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The Activating Immunoreceptor NKG2D and Its Ligands Are Involved in Allograft Transplant Rejection

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Although the linkage between innate and adaptive immunity in transplantation has been recognized, the mechanisms underlying this cooperation remain to be fully elucidated. In this study, we show that early “danger” signals associated with transplantation lead to rapid up-regulation of NKG2D ligands. A second wave of NKG2D ligand up-regulation is mediated by the adaptive immune response to allografts. Treatment with an Ab to NKG2D was highly effective in preventing CD28-independent rejection of cardiac allografts. Notably, NKG2D blockade did not deplete CD8+ T cells or NK1.1+ cells nor affect their migration to the allografts. These results establish a functional role of NKG2D and its ligands in the rejection of solid organ transplants. The Journal of Immunology, 2007, 179: 6416–6420.

An emerging concept in the field of transplantation is the linkage between innate and adaptive immunity (1, 2). The “danger” signals of surgical trauma and ischemia/reperfusion injury have been shown to induce a spectrum of responses within the host and the graft that ultimately augments the alloimmune response and prevent the induction of tolerance (3, 4). A family of MHC class I-related proteins has been recently identified that is up-regulated by a variety of conditions such as cellular stress, heat shock, infection, DNA damage, or transformation (5, 6). This family includes the proteins RAE-1, Mult1, and H60. These proteins bind to the activating receptor NKG2D, which is expressed on the cell surface of NK cells and CD8+ T cells (7). Expression of RAE-1 on transplanted tumor cells markedly increases the ability of a host’s NK cells to kill these targets (8).

Blockade of NKG2D with a neutralizing, nondepleting anti-NKG2D mAb has been shown to prevent NK cell-mediated bone marrow rejection in certain mouse strains, to enhance chemically induced tumor formation, and to prevent autoimmune diabetes in nonobese diabetic mice (8–10), demonstrating a role for NKG2D in these processes (11). The regulation of NKG2D ligands and the role of NKG2D in the setting of solid organ transplantation, however, are largely unknown.

In this study, we demonstrate that NKG2D ligands are up-regulated in a biphasic fashion in tissue grafts. The early phase appears to be solely dependent on the innate immune response, whereas the later phase is dependent on the adaptive immune response. Blockade of NKG2D, in the absence of CD28 or in the context of B7 blockade, results in striking prolongation of cardiac allograft survival. Our results establish a role for NKG2D and its ligands in solid organ allograft rejection.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from the National Cancer Institute or Jackson Laboratories. C57BL/6 Cd28−/− mice were obtained from Dr. J. Allison (University of California, Berkeley) and used at 10–14 wk of age. All mice were housed in a specific pathogen-free facility at University of California San Francisco (UCSF). All experimental procedures strictly adhered to protocol guidelines set forth by the UCSF Institutional Animal Care and Use Committee.

Reagents and Abs

Anti-mouse NKG2D mAb (CX5) was generated as previously described (12). Control rat IgG was purchased from Sigma-Aldrich. All purified Abs used for injections did not contain detectable endotoxin (<0.3 pg/injection). Mice were administered either 250 μg of CX5 or control rat IgG by i.p. injection on the day before transplant (day −1) and retro-orbital injections twice weekly for 4 consecutive weeks starting on day 3. Human CTLA4-Ig was kindly provided by Bristol-Meyers (13), 200 μg CTLA4-Ig were administered by i.p. injections on the day of transplant (day 0) and retro-orbital injections on days 3, 7, and 10.

Transplants

Syngeneic and allogeneic full-thickness tail-skin grafts were transplanted onto the dorsal thorax of C57BL/6 mice. Grafts were secured with 6-0 silk sutures placed at the corners and covered with Xeroform gauze and an adhesive bandage. RNA from skin grafts was extracted for quantitative real-time PCR analysis on days 0, 1, 3, 5, 7, and 9. Hearts were transplanted heterotopically into the abdomen, as previously described (14), and were monitored by transabdominal palpation on subsequent days. Rejection was defined as cessation of palpable contractions for 2 consecutive days and verified visually by laparotomy. Additional syngeneic and allogeneic heterotopic cardiac grafts in C57BL/6 mice were harvested on days 0, 3, 5, and 9 for real-time PCR analysis. For flow cytometric analysis, allografts were harvested at days 7–10.

Antibodies and flow cytometry

For detection of NKG2D, cells were stained with biotinylated anti-NKG2D (clone CX5) and APC-Cy7-conjugated streptavidin. Cells were costained for detection of NKG2D, cells were stained with biotinylated anti-NKG2D (clone CX5) and APC-Cy7-conjugated streptavidin. Cells were costained for 3 Address correspondence and reprint requests to Dr. Sang-Mo Kang, University of California, 513 Parnassus Avenue, Box 0780, San Francisco, CA 94143. E-mail address: kangs@surgery.ucsf.edu

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with FITC-conjugated anti-CD8 and PE-conjugated anti-NK1.1 (BD Pharmingen). To verify modulation of NKG2D after treatment, cells were stained 24 and 72 h with FITC-conjugated anti-NK1.1 and biotinylated anti-NKG2D (clone CX5 or clone MI-6) and APC-Cy7-conjugated streptavidin. Flow cytometry was performed on a FACSCalibur (BD Immuno-cytometry Systems) and data were analyzed using CellQuest software (BD Biosciences).

Quantitative RT-PCR

RT-PCR was performed using an ABI 7300 real-time PCR system (Applied Biosystems) instrument, according to the manufacturer’s instructions. Pan RAE-1-specific probes and primers (recognizing all known RAE-1 alleles) were used as previously described (12): sense, 5’-CTAGTGCCA CCTGGGAATTCA; anti-sense, 5’-CATCATTAGCTGATCTCCAGC TCA-3’; and the probe was 5’-FAM-CATCAGTGACAGTTACTTC TTCACCTTCTACACAGAGA-Tamra-3’. Mult1 primers used were: sense, 5’-GAGCCACCGAGAAGGCA-3’; anti-sense, 5’-CCAGTAT GGTCCCCAGATAGCT-3’; and the probe was 5’-VIC-TTGCCTGAT TCTGAGCCTTTTCATTCTGCT-Tamra-3’. H60 primers used were: sense, 5’-GAGCCACCGAGAAGGCA-3’; anti-sense, CCAGTAT GGTCCCCAGATAGCT-3’; and the probe was 5’-VIC-TTGCCTGAT TCTGAGCCTTTTCATTCTGCT-Tamra-3’. Total RNA was extracted using the RNeasy fibrous tissue mini kit from Qiagen according to the manufacturer’s protocol. cDNA was generated by using random hexamer primers and reagents purchased from Invitrogen Life Technologies. The cycling conditions for real-time PCR were: 50°C for 10 min, followed by 45 cycles at 95°C for 30 s and 60°C for 2 min. Data were analyzed by using the Sequence Detector v1.7 Analysis Software (Applied Biosystems).

Statistical analysis

PCR and flow cytometry results are expressed as the mean ± SD. Survival data are expressed as the mean with 95% confidence intervals (CI).4 Group comparisons were performed using the Student’s t test for survival data and Student’s t test for all other data. Significant differences were noted to be p < 0.05.

Results

Transplantation induces expression of NKG2D ligands in allografts

To examine whether NKG2D ligands are induced after transplantation, quantitative RT-PCR was used to detect expression of RAE-1 transcripts after full-thickness skin transplants in C57BL/6

FIGURE 1. Up-regulation of NKG2D ligand transcripts in transplants. A, Relative expression of RAE-1 transcripts (all isoforms) in syngeneic (C57BL/6) and allogeneic (BALB/c) full-thickness tail-skin grafts posttransplant. B, Regulation of RAE-1 transcripts in vascularized cardiac BALB/c allografts vs syngeneic grafts posttransplant. C, Mult1 transcripts in heart allografts vs syngeneic grafts after transplantation. D, H60 transcripts in BALB/c allografts. E, Late up-regulation of RAE-1 transcripts requires the adaptive immune response. Syngeneic C57BL/6 and allogeneic BALB/c skin transplants in C57BL/6 Rag1−/− recipients showed no difference in relative expression of RAE-1. Normalized RAE-1 expression (compared with nontransplanted control) ± SD. Representative data shown from a minimum of three independent experiments. *, p < 0.05 allogeneic vs syngeneic groups using Student’s t test.

4 Abbreviations used in this paper: CI, confidence interval; MST, mean survival time.
hosts (Fig. 1A). The expression of RAE-1 in the grafts was detected by quantitative RT-PCR because Abs suitable for use in immunohistochemistry are not available. RAE-1 transcript levels in syngeneic skin transplants exhibited an early phase of induction, peaking at day 3 and decreased by day 5. RAE-1 transcript levels in BALB/c allografts exhibited a similar early phase of induction, which also decreased by day 5. By day 7, however, RAE-1 transcripts in allografts were again up-regulated and continued to increase on day 9, corresponding to obvious signs of alloimmune rejection. RAE-1 transcripts in heart allografts were up-regulated in a similar biphasic fashion, with far greater expression by day 9 than in the syngeneic grafts (Fig. 1B). Mult1 and H60 transcripts were also up-regulated, albeit to a lesser degree than RAE-1 transcripts, in both cardiac allografts (Fig. 1, C and D) and skin allografts (not shown). Notably, H60 is not expressed by the C57BL/6 mouse strain; thus, all of the H60 up-regulation observed in the BALB/c allografts is due to induction on donor tissue, rather than by infiltration of host-derived cells.

Secondary expression of RAE-1 is due to the adaptive immune response

To investigate whether the late increase in RAE-1 transcription posttransplantation was caused by the adaptive immune system, we used Rag1−/− mice, which are deficient in T cells and B cells, as graft recipients. The early phase of RAE-1 induction was identical in syngeneic and allogeneic grafts, whether or not the grafts were placed onto Rag1+/+ or Rag1−/− recipients (Fig. 1E). Thus, the early phase of RAE-1 induction does not depend on T or B cells; however, the late rise in RAE-1 expression in allogeneic grafts was absent in Rag1−/− hosts (Fig. 1E). The late rise in RAE-1 expression is therefore dependent on the adaptive immune response. Reagents to detect RAE-1 by immunohistochemistry are not available; therefore, the cellular source of RAE-1 protein in the graft could not be determined, but conceivably might include both nonhemopoietic tissue cells as well as immune cells because activated lymphocytes and myeloid cells have previously been shown to express RAE-1.

Anti-NKG2D mAb prevents acute cardiac allograft rejection in Cd28−/− mice

The up-regulation of NKG2D ligands in allografts suggested that NKG2D might participate in allograft rejection. NKG2D, by virtue of its expression on activated CD8+ T cells and NK cells (15), could participate in the adaptive as well as the innate immune response to allografts. To test this hypothesis, we used the

FIGURE 2. Treatment in vivo with anti-NKG2D mAb prolongs cardiac allograft survival in Cd28−/− mice. Kaplan-Meier survival curve of BALB/c cardiac graft survival in days for C57BL/6 Cd28−/− mice treated with neutralizing anti-NKG2D mAb (n = 7) (250 μg CX5 twice weekly for 4 wk), control IgG (n = 5) or no Ab (n = 4). Duration of treatment is indicated by the shaded area in the figure. p < 0.005, anti-NKG2D mAb vs control IgG or no Ab groups by Mann-Whitney U test.

NKG2D IN TRANSPLANT REJECTION

This was then confirmed by a similar experiment using the representative mouse strain, which has been shown to be dependent on CD8+ T cells and NK cells for rejection (16, 17). C57BL/6 Cd28−/− mice were treated with a neutralizing anti-NKG2D mAb (CX5) for 4 consecutive weeks following transplant with BALB/c vascularized cardiac allografts (Fig. 2). Anti-NKG2D mAb-treated mice demonstrated a significantly greater
Anti-NKG2D treatment does not deplete CD8\textsuperscript{+} T cells or NK1.1\textsuperscript{+} cells

To assess the mechanism of anti-NKG2D-mediated prolongation in allograft survival, we analyzed peripheral and allograft-infiltrating leukocytes by flow cytometry (Fig. 3). In untreated mice, a population of activated CD8\textsuperscript{+} T cells expressing NKG2D was induced by transplantation (Fig. 3A). Anti-NKG2D treatment markedly diminished the proportion of NKG2D\textsuperscript{+} CD8\textsuperscript{+} T cells and NK1.1\textsuperscript{+} cells (Fig. 3A, top panels). Anti-NKG2D treatment did not deplete either CD8\textsuperscript{+} cells or NK1.1\textsuperscript{+} cells (Fig. 3A, bottom panel). The number of CD8\textsuperscript{+} T cells and NK1.1\textsuperscript{+} cells infiltrating the cardiac graft after transplantation was also unaffected by anti-NKG2D treatment (Fig. 3B). To rule out the possibility that the observed decrease in NKG2D expression was due to saturation of NKG2D by the treatment Ab, CX5-treated animals were analyzed using either the treatment Ab (CX5) or another anti-NKG2D Ab (MI-6) that recognizes an independent epitope (Fig. 3C). NK1.1\textsuperscript{+} cells from CX5-treated animals had reduced staining with both CX5 and MI-6. Thus, anti-NKG2D mAb treatment appeared to internalize or down modulate NKG2D expression on CD8\textsuperscript{+} cells and NK1.1\textsuperscript{+} cells, as demonstrated previously (10, 12, 19).

Anti-NKG2D treatment with CTLA4-Ig in C57BL/6 mice

To assess the effect of NKG2D blockade in a more clinically relevant model, we tested the ability of anti-NKG2D mAb to prolong allograft survival in wild-type mice, in conjunction with B7 blockade with the fusion protein CTLA4-Ig. Prior studies have demonstrated that CTLA4-Ig can prolong the survival of heart allografts (20, 21). We treated wild-type C57BL/6 mice with CTLA4-Ig alone or with both CTLA4-Ig and anti-NKG2D mAb (Fig. 4). Treatment with CTLA4-Ig alone, at the dose used in these experiments, extended fully allogeneic BALB/c heart allograft survival by 22 days (Fig. 4). Anti-NKG2D mAb treatment alone had no effect on heart allograft survival in wild-type recipients; however, the addition of anti-NKG2D mAb treatment to CTLA4-Ig extended heart allograft survival by an additional 52 days (p < 0.005, CTLA4-Ig vs CTLA4-Ig plus anti-NKG2D).

Discussion

Our results demonstrate that NKG2D ligands are up-regulated after transplantation, and that NKG2D\textsuperscript{+} NK1.1\textsuperscript{+} cells and CD8\textsuperscript{+} T cells infiltrate the graft. Blockade of NKG2D prolonged cardiac allograft survival in CD28-deficient mice and in wild-type mice if combined with B7 blockade, representing the first demonstration of a functional role for NKG2D-NKG2D ligand interactions in the alloimmune response against solid organ grafts. Blockade of NKG2D did not deplete CD8\textsuperscript{+} T cells or NK cells, nor prevent their infiltration into the cardiac allografts, suggesting that NKG2D blockade primarily inhibits effector function, rather than trafficking of lymphocytes to the allograft.

Early up-regulation of NKG2D ligands was similar in syngeneic and allogeneic grafts, suggesting that early up-regulation was due to nonspecific trauma and/or ischemia/reperfusion injury. In contrast, the late up-regulation of NKG2D ligands seen in the allogeneic grafts appears to be mediated primarily by the adaptive immune response, as demonstrated by the results in Rag1\textsuperscript{−/−} recipients. These data suggest that innate responses alone are insufficient to maintain or increase the expression of NKG2D ligands in transplanted tissue, after the initial trauma and ischemia/reperfusion injury. The adaptive immune response, once primed, appears to provide a later stimulus for NKG2D ligand up-regulation that would likely enhance effector function of both CD8\textsuperscript{+} T cells and NK cells infiltrating the graft. Thus, the NKG2D ligands may act to amplify adaptive responses within the graft, increasing graft injury.

The expression of NKG2D ligand mRNA has been described in human transplant samples under circumstances of acute allograft rejection, chronic allograft nephropathy, and renal acute tubular necrosis (22, 23), making it likely that the NKG2D ligand up-regulation demonstrated in our study is relevant to human transplantation. Notably, although we detected up-regulation of NKG2D ligand transcripts in skin allografts, anti-NKG2D mAb did not prolong survival of BALB/c skin grafts in C57BL/6 Cd28\textsuperscript{−/−} mice (not shown). The resistance of skin graft rejection to NKG2D blockade likely reflects the well-documented differences in susceptibility of skin and cardiac grafts to rejection (24). In the Cd28\textsuperscript{−/−} model, CD8\textsuperscript{+} T cells have been shown to be necessary for cardiac allograft rejection, but are dispensable for skin allograft rejection (25). Thus, it is likely that efficacy of anti-NKG2D treatment will be limited to processes wherein CD8\textsuperscript{+} T cell or NK1.1\textsuperscript{+} cells play an important role.

An unresolved question in our studies is whether the graft prolongation by anti-NKG2D Ab is mediated by NKG2D blockade on CD8\textsuperscript{+} T cells, NK1.1\textsuperscript{+} cells, or both. Within the NK1.1\textsuperscript{+} population, it is likely that NKG2D blockade is most relevant for NK cells, as NKT cells have been shown to be dispensable for cardiac rejection in the Cd28\textsuperscript{−/−} model (26). Systems to distinguish the effects of anti-NKG2D mAb on NK cells vs T cells are not currently available.

Prolonged administration of anti-NKG2D appears to be critical to in vivo efficacy because a previous study (26) that used only two doses of anti-NKG2D mAb found it to be ineffective in the same Cd28\textsuperscript{−/−} cardiac transplant model used in this study. We have similarly found that two doses of anti-NKG2D mAb do not prolong allograft survival in this model (data not shown). The requirement for prolonged treatment is consistent with the prior finding that continued treatment with anti-NKG2D was required to prevent autoimmune diabetes in the NOD diabetes model (10). In this regard, it is possible that extending anti-NKG2D mAb treatment might further prolong allograft survival. Our findings suggest a potential role for NKG2D blockade in clinical transplantation.

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Disclosures
The University of California, San Francisco, has licensed intellectual property rights relating to this research for potential therapeutic development.

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