficient B6 CD8<sup>-/-</sup> mice (Fig. 6A) consistent with an inhibitory effect of NK cells on alloreactive T cell priming. To define the cytokine production profile of allospecific CD4 T lymphocytes, expanded T cells were stained intracellularly for IL-4 and IFN-γ after PMA/ionomycin stimulation. Data shown in Fig. 6B illustrate that allospecific CD4 T cells primed in the absence of NK cell activation, either following Ab depletion (Fig. 6B, upper panel) or in mice with impaired NK cell activity (Fig. 6B, lower panel), exhibited strikingly similar profiles of cytokine-producing cells. The response was dominated by IL-4-producing cells (up to 30% of CD4 T cells), of which one-third also secreted IFN-γ. The frequency of IFN-γ<sup>+</sup> T cells was ~10% of CD4 T cells. Similar results were obtained when B6 CD8<sup>-/-</sup> mice were primed with semiallogeneic F<sub>1</sub> APCs (Fig. 5C). Because it has been reported that syngeneic DC could be killed efficiently by autologous NK cells (22), and that there is a bidirectional cross-talk between DC and NK cells (23), we tested the effect of NK cell depletion in mice immunized with semiallogeneic DCs. Data in Fig. 7 show that in this combination where NK cells are silenced by the expression of syngeneic MHC class I molecules, NK cell depletion had no significant effect on alloseactive CD4 T cell activation and differentiation. Thus, in agreement with our previous observations in the BALB/c genetic background, inhibition of NK cell activation in B6 recipients immunized with fully allogeneic DCs enhances allospecific CD4 T cell priming and promotes Th2 cell development.
NK cell-mediated regulation of allospecific CD4 T cell response following fully allogeneic skin graft transplantation

We next determined whether NK cell activation in the absence of self-MHC class I expression can also control allospecific CD4 T cell responses induced by tissue transplantation. To this end, we performed skin graft on CD8-deficient BALB/c recipients in combinations in which NK cells were activated (B6 donor) or not (CB6 F1 donor). As shown in Fig. 8A, the outcome of CD4 T cell-mediated graft rejection was similar in CD8-deficient recipient grafted with either a semi or fully allogeneic skin, and occurred between days 8 and 11. Then we analyzed the polarization of allospecific CD4 T cells from lymph nodes draining the graft before and during the rejection process. As shown in Fig. 8B, CD4 T cells from CD8-deficient BALB/c mice grafted with CB6 F1 skin produced large amounts of IL-4 in addition to IFN-γ upon restimulation with CB6 F1 APCs at both time points tested. In individual mice, the level of IL-4 synthesis was inversely correlated to IFN-γ secretion, indicating that strong Th2 polarization had occurred in CB6 F1-grafted recipients. In contrast, while IL-4 production was low or undetectable in recipient mice bearing fully allogeneic graft, the allospecific CD4 T cell response was characterized by the selective development of IFN-γ-producing cells.

We next analyzed the impact of NK cell activation on the development of CD4 T cells in fully allogeneic skin graft recipients. CD4 T cell-mediated rejection of BALB/c skin by CD8-deficient B6 mice was characterized by the selective development of IFN-γ-producing T cells (Fig. 9A). In contrast, NK cell depletion in vivo resulted in enhanced IL-4 and decreased IFN-γ production by T cells from the lymph nodes draining the BALB/c graft (Fig. 9A). Interestingly, a similar skewing of the H-2d-specific T cell response to the Th2 phenotype was observed in β2m- and CD8-deficient B6 mice. In contrast to PK136-treated mice, those injected with control mAb exhibited a Th1-polarized allogeneic T cell response. Finally, although the character of alloreactive CD4 T cell responses was dramatically affected by NK cell depletion, the kinetics of allogeneic BALB/c skin graft rejection in PK136-treated B6 CD8−/− mice was not modified, demonstrating that acute graft rejection can occur in the context of either a Th1- or Th2-polarized immune response (Fig. 9B). Taken together, these data demonstrate that donor DC-driven host NK cell activation controls alloreactive Th cell development during skin graft transplantation without affecting the kinetics of rejection.

Discussion

NK cells represent a key component of innate immune response and can mediate graft rejection following bone marrow transplantation (6, 18). However, little is known about the role of NK cells in alloreactivity of solid organs (4–7). In the present paper, we have evaluated whether NK cells can influence the response of alloreactive CD4 T lymphocytes, presumably through their interaction with donor-derived APCs. We have previously shown that allospecific CD4 T lymphocytes differentiate into type 2 cytokine-producing cells in the absence of CD8 T lymphocyte activation (10).
Strong Th2 responses were first observed in a MHC class I-deficient donor/recipient combination where β₂m-deficient BALB/c mice were immunized with β₂m-deficient semiallogeneic CB6 F₁ (H-2bxd) APCs. In contrast, immunization of wild-type (WT) BALB/c mice with CB6 F₁ APCs resulted in the selective development of H-2²-specific Th₁ cells (10). The absence of Th2 cell development was controlled by CD8 T cells, because immunization of CD8-deficient parental strains with CB6 F₁ APCs induced allospecific CD4 T cells producing a large amount of Th2-type cytokines (10). We initially thought of using CB6 F₁ APCs to prime alloreactive host CD4 T cells on the assumption that F₁ APCs expressed, in addition to allogeneic MHC products, self MHC class I molecules that bind to inhibitory receptors on host NK cells, thereby silencing inhibitory receptor-bearing cells. We now provide direct evidence that NK cells, through their interaction with allogeneic APCs, can quantitatively and qualitatively control allospecific CD4 T responses in vivo. This was tested in different combinations: 1) by injecting β₂m-deficient allogeneic APCs into normal mice, or 2) by immunizing CD8-deficient mice with fully allogeneic APCs. In the first combination, host NK cells were likely to be activated as a consequence of the lack of expression of self MHC class I molecules on donor-derived APCs, thus resulting in NK cell activation. In this combination, the allospecific CD4 T cell response was impaired and led to the development of a Th1-dominated immune response. Depletion of asialo-GM-1-positive cells before immunization with β₂m-deficient APCs restored a strong alloreactive CD4 T cell response characterized by the emergence of type 2 cytokine-producing cells. These data indicate that NK cells can regulate CD4 T cell activation and differentiation in vivo. This was further demonstrated in the second type of combination where inhibitory receptors on NK cells cannot be engaged by allogeneic donor APCs that unlike CB6 F₁ APCs do not express syngeneic MHC class I molecules. Again, NK cell depletion was found to enhance CD4 T cell priming and to unmask Th2 cell development in both CD8-deficient B6 and BALB/c-recip-ient mice. Furthermore, we showed that H-2²-specific Th2 responses of comparable magnitude were generated in NK-depleted B6 CD8⁻/⁻ mice and in B6 CD8⁻/⁻ β₂m⁻/⁻ double-deficient mice that have an impaired NK cell activity due to the lack of MHC class I molecules (11–13). Thus, our data demonstrate that in various donor/recipient combinations where NK cells are acti-vated, allospecific Th2 cell responses are abrogated or strongly diminished with the development of effector Th cells producing selectively IFN-γ but no type 2 cytokines. Inhibition of host NK cells was sufficient to induce alloreactive CD4 T cell responses dominated by type 2 cytokine-producing effector Th cells. This mechanism was not restricted to the BALB/c background and occurred also in B6 mice. However, in this latter strain, NK cell activation resulted in a complete inhibition of alloreactive CD4 T cell priming when DCs were used for immunization. In contrast, when skin grafts were applied as stimulus, unipolar Th1 cell responses were selectively observed in combinations where NK cells were activated in both BALB/c and B6 strains. For instance, in CD8-deficient BALB/c recipients, Th1 responsiveness was not significantly different between mice grafted with either allogeneic or semiallogeneic skin. Conversely, strong priming of IL-4-producing T cells was exclusively observed in the semiallogeneic combination. The impact of NK cell activation on Th1/Th2 differentiation was further demonstrated by grafting CD8-deficient B6 mice with allogeneic BALB/c skin. In this combination, turning NK cells off resulted in a decreased production of IFN-γ and an increased synthesis of IL-4 by alloreactive CD4 T cells from the lymph nodes draining the graft. Taken together, our data support the conclusion that NK cell activation reduces alloreactive CD4 T cell priming and thus, the subsequent development of both subsets of memory/effector T lymphocytes with a major impact on Th2 cells. Indeed, IL-4-producing cells expand exclusively when NK cells were turned off. In contrast, when CD4 T cell responses develop in combinations where NK cells are activated by donor-derived APCs, these Th cells exhibited a unipolar Th1 profile.

Our study unveils a novel mechanism by which NK may regu-late the adaptive immune response directed against transplantation Ags in the absence of appropriate interaction between inhibitory receptors on NK cells and MHC class I molecules on donor APCs. These observations are relevant to the clinical transplant situation because similar conclusions could be drawn by analyzing CD4 T cell responses to allograft transplantation. Allospecific CD4 T cells producing IL-4 could readily be detected only when CD8-deficient mice were grafted with semi-identical skin. In this combination, NK cells were not activated by donor-derived DCs because they could receive a silencing signal via self MHC molecules. In con-trast, rejection of fully allogeneic skin grafts was associated with the selective development of Th1 cells due to NK cell activation by donor-derived DCs. Indeed, we showed that inhibition of NK cells during CD4 T cell-mediated allogeneic skin graft rejection induced a skewing of the alloreactive T cell response to the Th2 pathway. Therefore, the impact of NK cell activation on the development of Th2 effector functions was similar when skin grafts or BM-DCs were used as stimulus. This conclusion is in agreement with the hypothesis that donor-derived APCs, e.g., DCs, play a central role in inducing CD4 T cell-mediated allograft rejection through direct presentation of donor allogeneic MHC class II molecules to host Th lymphocytes in the draining lymph nodes (8, 9, 24). However, despite the dramatic effect of NK cells on alloreactive Th1/Th2 cell development, there was no effect on the kinetics of skin graft rejec-tion. This is not surprising since it has been shown by others that both Th1 and Th2 responses are capable of causing acute transplant rejection with identical kinetics in recipient mice receiv-ing either cardiac (25) or skin (26) grafts. Furthermore, subsequent studies have established that rejections mediated by Th2 cells were characterized by marked eosinophilic infiltration of skin and heart transplants (27, 28). Finally, it has been reported by Le Moine et al. (29) in a model of MHC class II disparate skin grafts (B6f12 → B6) that IL-4, IL-5, and eosinophils were critically involved not only in chronic but also in acute (30, 31) skin graft rejection. These observa-tions are in agreement with our present study and support the conclusion that allospecific CD4 T cell development during solid organ transplant rejection is strongly biased toward Th2 phenotype as long as host NK and CD8 T cells are not activated.

DCs have been shown to play a crucial role in allorejection by migrating from the transplanted tissue to secondary lymphoid organs of the host where they can prime allospecific T cells and initiate graft rejection (8, 9). Accumulating evidence indicates that DCs are phenotypically heterogeneous, and represent a multilin-eage system of leukocytes with variable functions (32). These DC subsets appear to play a role in determining the specific cytokines secreted by Th cells. It has been hypothesized that DCs recruited in immune lymph node can be instructed by environmental stimuli to perform different functions (33, 34). It has been shown in hu-mans that IL-12 is produced by DCs within a narrow time window so that only recently activated DCs can promote Th1 cell develop-ment (33, 35). At later time points, DCs become exhausted in their capacity to secrete various cytokines including IL-12, thereby favoring conditions for priming of Th2 responses, which are de-pendent on IL-4 production by responding T cells (33–35). Transient IL-12 production by DCs in vivo have also been documented in mice following systemic stimulation with microbial products (36). To explain our data, we hypothesize that in a situation where
both the NK and CD8 T cell pathways are not operative, donor DCs may accumulate in immune lymph nodes, thereby favoring strong CD4 T cell priming and Th2 type responses. Thus, tissue resident NK cells and possibly also CD8 T cells present in the secondary lymphoid organs would inhibit the default Th2 differentiation of allospecific CD4 T cells by limiting the flux of incoming allogeneic DCs into the draining lymph nodes and/or by shortening DC half-life in situ. This may lead to incomplete kinetics of DC differentiation preventing allospecific CD4 T lymphocyte activation by a particular DC subset with Th2-prone capacity. This would explain why in these conditions, CD4 T lymphocytes selectively differentiate toward the Th1 phenotype.

It has recently been shown that inhibition of NK cells combined with CD28 costimulation blockade induced long-term survival of allogeneic vascularized cardiac grafts. In contrast, none of these treatments alone resulted in the acceptance of cardiac allografts. Thus, it has been suggested that NK cells may have a critical role in allorejection by providing help to T cells, such function being essential in the absence of CD28-mediated costimulation (37). Our data are contradictory to this hypothesis, and several reasons could explain this discrepancy. First, CD28-deficient mice have reduced humoral responses but normal cell-mediated immunity (38), indicating that CD4 T cells are more profoundly affected by CD28-B7 blockade than CD8 T lymphocytes. Indeed, it has been shown that blockade of CD28/B7 costimulation inhibits intestinal allograft rejection mediated by CD4+ but not CD8+ T cells (39). Furthermore, it has been shown that CD28-B7 costimulation plays an important role in generating the Th2 compartment (40–42). Altogether, this could explain why no Th2 cell priming was observed by turning NK cells off in CD28-deficient mice (37). Second, the CD4/CD8 ratio among graft infiltrating cells was strongly skewed toward CD8 T cells in CD28-deficient mice (37), suggesting that CD8 T cells appear to be the major effector T cell subset responsible for graft rejection. In contrast, it has been clearly established that rejection of skin and cardiac allografts in mice with functional CD28/B7 costimulation pathways was dependent upon CD4 T cells, whereas CD8 T cells were never necessary nor sufficient (43, 44). Therefore, the observation that NK cells in allogeneic graft might preferentially provide help to CD8 T cells might be a particularity of the CD28− phenotype and is most likely not relevant to the normal recipient situation where rejection mainly involves CD8 T cells (43, 44).

In conclusion, our data strongly support the hypothesis that NK cells rather than delivering help to allospecific CD4 T cells may actually limit their activation and differentiation in vivo through their interaction with allogeneic APCs. Indeed, it has been recently shown that donor NK cells from transplanted allogeneic bone marrow are able to eliminate host APCs, preventing donor T cell activation and the consequent graft-vs-host reaction (45). Therefore, our present observations should be taken into consideration in situations where NK cell-inactivating strategies are proposed to improve transplantation tolerance of allogeneic organs (37). According to our data, inactivation of both CD8 T cells and NK cells would promote allospecific CD4 T cell priming and type 2 cytokine production, resulting in an alternative pathway of solid organ rejection involving eosinophils (27, 28, 31) rather than transplantation rejection. We hypothesize that NK cells and/or CD8 T cells may limit the flux of graft-derived DCs and/or their kinetics of differentiation in immune lymph nodes, thereby preventing alloreactive CD4 T cell activation by a subset of terminally differentiated DCs displaying Th2-prone capacity. Current experiments are in progress to address this issue.

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