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Critical, but Conditional, Role of OX40 in Memory T Cell-Mediated Rejection

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Memory T cells can be a significant barrier to the induction of transplant tolerance. However, the molecular pathways that can regulate memory T cell-mediated rejection are poorly defined. In the present study we tested the hypothesis that the novel alternative costimulatory molecules (i.e., ICOS, 4-1BB, OX40, or CD30) may play a critical role in memory T cell activation and memory T cell-mediated rejection. We found that memory T cells, generated by either homeostatic proliferation or donor Ag priming, induced prompt skin allograft rejection regardless of CD28/CD154 blockade. Phenotypic analysis showed that, in contrast to naive T cells, such memory T cells expressed high levels of OX40, 4-1BB, and ICOS on the cell surface. In a skin transplant model in which rejection was mediated by memory T cells, blocking the OX40/OX40 ligand pathway alone did not prolong the skin allograft survival, but blocking OX40 costimulation in combination with CD28/CD154 blockade induced long-term skin allograft survival, and 40% of the recipients accepted their skin allograft for >100 days. In contrast, blocking the ICOS/ICOS ligand and the 4-1BB/4-1BBL pathways alone or combined with CD28/CD154 blockade had no effect in preventing skin allograft rejection. OX40 blockade did not affect the homeostatic proliferation of T cells in vivo, but markedly inhibited the effector functions of memory T cells. Our data demonstrate that memory T cells resisting to CD28/CD154 blockade in transplant rejection are sensitive to OX40 blockade and suggest that OX40 is a key therapeutic target in memory T cell-mediated rejection. The Journal of Immunology, 2006, 176: 1394–1401.

A normal T cell repertoire usually consists of both naive and memory T cells. Compared with their naive counterparts, memory T cells are endowed with special features that make such cells highly efficient in mounting an immune response. Memory T cells are located at both lymphoid and nonlymphoid sites (1), and they express unique homing receptors that can allow them to readily accumulate at inflammatory sites, including graft sites (2–5). Moreover, the memory response is much more intense, and on a per cell basis, memory T cells can generate far more effector T cells than naive T cells (6, 7). Importantly, the roles that govern naive T cell activation do not always apply to memory T cells (8–10). These features are clearly important in protective immunity against invading pathogens. However, the same features may also constitute a significant barrier to the induction of transplant tolerance. Indeed, in several models in which the transplant recipients harbor a high frequency of memory T cells, even memory T cells against conventional pathogens, induction of transplant tolerance proves to be extremely difficult (10).

Phenotypically and functionally, memory T cells are extremely heterogeneous (7, 11). Thus, understanding precisely the memory T cells that are alloreactive and the molecular pathways that can regulate their alloreactivity is becoming a critical issue in transplantation. Several recent studies suggest that certain alternative costimulatory molecules (e.g., ICOS, 4-1BB, OX40, CD30, etc.) may play a role in regulating effector/memory T cell functions. In contrast to CD28, which is constitutively expressed on naive T cells, such alternative costimulatory molecules are expressed only upon T cell activation (12). Their inducible nature of expression suggests that these alternative costimulatory molecules may play a particularly important role in the effector/memory phase of an immune response rather than the initial phase of T cell activation. Indeed, ICOS costimulation has been shown to play a key role in reactivation of effector/memory T cells (13). Also, engagement of CD27/CD70 costimulatory pathway enhances CD8+ effector/memory T cell functions (14, 15). In addition, 4-1BB costimulation has been shown to be critical for both survival and effector functions of memory CD8+ T cells (16). In certain models, dendritic cells expressing 4-1BB ligand can elicit much stronger effector/memory CTL responses than unmanipulated controls (17). More recently, OX40 costimulation has been shown to play a quintessential role in the memory T cell response (18, 19). Despite these observations, very little is known about the role of these alternative costimulatory molecules in regulating memory T cell-mediated rejection. Moreover, it remains uncertain whether signals from these alternative costimulatory molecules are responsible for tolerance resistance.

In the present study we investigated the role of such alternative costimulatory molecules (e.g., ICOS, 4-1BB, OX40, and CD30) in memory T cell-mediated rejection. We used the model of homeostatic proliferation as a tool to generate memory-like T cells (20) and the donor Ag priming method to generate alloantigen-specific memory T cells, and examined the role of such alternative costimulatory molecules in memory T cell-mediated rejection. Our study demonstrates that memory T cells resistant to CD28/CD154...
costimulatory blockade in skin allograft rejection are sensitive to OX40 blockade.

Materials and Methods

Mice

C57BL/6 (H-2b), DBA/2 (H-2h), and Rag-1-deficient mice in the C57BL/6 background were purchased from The Jackson Laboratory. All animals were housed in the animal facility at Beth Israel Deaconess Medical Center. Animal care and use conformed with the guidelines established by the animal care committee at our institution.

Monoclonal Abs and reagents

The following anti-mouse Abs used for cell surface staining were purchased from BD Pharmingen: CyChrome-CD4 (clone GK1.5), CyChrome-CD8α (clone 53-6.7), FITC-CD44 (clone IM7), PE-CD2DL (clone MEL-14), biotin-OX40 (clone OX86), biotin-CD30 (clone mCD30.1), biotin-ICOS (clone 7E.17G9), biotin-4-1BB (clone 1A2H), PE-streptavidin, and isotype control Abs. The anti-mouse IFN-γ capture Ab (clone R4-6A2), biotin-IFN-γ detection Ab (clone XMGI.2), HRP-streptavidin, and PE-anti-mouse Bcl-2 (clone 3F11) were also obtained from BD Pharmingen. PE-anti-mouse Bcl-xL (clone H-5) was obtained from Santa Cruz Biotechnology.

The human CTLA-4g fusion protein was provided by Dr. R. Peach (Bristol-Myers Squibb, Princeton, NJ). Anti-OX40 ligand (anti-OX40L)3 mAb (clone RM134L, rat IgG2b), anti-CD154 mAb (clone MR1, hamster IgG), anti-ICOS mAb (clone TK51, rat IgG), and anti-4–1BBL mAb (clone 17G9, rat IgG2b) were manufactured from their respective hybridoma lines by BioExpress and used for in vivo studies as previously reported (21, 22).

Generation of memory T cells by homeostatic proliferation

It is well known that naive T cells can be converted to memory T cells following homeostatic proliferation in syngeneic immunodeficient hosts (20). In our studies, T cells (2 × 10^6 cells) prepared from the peripheral lymph nodes of naive C57BL/6 mice were injected via the tail vein into Rag-1-deficient mice. The cells were allowed to undergo homeostatic proliferation for 4–6 wk before all analyses. In this model, extensive cell division occurred within the first 10 days after adoptive cell transfer, and the division process was usually complete 4 wk later. In some experiments the host mice were also treated with anti-OX40L at the time of cell transfer (0.25 mg i.p. on days 0, 2, 4, and 8, then once a week for 4 wk after adoptive cell transfer) before phenotypic analysis.

Generation of memory T cells by donor Ag priming

To generate alloantigen-specific memory T cells, C57BL/6 mice were primed with DBA/2 skin allografts. Four to 6 wk after skin allograft rejection, T cells were isolated from the peripheral lymph nodes of primed C57BL/6 hosts using the T cell enrichment columns (R&D Systems) and used as a source of alloantigen-specific memory T cells.

Flow cytometry

Spleen cells were prepared from Rag-1-deficient host mice at different time points after T cell transfer and homeostatic cell proliferation. The cells were then resuspended in PBS/0.5% BSA for surface staining with specific Abs (22). After staining, cells were fixed in 1% formaldehyde before analysis by flow cytometry. For intracellular staining of Bcl-2 and Bcl-xL expression, cells recovered from Rag-1-deficient hosts were stained with CyChrome-anti-mouse CD4 and FITC-anti-mouse CD44 first, then fixed and membrane permeabilized in Cytofix/Cytoperm solution (BD Pharmingen) at 4°C for 30 min. The cells were washed in Perm/Wash solution and resuspended in Perm/Wash solution (1 × 10^6 cells/100 μl) for staining with PE-conjugated Abs against Bcl-2 and Bcl-xL on ice. PE-conjugated isotype control Ab was included in the staining protocol as a control. All samples were acquired and analyzed using a FACSort equipped with CellQuest software (BD Biosciences).

ELISPOT

The ELISPOT assay was used to quantitate IFN-γ production as previously reported (23). Briefly, immunospot plates were coated with 4 μg/ml rat anti-mouse IFN-γ capture mAb (clone R4-6A2) in sterile PBS at 4°C overnight. The plates were washed and then blocked in PBS/1% BSA for 2 h at room temperature. CD4+ T cells from naive C57BL/6 mice or Rag-1-deficient mice with homeostatically proliferated T cells were isolated using the T cell enrichment column (R&D Systems). CD4+ T cells (4 × 10^5 cells/well) were then placed in each well in the presence or the absence of irradiated (3000 rad) allogeneic DBA/2 spleen cells as stimulators (8 × 10^4 cells/well) and cultured in complete RPMI 1640 (1% penicillin/streptomycin/glutamine) at 37°C for 24 h. In some experiments the CD4+ T cells (2 × 10^5 cells/well) were prepared from Rag-1-deficient mice with homeostatically proliferated cells but were treated with anti-OX40L mAb. After the incubation, the cells were discarded from the plates, followed by washing the plates in PBS/0.05% Tween 20 (PBS/T). Biotinylated rat anti-mouse IFN-γ detection mAb (clone XMGI.2; 0.5 μg/ml) was added to each well, followed by incubation at 4°C overnight. The plates were then washed with PBS/T, followed by 2-h incubation with HRP-streptavidin (1:2000 in PBS/BSA). After washing with PBS/T, the spots were developed using a 200 μg/ml 3-aminio-9-ethylcarbazole substrate reagent set (BD Biosciences). The resulting spots were counted using a computer-assisted ELISPOT image analyzer (T Spot Image Analyzer, Cellular Technology).

The in vivo CFSE model

Spleen and peripheral lymph nodes were harvested from C57BL/6 mice, and a single-cell suspension was prepared in HBSS. After RBC lysis, the cells were labeled with CFSE (Molecular Probes) as previously described (24). Briefly, the cells (10 × 10^6 cells/ml) were incubated with CFSE at a final concentration of 5 μM in serum-free HBSS for 6 min at room temperature. The labeling process was then terminated by adding FBS (10% of the total volume). Cells were washed twice in HBSS before i.v. injection into Rag-1-deficient mice. In some experiments the host mice were treated with anti-OX40L mAb at 0.25 mg i.p. on days 0, 2, 4, and 8 after adoptive cell transfer. The host mice were killed 10 days later for analysis.

Analysis of T cell apoptosis

T cells from naive C57BL/6 mice were adoptively transferred into Rag-1-deficient mice (2 × 10^6 cells/mouse) to allow homeostatic proliferation to occur. A cohort of Rag-1-deficient mice was treated with the anti-OX40L mAb or a control Ab at the time of cell transfer (0.25 mg i.p. on days 0, 2, 4, and 8 after adoptive cell transfer). At different time points after treatment, cells were recovered from the host mice and stained with CyChrome-conjugated anti-CD4 or anti-CD8, along with PE-annexin V in a calcium-rich, annexin-binding buffer (BD Pharmingen) on ice for 20 min. Cells were washed twice after staining and immediately analyzed by flow cytometry. Annexin V-positive cells were regarded as apoptotic cells (25).

Skin transplantation

Full-thickness tail skin grafts (~1 cm²) from DBA/2 mice were transplanted onto the thoracic wall of Rag-1-deficient mice with or without memory T cells. The skin grafts were secured with an adhesive bandage for the initial 7 days. Graft survival was assessed by daily visual inspection. Skin allograft rejection was defined as the complete necrosis and loss of viable tissue (22).

Treatment of skin transplant recipients

Skin allograft recipients were treated with CTLA-4g (0.5 mg i.p. on days 1 and 3) and anti-CD154 (0.5 mg i.p. on days 0, 1, 3, and 6). Some recipient mice were also treated with anti-ICOS mAb at 0.5 mg i.p. on days 0, 2, 4, and 8 after skin grafting. Anti-4–1BBL mAb or anti-OX40L mAb was given at 0.5 mg i.p. on days 0, 2, 4, and 8 after skin transplantation.

Statistical analyses

Survival analysis was plotted using the Kaplan-Meier method, and the significance of differences in survival between groups was assessed using the log-rank test. Analysis of ELISPOT data was performed using the Kruskal-Wallis one-way ANOVA. A value of p < 0.05 was considered as significant.

Results

Memory T cells generated by homeostatic proliferation mediate prompt skin allograft rejection regardless of CD28/CD154 blockade

Naive T cells can undergo extensive homeostatic proliferation when transferred into syngeneic immunodeficient hosts, and a key feature of this process is the conversion of naive T cells into a memory phenotype (20, 26). In our model, when 2 × 10⁶ naïve T
cells (i.e., CD44lowCD62Lhigh) from C57BL/6 mice were transferred into B6.Rag-1-deficient hosts, vigorous cell proliferation occurred around day 10 after cell transfer. The BrdU labeling assay revealed that this proliferative response was often complete ~4 wk after cell transfer (our unpublished observation).

As shown in Fig. 1A, in contrast to their naive counterparts, T cells recovered from Rag-1-deficient mice 4–6 wk after homeostatic proliferation showed a marked up-regulation of CD44 on the cell surface with concurrent down-regulation of CD62L expression, confirming their acquisition of memory phenotype (20, 26). After undergoing homeostatic proliferation, T cells readily produced large amounts of IFN-γ, one of the functional features that is often ascribed to conventional memory T cells (27). As shown in Fig. 1B, ELISPOT analyses showed that naive CD4+ T cells generated ~50 spots/400,000 cells upon a brief stimulation with DBA/2 alloantigens in vitro. In stark contrast, CD4+ T cells after homeostatic proliferation produced ~300 spots/400,000 cells under identical culture conditions (i.e., a 6-fold increase), suggesting that T cells after undergoing homeostatic proliferation also acquire the functional features of memory T cells.

To determine the capability of memory T cells generated by homeostatic proliferation to mediate skin allograft rejection and their sensitivity/resistance to CD28/CD154 costimulatory blockade, naive T cells (2 × 106) were adoptively transferred into Rag-1-deficient mice, and the cells were allowed to undergo homeostatic proliferation for 4–6 wk. The host mice were then grafted with the fully MHC mismatched DBA/2 skin allografts, and the skin allograft survival was determined. As shown in Fig. 1C, Rag-1-deficient mice harboring memory T cells uniformly rejected the DBA/2 skin allograft with a mean survival time (MST) of 14 days (n = 6). Treatment of the host mice with CTLA-4Ig and anti-CD154 at the time of skin grafting to block both CD28 and CD154 costimulatory pathways completely failed to prevent memory T cell-mediated rejection, and all skin allografts were rejected with an MST of only 15 days (n = 6). However, when Rag-1-deficient mice were transferred with naive T cells and at the same time grafted with the DBA/2 skin allografts and treated with the same CD28/CD154 blockade protocol, the skin allograft survival was markedly prolonged (MST, >60 days; p < 0.05; Fig. 1D). Thus, rejection by memory T cells developed after homeostatic proliferation is less dependent on CD28 and CD154 costimulation.

Memory T cells generated by homeostatic proliferation express high levels of OX40, 4-1BB, and ICOS costimulatory molecules

The CD28/CD154 blockade-resistant rejection in our model prompted us to examine whether other alternative costimulatory

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**FIGURE 1.** Naive T cells acquire phenotypic and functional features of memory T cells after undergoing homeostatic proliferation. A, Rag-1-deficient mice were transferred with 2 × 10⁶ naive T cells from C57BL/6 mice. Four to 6 wk later, T cells were recovered from Rag-1-deficient mice, and the expression of CD44 and CD62L was analyzed by FACS. □, Naive T cells; ■, memory T cells after homeostatic proliferation. B, ELISPOT analysis of IFN-γ production by naive CD4+ T cells and CD4+ cells after homeostatic proliferation. Irradiated allogeneic DBA/2 splenic cells were used as stimulator cells in the experiments. C, Skin allograft survival in Rag-1-deficient mice harboring homeostatic proliferation-generated memory T cells with or without CD28/CD154 blockade. D, Skin allograft survival in Rag-1-deficient mice transferred with naive T cells with or without CD28/CD154 blockade. HP, homeostatic proliferation.
molecules may play a role in the memory T cell-mediated rejection. For this purpose, we examined the cell surface expression of alternative T cell costimulatory molecules (OX40, ICOS, 4-1BB, and CD30) on T cells before and after the process of homeostatic proliferation. As shown in Fig. 2, when compared with the naive T cells, T cells recovered from the Rag-1-deficient mice 4–6 wk after homeostatic proliferation showed markedly up-regulated cell surface expression of OX40, ICOS, and 4-1BB. CD30 expression was not up-regulated on T cells after homeostatic proliferation, and its levels of expression were similar to those of naive T cells. This staining pattern suggests that costimulation mediated by OX40, ICOS, or 4-1BB may play a role in memory T cell-mediated rejection.

FIGURE 2. Expression of alternative costimulatory molecules by memory T cells developed after homeostatic proliferation. Rag-1-deficient mice were injected with $2 \times 10^6$ naive T cells from the peripheral lymph nodes of C57BL/6 mice. Four to 6 wk later, T cells were recovered from the host mice and stained for the expression of CD30, ICOS, 4-1BB, and OX40 costimulatory molecules. A. The upper panel shows the CD4$^+$ subset, and the lower panel shows the CD8$^+$ subset. Naive controls; , memory T cells generated by homeostatic proliferation. B. Comparative analysis of CD30, ICOS, 4-1BB, and OX40 expression by naive T cells and memory T cells as the percentage of positive cells among the total number of T cells acquired. Representative data from three individual experiments are shown.

FIGURE 3. Effect of blocking ICOS, 4-1BB, or OX40 costimulation with or without CD28/CD154 blockade on memory T cell-mediated rejection. A, Rag-1-deficient mice carrying memory T cells generated by homeostatic proliferation were grafted with the DBA/2 skin allograft and treated with anti-ICOS, anti-4-1BBL, or anti-OX40L mAb. Groups of mice were also treated with combined anti-ICOS, anti-4-1BBL, and anti-OX40L. Graft survival was determined and plotted. B, Rag-1-deficient mice harboring memory T cells generated by homeostatic proliferation were grafted with the DBA/2 skin allograft and treated with CTLA-4Ig and anti-CD154 mAb. Groups of recipient mice were also treated with anti-ICOS, anti-4-1BBL, or anti-OX40L in addition to CD28/CD154 blockade. Graft survival was determined and plotted.
Blocking OX40, but not ICOS or 4-1BB, costimulation prevents the CD28/CD154 blockade-resistant rejection mediated by memory T cells

To further define the role of OX40, ICOS, and 4-1BB costimulatory molecules, which are highly expressed on memory T cells after homeostatic proliferation, in memory T cell-mediated rejection, we transplanted DBA/2 skin allografts onto Rag-1-deficient mice with homeostatic proliferation-generated memory T cells. The recipient mice were then treated with anti-ICOS, anti-OX40L, or anti-4-1BB to block the ICOS/ICOS ligand (ICOS-L), OX40/OX40L, or 4-1BB/4-1BBL pathway, and the skin allograft survival was determined. As shown in Fig. 3A, treatment of recipient mice with anti-OX40L, anti-ICOS, or anti-4-1BBL mAb alone or in combination did not prolong the skin allograft survival; all skin allografts were rejected within 28 days after transplantation (n = 5–6), suggesting that blocking only such alternative costimulatory pathways is not sufficient to prevent memory T cell-mediated rejection in this model. Similarly, blocking the ICOS/ICOS-L pathway or the 4-1BB/4-1BBL pathway in combination with CD28/CD154 blockade also failed to prevent skin allograft rejection, and all skin allografts were rejected within 19 days after skin grafting (n = 3–4; Fig. 3B). In stark contrast, addition of anti-OX40L mAb to block the OX40/OX40L pathway to the CD28/CD154 blockade protocol markedly prolonged the skin allograft survival (MST, >85 days; n = 5; p < 0.01), and 40% of the hosts accepted their skin allograft for >100 days (Fig. 3B). This finding suggests that blocking the OX40/OX40L costimulatory pathway is uniquely important in preventing CD28/CD154 blockade-resistant rejection mediated by memory T cells.

Roles of OX40 in proliferation, survival, and effector functions of memory T cells

Memory T cells developed after homeostatic proliferation are clearly alloreactive and also require OX40 costimulation. To determine the mechanisms by which OX40 regulates memory T cell-mediated rejection, we first examined whether blocking OX40 costimulation would inhibit the conversion of naive T cells to a memory phenotype by blocking their homeostatic proliferation in vivo. As shown in Fig. 4A, treatment of Rag-1-deficient hosts with the anti-OX40L mAb at the time of cell transfer did not affect the homeostatic proliferation of transferred T cells, and the kinetics of cell proliferation in Rag-1-deficient mice were similar to those in control Ab-treated mice, suggesting that OX40 costimulatory signals are dispensable for homeostatic cell proliferation.

To determine whether OX40 blockade would induce apoptotic T cell death despite uninterrupted cell proliferation, T cells recovered from anti-OX40L-treated mice were examined for apoptotic cell death by annexin V staining. As shown in Fig. 4B, the survival of CD4+ T cells was not affected by blocking the OX40/OX40L pathway. However, a significant fraction of CD8+ T cells from anti-OX40L-treated mice stained positively for annexin V, suggesting that the CD8+ T cells are more sensitive to apoptotic cell death in this model after OX40 blockade.

Despite extended treatment with anti-OX40L mAb (0.25 mg i.p. on days 0, 2, 4, and 8, followed once every week for 8 wk), CD4+ T cells recovered from treated Rag-1-deficient mice still up-regulated the surface expression of CD44 (Fig. 5A). Furthermore, when CD4+ T cells were recovered from anti-OX40L-treated mice and analyzed for Bcl-2 and Bcl-xL expression, no marked differences were observed in the expression of such antiapoptotic molecules regardless of OX40/OX40L blockade (Fig. 5B). However, ELISPOT assay showed that memory CD4+ T cells recovered from anti-OX40L-treated hosts were severely impaired in their ability to produce IFN-γ in vitro. As shown in Fig. 5C, memory CD4+ T cells from control Ab-untreated mice readily produced large amounts of IFN-γ upon in vitro allograft antigen stimulation (~138 spots/200,000 CD4+ cells). In contrast, the ability of memory CD4+ T cells recovered from anti-OX40L-treated mice to produce IFN-γ was impaired (~35 spots/200,000 CD4+ cells). This finding suggests that OX40 signaling is critical to the functional competence of memory CD4+ T cells.

Critical involvement of OX40 in alloantigen-specific memory T cell-mediated rejection

To confirm that alloantigen-specific memory T cells also require OX40 costimulation in the rejection response, T cells from C57BL/6 mice presensitized with DBA/2 skin allografts 4–6 wk earlier were adoptively transferred into Rag-1-deficient hosts. The host mice were then transplanted with the DBA/2 skin grafts, and the role of OX40 costimulation in skin allograft rejection was examined. In contrast to naive T cells, T cells from donor Ag-presensitized mice readily produced large amounts of IFN-γ upon donor Ag restimulation in vitro (Fig. 6A), suggesting that such T cells have memory T cell features. As shown in Fig. 6B, such
memory T cells mediated prompt rejection of the DBA/2 skin grafts; treatment with CTLA-4Ig and anti-CD154 failed to prevent the rejection response. However, addition of anti-OX40L mAb to the CD28/CD154 blockade protocol markedly prolonged the skin allograft survival (MST, \( \text{H}^\text{11022} \), 80 days; \( \text{n} \), \( \text{H}^\text{11000} \), 5), demonstrating that alloantigen-experienced memory T cells are also sensitive to OX40 blockade.

**Discussion**

Memory T cells, although vital to the protective immunity against invading pathogens, are becoming a growing concern in the induction of transplant tolerance (2, 28). Several mechanisms collectively contribute to the memory pool; such mechanisms include deliberate immunizations, heterologous immunity (6, 10, 29), and homeostatic proliferation (20). Importantly, a significant proportion of such memory T cells are thought to be alloreactive, especially in humans. As most of our immunosuppressive drugs are designed to target the activation of naive T cells, and memory T cells are clearly different from naive T cells in their activation requirements, understanding the precise molecular pathways that control the memory T cell response is of central importance to the induction of transplant tolerance.

In the present study we examined the prerequisite of skin allograft rejection mediated by two types of memory T cells, i.e., memory T cells developed after homeostatic proliferation and memory T cells generated by donor Ag priming, and identified OX40 as a unique alternative costimulatory molecule in memory T cell-mediated rejection. Consistent with several previous reports (8, 9, 30), memory T cells can mediate prompt allograft rejection, and rejection mediated by such cells is CD28/CD154 blockade resistant (Fig. 1). Of the alternative costimulatory molecules examined in our study, OX40 is uniquely important in supporting CD28/CD154 blockade-resistant rejection. Clearly, in models where rejection is mediated by either homeostatic proliferation-generated memory T cells or memory T cells generated by alloantigen priming, blocking the OX40/OX40L pathway induced long-term skin allograft survival when combined with CD28/CD154 blockade. Blocking the ICOS/ICOS-L pathway and the 4-1BB/4-1BBL pathway, alone or in combination with CD28/CD154 blockade, completely failed to prevent the rejection response (Figs. 3 and 6). The failure of blocking the ICOS/ICOS-L or 4-1BB/4-1BBL pathway to prevent the rejection response is not due to the lack of expression of such costimulatory molecules, because memory T cells generated by homeostatic proliferation expressed high levels of ICOS and 4-1BB on the cell surface (Fig. 2). Also, the treatment protocols used in the present study to block the ICOS/ICOS-L or 4-1BB/4-1BBL pathway have demonstrated efficacy in other transplant models (31). Thus, it appears that the roles of such alternative costimulatory molecules in regulating the fate of memory T cells in transplant models are not redundant, albeit their roles in the activation of primary T cells can be overlapping.

OX40 appears to regulate multiple aspects of the T cell response. OX40 costimulation on effector T cells promotes cell survival, proliferation, and effector differentiation (32). Also, OX40 costimulation prevents T cell anergy and allows anergic T cells to acquire potent effector functions (33). Thus, blocking the OX40/OX40L costimulatory pathway or genetic mutations of OX40 or OX40L can repress certain T cell-mediated cytopathic conditions (21, 34–39). Furthermore, OX40 is instrumental in the generation...
costimulatory pathway has been shown to support the generation with CD28/CD154 blockade. In other models, the 4-1BB/4-1BBL effect in preventing skin allograft rejection, even in combination highly expressed on memory T cells, did not have any therapeuticduction (Fig. 5). Thus, input from the OX40 receptor is important cell death of CD8

Indeed, engagement of OX40 has been shown to induce the ex-
costimulation may be essential for the survival of memory T cells. The functions of fully mature memory T cells. In our model, blocking OX40 costimulation was blocked (Fig. 4). It is also possible that OX40 costimulation is required for activation and/or effector functions of fully mature memory T cells. In our model, blocking OX40 costimulation did not affect in vivo T cell proliferation and CD44 up-regulation (Fig. 5), suggesting that conversion of naive CD4+ T cells to a memory phenotype is likely to be OX40 inde-

FIGURE 6. Effect of OX40 blockade on alloantigen-specific memory T cell-mediated rejection. A, ELISPOT analysis of IFN-γ production in vitro by alloantigen specific memory T cells isolated from donor Ag-primed C57BL/6 mice. Representative data from two individual experiments are shown. B, Effect of blocking the OX40/OX40L costimulatory blockade on alloantigen-specific memory T cell-mediated rejection. Rag-1-deficient mice reconstituted with donor Ag-specific memory T cells from primed mice were transplanted with the DBA/2 skin graft and treated with anti-OX40L in addition to CD28/CD154 blockade. Graft survival was examined and plotted.

of memory recall response. Studies using OX40-deficient mice clearly showed that the most profound impact of OX40 deficiency on the immune response is the impaired generation of memory T cells (16, 40). In contrast, amplified OX40 signaling by transgenic expression of OX40L induces a marked expansion of memory T cells (37, 41). However, the precise mechanism by which OX40 regulates the memory T cell response remains to be defined. OX40 costimulation may be essential for the survival of memory T cells. Indeed, engagement of OX40 has been shown to induce the expression of Bcl-2 and Bcl-xL (18). We also observed precocious cell death of CD8+ T cells in the homeostatic proliferation model when OX40 costimulation was blocked (Fig. 4). It is also possible that OX40 costimulation is required for activation and/or effector functions of fully mature memory T cells. In our model, blocking OX40 costimulation did not affect in vivo T cell proliferation and CD44 up-regulation (Fig. 5), suggesting that conversion of naive CD4+ T cells to a memory phenotype is likely to be OX40 inde-

and/or survival of CD8+ memory T cells (16, 17, 42), but 4-1BB costimulation exhibits no obvious effect on the generation of CD4+ memory T cells (16). In our transplant model, rejection is mediated by both CD4+ and CD8+ memory T cells (Fig. 2); blocking 4-1BB-dependent CD8+ memory T cells only may be insufficient to prevent the rejection response in this model. The role of ICOS costimulation in memory T cell-mediated rejection also warrants further investigation. In certain models in which re-
jection is mediated by naive T cells, the nature of an immune response and the timing of ICOS blockade appear to be critical (31); whether this is also the case in memory T cell-mediated re-

There is compelling evidence that certain memory T cells, regard-
less of their Ag specificities, are alloreactive and can resist tolerance induction (10). However, it is noteworthy that not all memory T cells are alloreactive or inherently refractory to toler-
ance induction by all means. For example, a subset of memory CD4+ T cells from B10.D2 mice did not show any alloreactivity in a chronic graft-vs-host disease model (43). Furthermore, mem-

memory CD8+ T cells specific for a peptide Ag can be readily tolerized by i.v. injection of the soluble Ag (44). Thus, different memory subsets, especially those generated by heterologous immunity (6), may be intrinsically different in their alloreactivities and/or in their resistance to tolerance induction. Nonetheless, data from our study and that of others (30) clearly showed that memory T cells developed after homeostatic proliferation are alloreactive and also re-
sistant to tolerance induction. Importantly, such memory T cells in transplant rejection are very sensitive to OX40 blockade (Fig. 3). Similarly, the rejection response mediated by alloantigen-specific memory T cells is also sensitive to OX40 blockade (Fig. 6). This finding suggests that in certain transplant models in which allo-

reactive memory T cells are present at high levels, blocking the OX40/OX40L pathway may be critically important in preventing the rejection response.

In summary, our data demonstrate that memory T cell-mediated rejection resistant to CD28/CD154 blockade is sensitive to OX40 blockade and that, contrary to the traditional belief, memory T cells that are alloreactive are amendable in transplantation settings. The identification of OX40 as a key alternative costimulatory mol-
ecule in memory T cell-mediated rejection has important clinical implications. For example, attempts at inducing transplant toler-
ance using strategies involving broad T cell depletion will cer-
tainly result in homeostatic proliferation of residual T cells (30, 45). Memory T cells arising under such homeostatic conditions are likely to be alloreactive and also resistant to tolerance induction. Under such conditions, targeting the OX40/OX40L pathway may prove to be important in achieving transplantation tolerance.

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Disclosures

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References


