NK cells contribute to the skin graft rejection promoted by CD4\(^+\) T cells activated through the indirect allorecognition pathway

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Abstract

Rejection of solid organ allografts is promoted by T cells. Recipient T cells can directly recognize intact allo-MHC molecules on donor cells and can also indirectly recognize processed donor-derived allo-peptides presented by recipient antigen-presenting cells in the context of self-MHC molecules. Although CD4\(^+\) T cells primed through the indirect allorecognition pathway alone are sufficient to promote acute allograft rejection, it is unknown how they can mediate graft destruction without cognate recognition of donor cells. In this study, we analyzed the indirect effector mechanism of skin allograft rejection using a mouse model in which SCID recipients bearing MHC class II-deficient skin allografts were adoptively transferred with CD4\(^+\) T cells. Histologically, entire graft necrosis was preceded by mononuclear cell infiltration in the graft epithelia with epithelial cell apoptosis, indicating cell-mediated cytotoxicity against donor cells as an effector mechanism. Beside CD4\(^+\) T cells and macrophages, NK cells infiltrated in the rejecting grafts. Depletion of NK cells as well as blocking of the activating NK receptor NKG2D allowed prolonged survival of the grafts. Expression of NKG2D ligands was up-regulated in the rejecting grafts. These results suggest that NK cells activated through NKG2D contribute to the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells.

Introduction

Rejection of solid organ allografts is promoted by recipient T cells recognizing donor allo-antigens. Allorecognition can occur by two distinct, but not mutually exclusive, pathways (1). In the direct pathway, recipient T cells recognize intact allo-MHC molecules on donor antigen-presenting cells (APCs). In the indirect pathway, recipient T cells recognize donor-derived allo-peptides that have been processed and presented by recipient APCs in the context of self-MHC molecules. The direct pathway of sensitization generates CD4\(^+\) and CD8\(^+\) effector CTLs, either of which alone can mediate acute allograft rejection through cognate recognition of graft parenchymal cells (2). In addition, CD4\(^+\) T cells primed through the direct pathway provide help for CD8\(^+\) CTL generation and activate cells of the innate immune system, particularly macrophages and eosinophils, which may also contribute to the effector phase of allograft rejection (2).

CD4\(^+\) T cells primed through the indirect pathway were also shown to provide help for CD8\(^+\) CTLs (3). There was compelling evidence that indirectly primed CD4\(^+\) T cells alone are sufficient to promote skin allograft rejection (4–9). In this setting, however, graft destruction cannot be mediated by direct cytotoxicity of the effector CD4\(^+\) T cells through cognate recognition of graft parenchymal cells, because the determinants recognized by indirectly primed CD4\(^+\) T cells were not expressed on donor cells (4–9). Macrophage-dependent delayed-type hypersensitivity (DTH) and eosinophil-mediated tissue damage were suggested as the indirect effector mechanisms (2, 6–9). In addition, other cells of the innate immune system including NK cells and...
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neutrophils may also participate in allograft rejection (10–15). However, the role for the innate immune system in the skin allograft rejection promoted by indirectly primed CD4+ T cells has not been firmly established yet.

It was reported that indirectly primed (cross-primed) CD8+ T cells can also promote skin allograft rejection by themselves (16, 17). These studies demonstrated that the cross-primed CD8+ T cells mediate skin allograft rejection through cognate recognition of vascular endothelial cells of recipient-derived blood vessels that present donor allo-antigens in the context of self-MHC molecules (16, 17). The cross-primed CD8+ effector CTLs damage recipient-derived blood vessels feeding the grafts through direct cytotoxicity that eventually results in ischemic injury leading to graft necrosis (16, 17). It remains unknown, however, whether similar effector mechanism could be operative also in the skin allograft rejection promoted by indirectly primed CD4+ T cell, because vascular endothelial cells can directly activate CD8+ T cells (18) but are poor APCs for CD4+ T cells (19).

In this study, we analyzed the effector mechanism of skin allograft rejection promoted by indirectly primed CD4+ T cells. Here we show that skin allograft rejection promoted by indirectly primed CD4+ T cells is primarily mediated by NK cells rather than by other cells of the innate immune system or by ischemic injury through vascular damage. Indirectly primed CD4+ T cells may trigger activation of NK cells through the activating NK receptor NKG2D in the allografts by up-regulating NKG2D ligands on donor cells.

Materials and methods

Mice

C57BL/6 (H-2b) and BALB/c (H-2d) mice were purchased from Charles River Japan (Yokohama, Japan). C.B-17/scid/scid (SCID) mice (H-2d) were obtained from CLEA Japan (Tokyo, Japan). B6.129S-H2Kd1-Ea (MHC class II-deficient) mice (20) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) according to manufacturer’s instructions. Female mice were used at 6–10 weeks of age and maintained on a C57BL/6 background. Female mice were used at 6–10 weeks of age.

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Skin grafting, CD4+ T-cell purification and adoptive transfer

SCID mice were transplanted with full-thickness trunk skin allografts from MHC class II-deficient or wild-type C57BL/6 mice as described (21). In some experiments, alloimmune (MHC class II-deficient) and syngeneic control (BALB/c) skin grafts were placed side by side on the same recipient mice. The skin grafts were allowed to heal for at least 14 days before CD4+ T-cell reconstitution.

CD4+ T cells were prepared from BALB/c spleen cells using Mouse T Cell CD4 Subset Column Kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer’s instructions. The purity of the CD4+ T cells was >92% and contaminating CD8+ T cells were <1%, as determined by flow cytometry.

The SCID recipients bearing healthy skin grafts were reconstituted with $1 \times 10^7$ purified CD4+ T cells. The skin grafts were observed daily and defined as rejected when complete loss of the intact epithelia occurred. At the time of rejection, spleen cells were harvested and analyzed by flow cytometry to confirm the CD4+ T-cell reconstitution without detectable CD8+ T cells (<1%).

In vivo NK-cell depletion and NKG2D-blocking

To deplete NK cells in vivo, recipient mice were injected intra-peritoneally (i.p.) with 200 μg of rabbit anti-asialo GM1 antibody (Wako Chemicals, Osaka, Japan) or control rabbit Ig every third day from 2 days before CD4+ T-cell reconstitution until rejection. NK-cell depletion was confirmed by flow cytometric analysis of peripheral blood lymphocytes and spleen cells.

To block NKG2D in vivo, recipient mice were injected i.p. with 250 μg of neutralizing hamster anti-mouse NKG2D mAb (clone HMG2D) or control hamster Ig twice a week from 2 days after CD4+ T-cell reconstitution until rejection. The HMG2D mAb was generated by immunizing an Armenian hamster with mouse NKG2D-Fc fusion protein (R&D Systems). This mAb blocks NKG2D-mediated killing of retinoic acid early inducible-1 (RAE-1)-transfected RMA cells by NK cells in vitro and reverses the rejection of RAE-1-transfected RMA cells in C57BL/6 mice in vivo (H. Yagita, unpublished data). NK cells were not depleted by in vivo treatment with the HMG2D mAb (Supplementary Figure 1, available at International Immunology Online).

Histology

The skin grafts were dissected 3, 10, 14, 16, 18 and 22 days after CD4+ T-cell reconstitution or 14 and 100 days after transplantation on unconstituted SCID mice. Formalin-fixed paraffin sections were stained with hematoxylin and eosin. Apoptosis was analyzed by TUNEL assay using In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany).

Graft-infiltrating cells

Graft-infiltrating cells were isolated as described previously (22). Briefly, the skin grafts were dissected 14 days after CD4+ T-cell reconstitution and digested in Iscove’s modified Dulbecco’s medium containing 0.1 mg ml$^{-1}$ DNase I, 3.3 mg ml$^{-1}$ collagenase type I and 1 mg ml$^{-1}$ hyaluronidase type IV-S (all from Sigma-Aldrich, St Louis, MO, USA) in a shaking water bath at 37°C for 90 min. The digested skin was sequentially filtered through 70- and 30-μm nylon meshes.

Flow cytometry

Cells were re-suspended in PBS supplemented with 2% FCS and 0.1% NaN3. After pre-incubation with anti-CD16/32 mAb (clone 2.4G2; BD Biosciences, San Jose, CA, USA), cells were stained with saturating amounts of the following mAbs: b). T, PE-conjugated anti-CD4 (H129.19, BD Biosciences), FITC-conjugated anti-CD8a (53-6.7, BD Biosciences), b). T, PE-conjugated anti-CD49b (DX5, BD Biosciences), b). T, conjugated anti-CD4 (clone 2.4G2, BD Biosciences) and FITC- or PE-conjugated isotype control. Graft-infiltrating cells were analyzed by FACScan flow cytometry.
control mAbs. Biotin-conjugated mAbs were visualized with streptavidin–PE (Southern Biotechnology, Birmingham, AL, USA) or streptavidin–Quantum Red conjugate (Sigma-Aldrich). After gating on forward and side scatters and propidium iodide, viable cells were analyzed using the FACScan flow cytometer with the CellQuest Pro software (BD Biosciences).

RT–PCR
Total RNA was extracted from the cells and tissues and reverse transcribed using random hexamers (23). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression levels in each cDNA preparations were semi-quantified as described (23). The amount of cDNA was normalized according to the GAPDH mRNA levels, and fold serial dilutions of cDNA were amplified using RAE-1-specific primers. The GAPDH and RAE-1 primers were described elsewhere (23, 24). PCR conditions for 30 cycles were 94°C for 45 s, 55°C for 45 s and 72°C for 1.5 min. PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide. RAW264.7 (25) and Pam 212 (23) tumor cells were used as positive and negative controls for RAE-1 expression, respectively.

Statistical analysis
Differences in the numbers of graft-infiltrating-cell subpopulations were evaluated by t-test. Mann–Whitney’s U-test was used to compare the graft survival time. Differences were considered significant if two-tailed P-values were <0.05. All statistical analyses were performed using the JMP software (SAS Institute, Cary, NC, USA).

Results
Skin allograft rejection promoted by indirectly primed CD4+ T cells exhibits histological features indicating cell-mediated cytotoxicity against graft epithelial cells as an effector mechanism
To analyze the effector mechanism of skin allograft rejection promoted by indirectly primed CD4+ T cells, we used an adoptive transfer model with SCID mice bearing MHC class II-deficient skin allografts (5), in which graft rejection is mediated exclusively by transferred CD4+ T cells that can recognize allo-antigens only through the indirect pathway. In the original report (5), adoptive transfer of 5 × 10⁶ CD4+ T cells into SCID recipients bearing MHC class II-deficient skin allografts was shown to cause slow rejection or sometimes to be insufficient for rejection. We confirmed that this was, at least in part, because of the insufficient reconstitution of SCID recipients by CD4+ T cells (data not shown). Therefore, we increased the number of transferred CD4+ T cells in this study. When we transferred 1 × 10⁷ CD4+ T cells, all SCID recipients were successfully reconstituted by CD4+ T cells and all grafts were uniformly rejected within 22 days, while all grafts survived indefinitely (>100 days) in unreconstituted SCID mice. Macroscopic rejection based on visual inspection usually started 16–18 days after CD4+ T-cell reconstitution, at that time entire graft necrosis was observed histologically (data not shown). Histological analysis of the rejecting allografts at an earlier time point (14 days after CD4+ T-cell reconstitution) revealed epidermal thickening, mononuclear cell infiltration in the graft epidermis and hair follicles and epithelial cell apoptosis (Fig. 1A). Eosinophils or neutrophils were not observed in the rejecting allografts (Fig. 1A). Syngeneic control skin grafts placed on the same recipient mice as well as surviving allografts on unreconstituted SCID mice exhibited essentially normal histology (Fig. 1B and C). Because entire graft necrosis was preceded by intra-epithelial infiltrates with epithelial cell apoptosis, it is unlikely that the allograft rejection was primarily mediated by DTH or ischemic injury through vascular damage. Rather, these histological findings indicate cell-mediated direct cytotoxicity against graft epithelial cells as an effector mechanism.

NK cells infiltrate in the rejecting skin allografts
To identify the cytotoxic effector cells other than CD4+ T cells that mediated epithelial cell damage, we analyzed graft-infiltrating cells isolated from the rejecting skin allografts 14 days after CD4+ T-cell reconstitution. Flow cytometric analysis of the graft-infiltrating cells revealed that beside CD4+ T cells and macrophages, considerable numbers of NK cells infiltrated in the rejecting allografts (Fig. 2, upper panels). No CD8+ T cells were detected in the graft-infiltrating cells (data not shown).

In vivo depletion of NK cells delays the skin allograft rejection promoted by indirectly primed CD4+ T cells
Recent studies demonstrated contribution of NK cells to rejection of vascularized cardiac and liver allografts (12–15). To determine whether NK cells also participate in the skin allograft rejection promoted by indirectly primed CD4+ T cells, NK cells were depleted by in vivo treatment with anti-asialo GM1 antibody. Survival of the skin allografts from wild-type mice, which can be recognized by CD4+ T cells through both direct and indirect pathways, was not affected by NK-cell depletion (Fig. 3A). In contrast, survival of MHC class II-deficient skin allografts, which can be recognized by CD4+ T cells only through the indirect pathway, was significantly prolonged by NK-cell depletion (Fig. 3B).

Although anti-asialo GM1 antibody reacts not only with NK cells but also with a subset of alloreactive CTLs (26), flow cytometric analysis of the graft-infiltrating cells revealed that NK cells were selectively depleted by the in vivo treatment with anti-asialo GM1 antibody (Fig. 2, middle panels). Despite nearly complete depletion of NK cells in the peripheral blood and spleen of anti-asialo GM1 antibody-treated mice (data not shown), a small number of NK cells could still be detected in the skin allografts (Fig. 2, middle panel). The incomplete depletion of NK cells may explain the prolonged survival, but not permanent acceptance, of the grafts by the in vivo treatment with anti-asialo GM1 antibody (Fig. 3B). Supporting this, the rejecting allografts in anti-asialo GM1 antibody-treated mice exhibited histological features similar to that of untreated mice (Supplementary Figure 2, available at International Immunology Online). Alternatively, other cells of the innate immune system, in particular, macrophages, which infiltrated
in the rejecting allografts (Fig. 2), might also contribute to the graft rejection as suggested by the prior studies (6–9). Nevertheless, these results indicate that NK cells play an essential role in the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells.

**In vivo blocking of NKG2D delays the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells**

Activation of NK cells is regulated by the balance between positive and negative signals through various activating and inhibitory receptors (27). Recent studies demonstrated critical roles for the activating NK receptor NKG2D (28) in NK-cell-mediated rejection of bone marrow grafts (29) as well as in vascularized cardiac allograft rejection in the absence of CD28-co-stimulation (30) that is mediated by NK cells and CD4\(^+\) and CD8\(^+\) T cells (12, 13, 31). To determine the role for NKG2D in NK-cell activation in the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells, NKG2D was blocked by *in vivo* treatment with neutralizing anti-NKG2D mAb. As shown in Fig. 4, NKG2D-blocking significantly prolonged survival of MHC class II-deficient skin allografts in SCID recipients reconstituted with CD4\(^+\) T cells. *In vivo* treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (Fig. 2, lower panels). Therefore, NKG2D plays a critical role in NK-cell activation also in the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells. As similar to NK-cell depletion, NKG2D-blocking could not induce permanent graft acceptance (Fig. 4), probably because of incomplete blocking by the *in vivo* antibody treatment, involvement of activating NK receptors other than NKG2D or contribution of effector cells other than NK cells.

**Expression of NKG2D ligands is up-regulated in the rejecting skin allografts**

In mice, members of the RAE-1 protein family, minor histocompatibility antigen H60 and murine UL-16-binding protein-like transcript-1 act as ligands for NKG2D (28, 32). Most normal healthy tissues including the skin do not express NKG2D ligands, but their expression is up-regulated in a wide variety of conditions (24, 28, 32). Expression of NKG2D ligands was demonstrated in human renal and pancreatic allografts (33) as well as in mouse skin and cardiac allografts (30, 34). To determine whether NKG2D ligands are also induced in the rejecting skin allografts in our model, we analyzed RAE-1 mRNA expression, because MHC class II-deficient donor mice on a C57BL/6 background can express RAE-1\(\alpha\) and RAE-1\(\epsilon\), but not H60, and expression of murine UL-16-binding protein-like transcript-1 is regulated at a level other than transcription (28, 32). RT-PCR using the primers capable of amplifying all five RAE-1 family members revealed that before CD4\(^+\) T-cell reconstitution, RAE-1 was not expressed in the allografts or syngeneic grafts placed on the same recipient mice (Fig. 5). Up-regulation of RAE-1 expression was observed only in the rejecting allografts 10–14 days after CD4\(^+\) T-cell reconstitution, but not in the syngeneic grafts placed on the same recipient mice (Fig. 5).
In this study, we have shown that NK cells contribute to the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells and that NKG2D plays an essential role in the NK-cell activation in the rejecting allografts. Although the crucial role for NK cells in rejection of bone marrow grafts is well established, involvement of NK cells in rejection of solid organ allografts has been shown only in limited studies (10–15). Among these studies, the most compelling evidence for NK-cell contribution in acute allograft rejection was provided by the vascularized cardiac allograft model in CD28-deficient mice (12, 13). The cardiac allograft rejection in CD28-deficient mice depends on NK cells (12, 13) as well as both CD4\(^+\) and CD8\(^+\) T cells (31), but the precise role for NK cells in this model is unclear. It was proposed that NK cells may participate in the sensitization phase of rejection by providing help for CTL generation that in normal mice would be provided by CD28 (10–13). Using the same model, Kim et al. (30) recently demonstrated that in vivo treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (lower panels). Representative profiles from three independent experiments are shown. Mean ± SD (%) of each sub-populations (control versus anti-asialo GM1 antibody-treated versus anti-NKG2D mAb-treated mice, \(n = 3\)) were 24 ± 6 versus 31 ± 8 versus 24 ± 9 for CD4\(^+\) T cells, 26 ± 4 versus 25 ± 5 versus 26 ± 5 for macrophages. Statistically significant difference in sub-population of the graft-infiltrating cells was evident only for NK cells in control versus anti-asialo GM1 antibody-treated mice (\(P = 0.008\)).

**Fig. 2.** Graft-infiltrating cells. Graft-infiltrating cells were isolated from MHC class II-deficient allografts on SCID recipients 14 days after CD4\(^+\) T-cell reconstitution. Cells were stained with streptavidin–PE alone (negative control) or biotin-conjugated anti-CD4, anti-CD49b and anti-F4/80 mAbs. In control mice without antibody treatment, graft-infiltrating cells consisted of CD4\(^+\) T cells, CD49b\(^+\) NK cells and F4/80\(^+\) macrophages (upper panels). In vivo treatment with anti-asialo GM1 antibody selectively depleted NK cells in the graft-infiltrating cells (middle panels). In vivo treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (lower panels). Representative profiles from three independent experiments are shown. Mean ± SD (%) of each sub-populations (control versus anti-asialo GM1 antibody-treated versus anti-NKG2D mAb-treated mice, \(n = 3\)) were 24 ± 6 versus 31 ± 8 versus 24 ± 9 for CD4\(^+\) T cells, 26 ± 4 versus 25 ± 5 versus 26 ± 5 for macrophages. Statistically significant difference in sub-population of the graft-infiltrating cells was evident only for NK cells in control versus anti-asialo GM1 antibody-treated mice (\(P = 0.008\)).

**Discussion**

In this study, we have shown that NK cells contribute to the skin allograft rejection promoted by indirectly primed CD4\(^+\) T cells and that NKG2D plays an essential role in the NK-cell activation in the rejecting allografts.

Although the crucial role for NK cells in rejection of bone marrow grafts is well established, involvement of NK cells in rejection of solid organ allografts has been shown only in limited studies (10–15). Among these studies, the most compelling evidence for NK-cell contribution in acute allograft rejection was provided by the vascularized cardiac allograft model in CD28-deficient mice (12, 13). The cardiac allograft rejection in CD28-deficient mice depends on NK cells (12, 13) as well as both CD4\(^+\) and CD8\(^+\) T cells (31), but the precise role for NK cells in this model is unclear. It was proposed that NK cells may participate in the sensitization phase of rejection by providing help for CTL generation that in normal mice would be provided by CD28 (10–13). Using the same model, Kim et al. (30) recently demonstrated that in vivo treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (lower panels). Representative profiles from three independent experiments are shown. Mean ± SD (%) of each sub-populations (control versus anti-asialo GM1 antibody-treated versus anti-NKG2D mAb-treated mice, \(n = 3\)) were 24 ± 6 versus 31 ± 8 versus 24 ± 9 for CD4\(^+\) T cells, 26 ± 4 versus 25 ± 5 versus 26 ± 5 for macrophages. Statistically significant difference in sub-population of the graft-infiltrating cells was evident only for NK cells in control versus anti-asialo GM1 antibody-treated mice (\(P = 0.008\)).

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after transplantation alone cannot lead to NK-cell-mediated graft rejection in unreconstituted SCID recipients. Therefore, sustained expression of NKG2D ligands in the allografts that is mediated by T-cell-dependent adaptive immune response may be required for NK-cell activation leading to graft destruction. In our model, indirectly primed CD4+ T cells may induce inflammation by DTH and/or ischemic injury through vascular damage that may up-regulate and maintain the expression of NKG2D ligands in the allografts. It is also possible that inflammation induced by indirectly primed CD4+ T cells may be required for recruitment of NK cells into the allografts in addition to NK-cell activation through up-regulation of NKG2D ligands on donor cells.

In summary, this study demonstrates that indirectly primed CD4+ T cells, which cannot directly interact with donor cells, can induce sustained expression of NKG2D ligands in the skin allografts. This may trigger activation of NK-cell cytotoxicity against graft epithelial cells through NKG2D that eventually results in graft destruction. This conclusion implies that NK-cell depletion or NKG2D-blocking may be an additional strategy for the therapeutic intervention of allograft rejection. However, contribution of NK cells to tolerance induction rather than graft rejection has also been demonstrated in the prior studies (10, 11). Another implication of this study is that CD4+ T cells may collaborate with NK cells to eliminate infected or tumor cells with which CD4+ T cells cannot directly interact due to down-regulation or lack of MHC class II molecules, because the indirect pathway of allorecognition is analogous to the physiological pathway of foreign antigen recognition.

Supplementary data

Supplementary figures are available at International Immunology Online.

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Abbreviations

APC antigen-presenting cell
DTH delayed-type hypersensitivity
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