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A Diometric Role for OX40 in the Response of Effector/Memory CD4+ T Cells and Regulatory T Cells to Alloantigen

Gillian Kinnear,* Kathryn J. Wood,* Farnaz Fallah-Arani, † and Nick D. Jones‡

OX40 is a member of the TNFR superfamily that has potent costimulatory properties. Although the impact of blockade of the OX40–OX40 ligand (OX40L) pathway has been well documented in models of autoimmune disease, its effect on the rejection of allografts is less well defined. In this article, we show that the alloantigen-mediated activation of naïve and memory CD4+ T cells results in the induction of OX40 expression and that blockade of OX40–OX40L interactions prevents skin allograft rejection mediated by either subset of T cells. Moreover, a blocking anti-OX40 had no effect on the activation and proliferation of T cells; rather, effector T cells failed to accumulate in peripheral lymph nodes and subsequently migrate to skin allografts. This was found to be the result of an enhanced degree of cell death among proliferating effector cells. In clear contrast, blockade of OX40–OX40L interactions at the time of exposure to alloantigen enhanced the ability of regulatory T cells to suppress T cell responses to alloantigen by supporting, rather than diminishing, regulatory T cell survival. These data show that OX40–OX40L signaling contributes to the evolution of the adaptive immune response to an allograft via the differential control of alloreactive effector and regulatory T cell survival. Moreover, these data serve to further highlight OX40 and OX40L as therapeutic targets to assist the induction of tolerance to allografts and self-Ags. The Journal of Immunology, 2013, 191: 1465–1475.

The provision of costimulatory signals is critical for optimal T cell activation (1). OX40 (CD134) is a 50-kDa transmembrane protein belonging to the TNFR superfamily that possesses such costimulatory properties (2, 3). OX40 is upregulated on T cells following activation (4), whereas regulatory T cells (Treg) constitutively express OX40 (5, 6). This is in agreement with the proposed role for OX40–OX40 ligand (OX40L) interactions (i.e., in the propagation of the response, rather than in initial T cell priming). OX40L is predominantly restricted to APC, such as B cells, macrophages, microglia, dendritic cells, and endothelial cells (7–13). Signaling via OX40–OX40L interactions during effector T cell responses was shown to lead to enhanced T cell survival (14, 15), cytokine production (16), and increased numbers of memory CD4+ T cells (17). These data were corroborated using human CD4+ T cells cultured with an OX40–Ig fusion protein in a mouse model of cardiac transplantation (20). Similarly, blocking the OX40–OX40L pathway (using an OX40–Ig fusion protein) in a mouse model of cardiac transplantation was shown to be ineffective at prolonging allograft survival across a full MHC mismatch. However, prolonged cardiac allograft survival was observed (median survival time [MST]: 14 d versus >100 d) when donor and recipient were mismatched at only minor histocompatibility loci (21). These data provide a clear precedent for the use of the OX40–OX40L costimulatory pathway in rejection; however, this appears to be contingent on suboptimal or low-frequency T cell responses. This is borne out by the finding that when OX40 blockade is used in combination with interruption of other costimulatory pathways (e.g., CD40/CD154, CD28/CD80/CD86), a more pronounced impact on skin allograft survival was observed due to perturbation of the expansion or persistence of alloreactive effector T cells (17, 22–24).

It also was suggested that OX40–OX40L has a diometric role on effector T cells and Foxp3+ Tregs (i.e., OX40 signaling enhances effector T cell responses, whereas it inhibits the generation of inducible Treg [iTreg] from naïve CD4+ T cells). So and Croft (25) demonstrated that OX40 signaling and a low dose of Ag (moth cytochrome C) suppressed the differentiation of naïve TCR-transgenic CD4+ T cells into Foxp3+ Treg. More recently, Xiao et al. (26) elegantly showed that engagement of OX40 in naïve recipients results

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G.K. designed and performed experiments, analyzed data, and wrote the manuscript; K.J.W. provided reagents and was involved in writing the manuscript; F.F.-A. provided reagents and edited the manuscript; and N.D.J. provided reagents, designed experiments, analyzed data, and was involved in writing the manuscript.

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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; CBA, CBA/Ca; cLN, contralateral lymph node; dLN, draining lymph node; DST, donor-specific transfusion of whole blood; iTreg, inducible regulatory T cell; mLN, mesenteric lymph node; MST, mean survival time; OX40L, OX40 ligand; PEG, polyethylene glycol; TM, memory T cell; Treg, regulatory T cell.

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in expansion of Treg, although these in vitro–expanded cells function as poor suppressor cells as the result of a deficiency in IL-2.

In addition to OX40 signaling impacting naïve T cell and Treg responses, OX40 signaling was shown to be required to sustain memory T cell (Tm) responses. For example, Gaspal et al. (27) elegantly demonstrated that OX40 signaling, in concert with CD30 signals, was required for productive secondary Ab responses. CD30–/–/OX40–/– T cells had similar proliferation compared with wild-type controls, but these double-deficient T cells failed to survive in vivo (27).

Tm participate in the response to allografts and are not restricted to patients who have received prior sensitization with allotogaen in the form of a transplant, blood transfusion, or pregnancy. Indeed, it was shown that a subset of pre-existent Tm, generated as a result of a previous encounter with either infectious or environmental antigens, cross-react to allotogaen (a process termed “heterologous immunity”) (28). Moreover, the presence of high numbers of donor-reactive Tm prior to transplantation was found to be detrimental to transplant survival, regardless of whether this is induced by co-stimulatory molecule blockade or conventional immunosuppressive agents (29, 30). In addition, Hong et al. (31) showed that a combination of immune-modulating agents (i.e., anti-OX40, anti-CD154, anti-IL-2 complex, and rapamycin) failed to significantly prolong islet allograft survival mediated by Tm, whereas significant allograft survival was observed when mediated by naïve T cells. Therefore, the development of drugs that could inhibit Tm, as well as other cells in the immune repertoire, would clearly be beneficial in preventing allograft rejection as well as autoimmune disease.

In this study, we use a model of skin allograft rejection to demonstrate that blockade of the OX40-OX40L costimulation pathway prevents the activation and accumulation of naive and memory CD4+ T cells in response to allotogaen. In contrast, OX40 blockade promotes the survival of Treg following reactivation and enhances suppression of T cell responses to allotogaen in vitro and in vivo. Therefore, these data demonstrate that OX40–OX40L interactions differentially control the survival of effector/memory CD4+ T cells and Treg in response to allotogaen.

Materials and Methods

Mice

TEa TCR-transgenic mice (C57BL/6 background; H2b) produce CD4+ T cells that are reactive to a 17-mer peptide of the BALB/c H2b-2 chain presented in the context of H2A1/a (i.e., presented by B6 H2c class II via the indirect pathway) (32). The TEa mice were a kind gift from Dr. W. Gao and Prof. T. Strom (Harvard Medical School, Boston, MA). CBA.Ca RAG-1- and CD4–/– (Tdb CD4+ T cells) mice were bred from Prof. D. Kiossis (Mill Hill, London, U.K.). C57BL/6 RAG-1- and CD4–/– (Tdb CD4+ T cells) mice were bred and housed in the Biomedical Services Unit at John Radcliffe Hospital. All mice were 6–12 wk of age at the time of the first procedure, and all studies were performed in accordance with the Animals (Scientific Procedures) Act 1986.

Skin transplantation

Full-thickness tail skin (1 cm²) was transplanted onto the left flank of recipient mice 1 d prior to adoptive transfer of cells, as previously described (33). Grafts were covered with sterile dressings for 7 d, and graft viability was assessed daily. Rejection was defined as complete loss of viable donor skin.

Ab treatment in vivo

Mice were treated s.c. with PEPEgaled Fab Control (A33 Fab PEG) or anti-OX40 Fab PEG (710 Fab PEG; both from UCAM Pharma) at 2.5 mg/kg twice weekly for 4 wk posttransplantation. The anti-OX40 Fab PEG was shown to be able to completely block OX40L binding to OX40 on activated mouse T cells (data not shown), and it has no agonistic properties. PEPEgaled Fab fragments were chosen for use in these studies because addition of a polyethylene glycol (PEG) molecule extends the in vivo t½ of these reagents and lowers the immunogenicity, allowing them to be used effectively in experimental and clinical settings.

To generate allotogaen-reactive Treg, wild-type CBA mice were treated with 200 μg nondepleting anti-CD4 mAb (YTS177) i.v. on days −28 and −27. In addition, on day −27, mice received 250 μg C57BL/6 whole blood (i.v.). On day 0, mice received a population of CD4+CD25+ T cells capable of suppressing donor allotogaen–specific T cells (34, 35).

Generation of Tm

TEa Tm were generated by injecting 1 × 10⁶ sorted naïve TEa CD4+ T cells into B6 Rag2−/− mice on day −1. On days 0, 7, and 14, mice received 1 × 10⁶ BALB/c.B6F1 splenocytes. CBA polyclonal Tm were generated by allogeneic splenocyte challenge (B6) on days 0 and 7. Following the final allotogaen challenge, all mice were left for 50–100 d before being used in subsequent experiments. Upon harvest, spleen and mesenteric lymph nodes (mLN) were removed from mice containing Tm, and the percentage of live Tm was determined by flow cytometry. Typically >95% of T cells were Tm (expressed CD44; data not shown).

Lymphocyte isolation, cell sorting, and adoptive transfer

Naïve (CD44−) or memory (CD44+) CD4+ T cells were purified from the spleen and mLNs of naïve or allotogaen-primed wild-type mice (CBA) or B6 Rag−/− mice (reconstituted with TEa CD4+ T cells). Single-cell leukocyte suspensions were generated, and erythrocytes were removed by osmotic shock. Leukocytes were stained with CD4allophycocyanin, CD44-PE, CD45RB-Pacific Blue (or Viox for isolation of TEa T cells) (eBioscience) before the naïve CD4+ T cell population was sorted using a FACSAria cell sorter (BD Biosciences). Typically, CD4+ T cells were sorted to >98% purity. Cells for adoptive transfer were resuspended in PBS and injected i.v. into syngeneic Rag−− mice 1 d prior to skin transplantation.

Labeling of cells for analysis of proliferation

Leukocytes were resuspended to either 5 × 10⁶ or 1 × 10⁶ cells/ml in serum-free media before incubation for 10 min with 5 μM CFSE (Molecular Probes, Leiden, The Netherlands) or CellTrace Violet (Invitrogen), respectively. Cells were washed in ice-cold RPMI 1640 (Invitrogen Life Technologies) and then resuspended in PBS (Oxford; Oxford) prior to adoptive transfer.

Flow cytometry analysis

A single-cell suspension was prepared from spleen and mLNs, and cell surface staining was conducted as previously described (15). All samples were acquired immediately on a FACSCanto and analyzed using FACS-Diva software (both from BD Biosciences). For intracellular staining, cells were surface stained, as above, and then washed in FACS buffer. Cells were then incubated in 500 μl Fix/Perm Buffer (Transcription Factor Buffer Set; BD Biosciences) for 1 h at 4°C. This incubation was followed by two wash steps in wash buffer (Transcription Factor Buffer Set) before incubation with FoxP3-allophycocyanin and Bcl-2- FITC for 30 min at 4°C. After incubation, cells were washed once in permeabilization buffer. The absolute number of TEa CD4+ T cells was determined using fluorescent beads (CalibRITE beads; BD Biosciences), as described previously (36).

Isolation of CD25+ and CD25− CD4+ T cells

A single-cell suspension enriched for T cells was prepared and enriched for CD4+ T cells. After CD4+ purification by Dynal beads, cells were stained with anti–CD25-PE (eBioscience) for 30 min at 4°C. Cells were washed once in PBS/2% FCS before incubation with anti-PE beads (Miltenyi Biotech, Cologne, Germany). Cells were washed and loaded onto a MACS column on a MACS magnet. CD25+ CD4+ T cells were isolated by negative selection (collected from flow through), and CD25− CD4+ T cells were isolated by positive selection (those attached to the column), according to the manufacturer’s instructions.

CD11c+ isolation

A single-cell suspension (~2 × 10⁶/ml in PBS/2% FCS) from spleens of naïve B6 mice was incubated with anti-B220 (RA36β2) at a final concentration of 10 μg/ml for 45–60 min at 4°C. Cells were washed once and resuspended in 10% FCS/PBS. Sheep anti-rat–coated Dynabeads (Dynal, Oslo, Norway) were added to a ratio of one bead/cell and incubated on a rotating wheel at 4°C for 30 min. Residual cells were incubated with anti-CD11c (Milenyi Biotech) for 30 min at 4°C, before being isolated by positive selection using a magnet. The resulting enriched cell population was then counted, checked for purity, and resuspended at the required concentration for further use.
In vitro suppression assay

A total of $5 \times 10^5$ CD4+CD25- responder T cells (isolated from naive CBA mice) was labeled with violet proliferation dye (Invitrogen) and incubated with purified CD11c+ cells and various numbers of CFSE-labeled Treg. Treg were isolated from mice that had received 177/DST and either anti-OX40 or PEG control on days −28 and −25. Proliferation of the CD4+CD25- responder T cells was assessed by flow cytometry on day 5.

Histology

Skin grafts were embedded into paraffin wax and cut into 4-μm sections before being loaded onto an Autostainer (Lab Vision, Chicago, IL) for staining with rabbit anti-CD3 Ab (Abcam) at room temperature. After washing, sections were incubated with a biotinylated donkey anti-rabbit secondary Ab (Jackson ImmunoResearch) and then labeled streptavidin-biotin (Dako) before being incubated with diaminobenzidine for 5 min. The cell nuclei were counterstained with hematoxylin. The sections were then dehydrated through graded ethanol, cleared in Histo-Clear (Fischer Scientific), and cover slipped before being analyzed. Automatic image analysis was performed using Definiens Tissue Studio software (Definiens, Munich, Germany).

Statistical analysis

Data were analyzed with the Student t test using Prism software (version 5; GraphPad) and are expressed as mean ± SD. Kaplan–Meier survival graphs were constructed, and log-rank comparisons of the groups were used to calculate p values. The p values < 0.05 were considered statistically significant.

Results

CD4+ T cells upregulate OX40 after stimulation with alloantigen in vitro, whereas Treg constitutively express OX40

Before the role of OX40–OX40L interactions in CD4+ T cell responses to alloantigen was investigated, we sought to determine the expression pattern of OX40 and OX40L on CD4+ T cells (naive and memory) by stimulating naive CBA splenocytes with allogeneic irradiated B6 splenocytes in vitro. Polyclonal naive CD4+ T cells (gated on total CD4+ T cells) were found to upregulate expression of OX40 from day 2 of culture, with maximal expression on day 5 (Fig. 1A), whereas no expression of OX40L was found at any time point following activation (Fig. 1A). Similarly, polyclonal Tm (CD3+CD4+CD44+) stimulated under the same conditions also expressed OX40 from day 3 (Fig. 1B), but they did not express OX40L at any time (Fig. 1B). Treg also showed a similar profile of expression for OX40 (data not shown).

The expression of OX40 and OX40L was also analyzed on CD4+CD25+FoxP3+ T cells (Treg). Treg sorted from CBA mice were found to express OX40 without activation (Fig. 1C) but did not express OX40L (Fig. 1C). After activation with alloantigen, Treg expressed OX40 to similar levels but remained OX40L- (data not shown).

Blockade of OX40–OX40L interactions attenuates skin allograft rejection mediated by naive and memory CD4+ T cells

It was shown that both naive T cells and Tm express OX40 and that OX40 signaling is required for allograft rejection in certain settings (15, 17). In light of these data, we next wanted to test whether anti-OX40 could prevent the rejection of skin allografts in a model in which rejection was elicited by traceable, alloantigen-reactive CD4+ T cells. To this end, $1 \times 10^5$ naive or memory TCR-transgenic (TEa) CD4+ T cells were adoptively transferred into syngeneic Rag−/− mice, and the mice received a skin allograft (BALB/cB6 F1) 1 d later. Mice were treated twice weekly with PEG control or anti-OX40 from the time of transplant until day 28 posttransplant. Mice reconstituted with naive TEa T cells and given PEG control acutely rejected their skin allografts, whereas...
allograft survival was significantly prolonged in mice treated with anti-OX40 (MST: 49 versus 18.5 d; \( p < 0.0004 \)) (Fig. 2A). Similarly, mice reconstituted with TEa Tm and treated with PEG control rejected their skin allografts acutely, whereas treatment with anti-OX40 markedly prolonged skin allograft survival (MST: 89.5 versus 18 d; \( p < 0.0007 \); Fig. 2C).

Although OX40 blockade resulted in significant attenuation of skin allograft rejection mediated by naive or memory TEa T cells, we sought to confirm these results using polyclonal naive T cells and Tm to rule out any effects that could be attributable to the use of TCR-transgenic T cells. To this end, naive or memory polyclonal CD4+ T cells were transferred into syngeneic Rag-/- mice. The following day, mice received an H2Kb (B6) skin allograft together with either anti-OX40 or PEG control (twice weekly for 4 wk).

Administration of anti-OX40, given from the time of skin transplant, significantly prolonged allograft survival in mice that had received naive polyclonal CD4+ T cells compared with the PEG control group (MST: 97 versus 14 d; \( p < 0.0006 \); Fig. 2B). This dramatic impact of OX40 blockade on graft survival was also seen in a second strain combination when polyclonal B6 CD4+ T cells were adoptively transferred into syngeneic Rag-/- recipients and mice received an allogeneic skin transplant (CBA; MST: 67 versus 13 d; \( p < 0.0067 \); Supplemental Fig. 1). Mice reconstituted with polyclonal CD4+ Tm and treated with PEG control rejected skin allografts acutely. In contrast, skin allograft survival was significantly prolonged when mice received allograft and anti-OX40 (MST: 51.5 versus 15.5 d; \( p < 0.0005 \); Fig. 2D).

Taken together, these data demonstrate that the OX40–OX40L pathway plays a key role in facilitating the rejection of allografts by both naive and memory CD4+ T cells, which is consistent with other studies.

**Anti-OX40 prevents accumulation of naive and memory CD4+ T cells in the dLN following allogeneic skin transplantation**

It was suggested that OX40–OX40L costimulation promotes effector T cell survival (14, 37); therefore, we next examined whether this was also the case in the context of T cell responses to allografts. Our previous studies showed that large numbers of primed effector/memory CD4+ T cells could be detected specifically in the dLN by 15 d after allogeneic skin transplantation (data not shown). Therefore, we next assessed the impact of OX40–OX40L interactions on the clonal expansion of donor-reactive TEa T cells following allogeneic skin transplantation in vivo. A total of 1 \( \times \) 10^5 TEa T cells was adoptively transferred into syngeneic Rag-/- mice; 1 d later, mice received either a syngeneic or allogeneic skin graft. Mice receiving an allograft were either given PEG control or anti-OX40 twice weekly until harvesting at day 15.

In the presence of PEG control, a significant expansion of primed effector/memory TEa T cells was found in the dLN after an
allogeneic skin graft (TEa cell number: 198,562 ± 91,555), whereas little expansion occurred in the nondraining contralateral lymph node (cLN) (TEa number: 9,628 ± 7,212) (Fig. 3A). The expansion of TEa T cells in the dLN was shown to be allospecific, because there was no expansion of TEa T cells in the dLN after syngeneic skin graft transplantation (Fig. 3A). In contrast, anti-OX40 treatment prevented the accumulation of TEa T cells in the dLN (TEa number: 23,146 ± 18,585; 91 ± 5% inhibition; p < 0.0095; Fig. 3A) 15 d after skin allografting. Similar results were obtained after adoptive transfer of TEa Tm (Supplemental Fig. 2A).

Following allogeneic skin transplantation, alloreactive T cells become activated, proliferate, and accumulate in the dLN before migrating to the allograft and to the spleen where they reside as long-lived effector/memory T cells. Therefore, as expected, an expanded population of TEa T cells was found in the spleen after allogeneic skin transplantation; however, this redistribution of effector/memory TEa T cells was also inhibited by anti-OX40 treatment at day 15 (anti-OX40: 19,278 ± 11,296 versus PEG control: 213,722 ± 18,008 TEa T cells; 92 ± 5% inhibition; p < 0.0001; Fig. 3A).

Alloreactive effector T cells do not require OX40 blockade for optimal proliferation

The attenuated T cell accumulation seen on administration of anti-OX40 could be due to a requirement for OX40 for optimal proliferation or OX40 may be required to maintain the survival of activated effector cells. To distinguish between these possibilities, we looked at the proliferation of alloreactive T cells (as judged by the loss of CFSE) following transplantation in the presence or absence of anti-OX40 blockade. TEa T cells proliferated specifically in the dLN by 15 d after transplantation (Fig. 3B). Interestingly, despite a marked diminution in the number of TEa T cells (Fig. 3A), anti-OX40 did not affect the initial proliferation of alloreactive T cells (Fig. 3B). TEa T cells in mice that had received a syngeneic skin graft or in the cLN of allograft recipients maintained high levels of CFSE, consistent with the fact these cells had not proliferated or expanded by homeostatic proliferation (Fig. 3B). Experiments in which TEa Tm, rather than naive T cells, were transferred yielded similar results (Supplemental Fig. 2B).

Anti-OX40 inhibits T cell infiltration of skin allografts

Anti-OX40 inhibited the accumulation, but not the initial proliferation, of CD4+ T cells in the dLN following allogeneic skin transplantation. To rule out the possibility that anti-OX40 had enhanced the migration to other lymphoid tissues, and in particular to the allograft, we harvested skin allografts from mice treated with anti-OX40 or PEG control at day 15. Skin allografts were analyzed for the presence of CD3+ T cells by immunohisto-
chemistry, because the only T cells present in these mice were the transferred naive or memory TEa T cells (Fig. 3C, 3D, Supplemental Fig. 2C, 2D). We found a dramatic reduction in the number of TEa cells that had infiltrated skin allografts after treatment with anti-OX40 (Fig. 3C–E), which is consistent with anti-OX40 preventing the survival of effector CD4+ T cells, rather than inducing enhanced migration of effector T cell to the skin allografts.

OX40 is required for the survival of activated, alloreactive CD4+ T cells

To confirm this finding, we next looked ex vivo at dLN taken from mice that had received skin allografts. To this end, $1 \times 10^5$ TEa T cells were adoptively transferred into syngeneic Rag-/- recipients, and mice received a skin allograft on the following day. Mice were treated with either PEG control or anti-OX40 twice weekly, before being sacrificed on day 13 for analysis by flow cytometry. This time point provided an opportunity to observe cell death in the dLN before the cells were deleted.

Cells from the dLN were analyzed by annexin V and 7-aminoactinomycin D (7AAD) viability stains to assess the proportion of live/dead TEa T cells. Compared with control mice, the number and percentage of live cells were reduced after anti-OX40 treatment (Fig. 4A, 4B). OX40 blockade increased the proportion of preapoptotic (AnnexinV+7AAD-) TEa T cells (Fig. 4C), but it had no impact on the proportion of apoptotic (AnnexinV+7AAD+) TEa T cells in the dLN (Fig. 4D). These data correlated with our in vitro findings in CD8+ T cells (15), as well as with data from other studies in CD4+ T cells (14, 37), suggesting that OX40 signals were required for the survival of activated, alloreactive CD4+ T cells.

OX40 blockade enhances the potency of Treg

It was suggested that there may be a differential impact of OX40–OX40L signaling on effector/memory T cells and Treg. Therefore, we next wanted to investigate the impact of OX40 blockade on alloreactive Treg responses. Previously, our laboratory developed a protocol to generate alloreactive Treg in vivo by the administration of a nondepleting anti-CD4 mAb (YTS177; 177) and donor-specific transfusion of whole blood (DST) to mice (35, 38).

Using this protocol, we determined whether OX40 blockade enhanced or attenuated the number or activity of Treg generated by 177/DST in vivo. To this end, alloreactive Treg were generated in vivo following the administration of 177/DST in the presence or absence of anti-OX40 (days -28 and -25). Treg were isolated on day 0 and tested for their relative ability to suppress an alloreactive T cell response in vitro or skin allograft rejection in vivo (Fig. 5).

The addition of alloreactive PEG control Treg markedly inhibited the proliferation of CD4+CD25+ responder T cells to CD11c+ allogeneic stimulator cells at a 1:1 ratio (Fig. 5B). With decreasing numbers of PEG control Treg (i.e., increased ratio of naive/Treg), suppression was lost in a dose-dependent manner (Fig. 5B). Importantly, Tregs generated in the absence of OX40 signaling showed enhanced suppression compared with PEG control Treg at ratios of 1:1 and 1:4 (Fig. 5B). Tregs generated in the presence or absence of anti-OX40 had comparable levels of FoxP3 (data not shown).

Similarly, 177/DST+PEG control–generated Tregs were able to control skin allograft rejection at a 1:1 ratio but not at a 4:1 (effector/Treg) ratio with naive T cells when transferred to Rag-/- mice (Fig. 5C). However, blockade of OX40 at the time of 177/DST resulted in the generation of Treg that remained able to prevent skin allograft rejection when transferred in a 4:1 ratio with naive T cells (MST: >100 versus 16.5 d; $p < 0.0003$; Fig. 5C). These data clearly demonstrate that blockade of OX40 interactions increased, rather than decreased, the potency of alloreactive CD4+ CD25+ Treg.

**Alloreactive Treg generated in the absence of OX40 signaling demonstrate enhanced survival compared with Treg generated in an OX40-sufficient environment**

OX40 blockade significantly enhanced cell death in CD4+ (Fig. 4) and CD8+ alloreactive (15) effector T cells; this led us to investigate whether OX40 blockade had a distinct impact on the survival of Treg, which may explain the resultant increase in potency. To address this, the number of live Treg (that had been generated by 177/DST in the presence or absence of OX40–OX40L interactions) present after 5 d of culture with syngeneic responder

\[ \text{Number of Live Treg} \]

\[ \text{PEG Control} \]

\[ \text{Anti-OX40} \]

\[ \text{Treg} \]

\[ \text{Suppression} \]

\[ \text{FoxP3} \]

**Figure 4.** Anti-OX40 increases cell death in the dLN after skin allograft transplantation. A total of $1 \times 10^5$ naive TEa CD4+ T cells was adoptively transferred into syngeneic Rag-/- mice. One day later, mice received an allogeneic skin graft and either anti-OX40 or PEG control, administered s.c. at 100 mg/kg, and were sacrificed on day 13. dLN were analyzed for live (A, B), preapoptotic (AnnexinV+7AAD-) (C), or apoptotic (AnnexinV+7AAD+) (D) CD4+ T cells. Results are mean ± SD, performed in triplicate. *$p < 0.05$, **$p < 0.01$.**
Importantly, Treg from anti-OX40–treated or control-treated animals were used at the same number per well. Treg generated in the absence of OX40 signaling had a significant survival advantage both in terms of number (anti-OX40, 34,527 ± 3368, PEG control, 5934 ± 384; Fig. 6A) and percentage of live cells (anti-OX40: 46.4 ± 0.8%, PEG control: 12.3 ± 1.1%; Fig. 6B) compared with Treg generated in PEG control–treated mice. This survival advantage was evident at all ratios of responders to Treg following reactivation with allogeneic splenocytes in vitro.

Finally, given that Treg generated in the absence of OX40–OX40L interactions demonstrated enhanced survival following reactivation (Fig. 6A, 6B), we examined the expression of the antiapoptotic molecule Bcl-2 in such cultures. Treg generated in the absence of OX40 signaling had a significantly higher number of Bcl-2+ Treg at day 4 (2410 ± 255 versus 844 ± 559; p < 0.0116) and day 5 (7943 ± 594 versus 3466 ± 627; p < 0.0009) compared with 177/DST+PEG control–generated Treg (Fig. 6C).

**Discussion**

Alloimmune responses are complex and require orchestration of a number of cell types to elicit rejection. Given the significant role played by costimulatory molecules in T cell activation, costimulation blockade provides a promising adjunctive or alternative therapy to the currently licensed immunosuppressive drugs used to prevent graft rejection and autoimmunity (1). A number of co-

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**FIGURE 5.** Treg generated in the absence of OX40 signaling have a greater suppressive capacity in vivo. (A) Naive mice received 200 μg of nondepleting anti-CD4 mAb (YTS177) and DST on days −28 and −27. Anti-OX40 or PEG control was administered s.c., at 100 mg/kg, on days −28 and −25. Tregs (CD4+CD25+) were purified on day 0 from mice treated with 177/DST and either labeled with CFSE for use in an in vitro suppression assay or cotransferred in vivo with CD4+CD25− T cells (naive T cells) purified from unmanipulated CBA mice. Purified Treg and naive T cells were adoptively transferred into syngeneic Rag−/− recipient mice at various ratios. The following day, mice received a skin allograft (H2b). (B) Naive responder T cells (CD4+CD25−) were cultured with stimulator B6 CD11c+ cells, with and without various ratios of Tregs (purified from mice that had received 177/DST with and without anti-OX40). Cultures were analyzed after 6 d of culture by flow cytometry. Results are mean cell number ± SD, performed in triplicate (n = 6 mice/group [pooled]), and are representative of two independent experiments. (C and D) Kaplan–Meier survival curves show skin allograft rejection kinetics. (C) Allograft survival after reconstitution with 1 × 10⁵ naive T cells (effectors) and 1 × 10⁵ Treg (1:1 ratio). (D) Allograft survival after transfer with 4 × 10⁵ naive T cells (effectors) and 1 × 10⁵ Treg (4:1 ratio). In (C) and (D), a group of mice was included that received naive T cells alone. (n = 4–8 mice/group and data pooled between two independent experiments.) **p < 0.01, ***p < 0.0001.
stimulatory molecules that are required for optimal naive T cell responses are also expressed by Treg (e.g., CD28 signaling was reported to be required for the optimal suppressive function of Treg) (39). However, not all costimulatory molecules promote Treg-mediated suppression; for example, signaling through GITR (a TNFR superfamily member), which is expressed on Treg (like OX40), was shown to result in a loss of Treg function (40, 41). Importantly blockade of OX40 and GITR has a similar impact on effector T cells and results in a delay in disease onset or attenuated severity in models of diabetes (42, 43). Therefore, data such as these led to the possibility that certain costimulatory molecules may be used in different ways by conventional T cells and Treg.

In agreement with the literature, OX40 was found to be expressed on activated, but not naive, CD4+ T cells (Fig. 1). Furthermore, the delayed kinetics of expression (OX40 was not expressed until 3 d after activation) was consistent with a role for OX40–OX40L in effector T cell and Tm generation (i.e., in the propagation of the response, rather than in initial T cell priming). Blocking OX40 markedly impacted the ability of alloreactive naive and memory CD4+ T cells to elicit rejection of skin allografts in vivo (Fig. 2). Furthermore, blockade of the OX40–OX40L pathway prevented the accumulation of alloreactive CD4+ T cells in the dLN (Fig. 3, Supplemental Fig. 2). These in vivo studies demonstrate that CD4+ T cells undergo enhanced activation-induced cell death in the absence of OX40 signaling (Fig. 4). Therefore, OX40–OX40L interactions appeared to provide critical survival signals for effector/memory CD4+ T cells rather than being essential for initial T cell activation and differentiation, which is in agreement with other studies (14, 15, 37). These data are also in agreement with studies blocking OX40–OX40L in autoimmune diseases, such as experimental autoimmune encephalomyelitis: Nohara et al. (44) used a proteolipid protein–induced model of experimental autoimmune encephalomyelitis, and administration of anti-OX40L ameliorated disease. These studies showed a dramatic reduction in the infiltration of mononuclear and CD4+ T cells into the CNS.

Our experiments with alloreactive naive and memory CD4+ T cells also agree with published data whereby administration of agonistic OX40 enhanced primary T cell expansion and survival, which, in turn, increased the frequency of Tm (46, 50, 51). These data suggested that the absence of OX40 gave rise to a defect in survival or in their ability for long-term division. The impaired survival of OX40−/− T cells could be reversed by the addition of peptide inhibitors of caspases (pan-caspase inhibitor or specific inhibitors to caspase-3 or -9) (14), suggesting that signaling via OX40–OX40L prevents caspase-mediated apoptosis. However, in our studies we did not discriminate between caspase-dependent and -independent apoptosis.

The upregulation of antiapoptotic molecules (Bcl-2L and Bcl-2) to promote survival after T cell Ag recognition and OX40 co-stimulation is not unique to OX40; it also was demonstrated when...
costimulation was provided by other Ig and TNFR family members, such as CD28 and 4-1BB (53, 54). Our data clearly showed that OX40 signaling provided a critical survival signal for naive effector CD4\(^+\) T cells but, importantly, was also key for memory CD4\(^+\) T cells. Tm are now accepted as being a barrier to tolerance induction, because a number of strategies (e.g., CD28 and/or CD154 blockade) that are successful at preventing allograft rejection by naive T cells (using mouse models in pathogen-free conditions) (55–57) fail to induce tolerance in rodents that have been presensitized with donor alloantigen (28, 58). In addition, Heeger et al. (29) showed that alloreactive Tm were present in patients prior to kidney transplantation and that increased numbers of Tm were associated with an increased incidence of acute rejection. These and other reports provide evidence that Tm are critical to the success of allograft survival; therefore, developing strategies to inhibit Tm would be beneficial in preventing allograft rejection and tolerance induction.

It is particularly noteworthy that the absence of OX40–OX40L signaling resulted in significantly prolonged allograft survival, despite the availability of numerous other costimulatory ligands, such as CD80/CD86 (59) and CD40 (60, 61), on donor APC that could provide survival signals to the T cells (59). Therefore, it appears that OX40 plays a dominant and indispensable role in the continued survival of alloantigen-activated T cells and the ability to mediate graft rejection. However, it must be noted that the prolongation of skin allograft survival in the absence of OX40–OX40L signaling was limited to situations in which a small number of T cells was transferred, because anti-OX40 failed to impact allograft survival in immunocompetent mice (data not shown).

Importantly, although anti-OX40 continued to be administered, allografts remained free from rejection; however, grafts were rejected after the cessation of therapy (Fig. 2). Therefore, it appears that blockade of OX40–OX40L interactions failed to induce tolerance. Other evidence for the lack of tolerance induction is borne out of the observation that skin allografts were rejected after the anti-OX40 therapy was discontinued and the reagent cleared from the systemic circulation (data not shown).

The failure of OX40 blockade to induce tolerance could be due to a number of factors. First, not all activated CD4\(^+\) T cells expressed OX40 (Fig. 1), suggesting that some T cells are not dependent on the OX40–OX40L pathway. Numerous other costimulatory molecules within the Ig and TNFR superfamilies can make up for the lack of OX40 signaling because of the high degree of redundancy within the system.

Secondly, in this adoptive-transfer model, not all T cells encounter alloantigen simultaneously following transplantation (e.g., naive alloreactive T cells can be detected up to 50 d posttransplantation, despite allograft rejection [N.D.J., unpublished observations]). Because naive T cells do not express OX40, these cells may be able to elicit rejection upon alloantigen recognition and activation following the clearance of anti-OX40.

Lastly, an important caveat of this adoptive-transfer model is that purified naive CD4\(^+\) T cells were transferred to Rag\(^{-/-}\) knockout mice; therefore, these animals have no alloreactive Treg. Indeed, the TCR-transgenic T cells used in these studies were devoid of T cells with a regulatory phenotype (data not shown), and the polyclonal T cells were isolated as CD4\(^+\)/CD45R\(^{b+}\) cells to ensure that Tm and Treg were excluded. Although a strict sorting strategy was used, it is possible that T cells could be converted into Treg (so-called "iTreg") by OX40 blockade, although we suggest that this is unlikely because all of the allografts were rejected following clearance of anti-OX40 (Fig. 2). Treg were shown to be critical for the maintenance of long-term graft survival following costimulatory molecule blockade in immunocompetent mice, raising the possibility that tolerance would never be attainable in this model because of their absence (62).

The data presented in this article raise the question whether anti-OX40 could impact Treg function, because Treg constitutively express OX40, unlike conventional T cells. We demonstrate that the exposure of Treg to OX40 blockade and alloantigen generates Treg that are more potent at suppressing T cell responses to alloantigen in vitro and in vivo compared with those generated in the presence of OX40 signaling.

In our studies, OX40 blockade promoted, rather than suppressed, the ability of Treg generated in vivo under the cover of anti-CD4 and DST to regulate allospecific immune responses by increasing the survival of Treg (Figs. 5, 6). Other groups also showed that OX40–OX40L can impact Treg function (26, 63), whereas signaling via OX40–OX40L in effector T cells results in enhanced survival and generation of productive Tm responses (4, 46, 64). These data suggest that OX40–OX40L interactions have a dichotomous role in effector/memory T cells and Treg. Burocchi et al. (65) also suggested a diametric role for OX40–OX40L, whereby signaling via OX40 inhibited Treg-mediated suppression and enhanced effector T cell activation in a tumor model. Other in vitro data suggested that signaling through OX40–OX40L results in a reduction in Foxp3 expression (26, 63). Our data, although consistent with these reports, additionally reveal that OX40 signaling diminishes the survival of activated Treg, which clearly impacts on their potency to suppress allospecific immune responses in vitro and in vivo (Figs. 5, 6).

There is now strong supportive evidence that Foxp3\(^+\) T cells can be converted into Foxp3\(^-\) T cells (iTreg) that have suppressive function in the periphery (66, 67). Indeed, it was shown that YTS177/DST-generated alloreactive Treg are generated, in part, through conversion of naive T cells to iTreg and, in part, through the expansion/priming of naturally occurring Treg that are cross-reactive with donor alloantigen (68, 69). Therefore, further experiments will be required to ascertain whether anti-OX40 impacts naturally occurring Treg, iTreg, or both; however, So and Croft (25) provided evidence that OX40 signaling could antagonize Foxp3 induction, suggesting that OX40 signaling may impact the induction of Foxp3 expression and, therefore, iTreg generation.

Enhanced Treg survival may only be part of the story; OX40 blockade may have other effects on Treg function that were not examined in our studies and could also increase Treg potency. Therefore, further studies would be required to dissect the role of OX40–OX40L on molecules that have been associated with Treg function, such as IL-10 and TGF-β. Analysis of Treg isolated from tumor sites demonstrated that tumor-resident Treg exhibited a reduction in IL-10 production from Treg isolated from the lamina propria (70). These data suggest that further analyses of molecules, such as TGF-β, IL-10, and CTLA-4, could provide further information on the mechanisms by which OX40–OX40L impact Treg and their suppressive capacity.

OX40 signaling has been described to alter Treg positioning and homing. Following administration of agonistic OX40, Treg exhibited an increase in the expression of CCR8 and CD103 and a decrease in CCR4 (65). However, these findings have not been replicated in disease models. For example, OX40\(^{-/-}\) Tregs express comparable levels of CD103 to wild-type Tregs, and in a T cell transfer–induced colitis model OX40\(^{-/-}\) Tregs were able to accumulate in the mLN and lamina propria (71), thus making the interpretation of the transcriptome data more complex. These analyses examine patterns of expression at a specific time during OX40 signaling but do not assess the long-term impact of OX40 signaling.
In summary, we showed that OX40–OX40L interactions are important for the sustained expansion/survival of alloreactive naive and memory CD4+ T cells during skin allograft rejection. However, in clear contrast, mice exposed to alloantigen in the absence of OX40 signaling generate alloreactive Treg that demonstrate increased potency in their ability to suppress alloreactive T cell responses as a result of enhanced survival. This differs from other reports that showed that signaling via OX40–OX40L results in a downregulation of Foxp3 or induction of iTreg (63, 72). These data confirm a clear diaminic role for OX40 in the response of effector/memory T cells and Treg to allografts that could be exploited as part of tolerance-induction therapies to combat transplant rejection and re-establish tolerance in autoimmunity. In addition, blockade of OX40–OX40L proved successful in mouse models of autoimmune diseases (19, 44) and allergic inflammation (48, 73), thereby providing other disease areas in which the blockade of OX40 may have a therapeutic effect. In particular, there is a growing body of evidence that OX40 signaling affects Th2 (74) and Th9 differentiation (75); therefore, targeting OX40 (48, 73), thereby providing other disease areas in which the models of autoimmune diseases (19, 44) and allergic inflammation addition, blockade of OX40–OX40L proved successful in mouse transplant rejection and re-establish tolerance in autoimmunity. In effector/memory T cells and Treg to allografts that could be demonstrated increased potency in their ability to suppress alloreactive T cell responses as a result of enhanced survival. This differs from other reports that showed that signaling via OX40–OX40L results in a downregulation of Foxp3 or induction of iTreg (63, 72). These data confirm a clear diaminic role for OX40 in the response of effector/memory T cells and Treg to allografts that could be exploited as part of tolerance-induction therapies to combat transplant rejection and re-establish tolerance in autoimmunity. In addition, blockade of OX40–OX40L proved successful in mouse models of autoimmune diseases (19, 44) and allergic inflammation (48, 73), thereby providing other disease areas in which the blockade of OX40 may have a therapeutic effect. In particular, there is a growing body of evidence that OX40 signaling affects Th2 (74) and Th9 differentiation (75); therefore, targeting OX40 in allergic inflammation may also be beneficial.

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Disclosures

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References


