Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD),
and graft rejection in allogeneic bone marrow transplant (BMT) recipients

Bruce R. Blazar*, 1, Arlene H. Sharpe†
Andy I. Chen†
Angela Panoskaltsis-Mortari*
Christopher Lees*
Hisaya Akiba¶
Hideo Yagita¶
Nigel Killeen‖
Patricia A. Taylor*

From the *University of Minnesota Cancer Center and Department of Pediatrics, Division of Bone Marrow Transplantation, Minneapolis, MN 55455; †Department of Pathology, Brigham and Women’s Hospital, Boston, MA 02115; ¶Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan; ‖Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

1 BRB and AHS are co-first authors.

Correspondence to: Bruce R. Blazar, M.D.; University of Minnesota Hospital; Box 109 Mayo Bldg; 420 S.E. Delaware St; Minneapolis, MN 55455; telephone: 612-626-2734; FAX: 612-624-3913; email: blaza001@tc.umn.edu

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Abstract

OX40 (CD134) is expressed on activated T-cells; its ligand, OX40 ligand (OX40L) is expressed on dendritic cells, B-cells, and activated endothelial cells. To determine how OX40/OX40L interaction affects GVHD, we utilized antagonistic anti-OX40L mAbs or OX40−/− donor or OX40L−/− recipient mice. Similar degrees of GVHD reduction were observed with each approach. Despite the fact that OX40 is upregulated on both CD4+ and CD8+ T-cells isolated during GVHD, the major effects of OX40 ligation were on CD4+ and not CD8+ T-cell-mediated alloresponses as assessed in both GVHD and engraftment model systems. GVHD inhibition by blockade of the OX40:OX40L pathway did not require CD28 signaling. Some studies have indicated OX40 is essential for inducing Th2 responses. However, in vivo blockade of OX40/OX40L interactions reduced GVHD mortality induced by either Stat-6−/− (Th2-defective) or Stat-4−/− (Th1-defective) MHC disparate splenocytes, indicating that the GVHD ameliorating effects did not require Stat-4 or Stat-6 signaling. Although OX40L has been reported to be expressed on activated T-cells, no effects on GVHD were observed when OX40L−/− vs OX40L+/+ T-cells were infused in different models. These data provide insights as to the mechanism(s) responsible for OX40/OX40L regulation of GVHD.

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Introduction

T-cells receiving signals via the antigen-specific TCR require a second, costimulatory signal to stabilize cytokine mRNA and induce anti-apoptotic proteins\(^1\). Members of the immunoglobulin supergene and tumor necrosis factor (TNF)/TNF receptor families can function as costimulatory molecules. The latter include CD40L (CD154), 4-1BB receptor (CD137), CD30 and OX40 (CD134)\(^3\). OX40 is expressed on activated CD4\(^+\) and CD8\(^+\) T cells in mice and humans. OX40L (CD134L) is a type II membrane protein expressed on APCs including dendritic cells (DCs), B-cells and macrophages that have been activated by known inductive stimuli such as CD40 or proinflammarory mediators (eg. LPS)\(^4\). In addition, OX40L expression has been reported on endothelial cells, microglial cells, and T-cells\(^8\-11\). Signaling of OX40 receptor on antigen-specific CD4\(^+\) T cells results in production of Th1 and Th2 cytokines, upregulation of anti-apoptotic proteins,\(^12\-16\) clonal expansion and development into memory cells\(^11\,14\,17\-22\).

Because OX40 is upregulated following T-cell activation, studies were performed to assess the role of OX40/OX40L interaction on graft-versus-host disease (GVHD) which is mediated by alloantigen-activated donor T-cells. Tittle et al. observed that increased numbers of alloreactive CD4\(^+\) T-cells that co-expressed OX40 were present in the peripheral blood, lymphohematopoietic organs, and liver of non-irradiated F1 rat recipients of parental donor grafts that were experiencing a GVHD reaction\(^23\). Chen et al. showed comparable allogeneic MLR responses using OX40L\(^-\)/- dendritic cells (DCs) as stimulators of CD4\(^+\) T-cell proliferation and Pippig et al. demonstrated that OX40\(^-\)/- T-cells have a similar allogeneic MLR responses as OX40\(^+/+\) T-cells\(^11\,13\). Stuber et al. have shown that blocking OX40/OX40L interactions with an OX40-Ig fusion protein can diminish the intestinal manifestations of acute GVHD using an analogous non-irradiation semi-allogeneic system in mice as described by Tittle et al. in rats\(^23\,24\). Tsukada et al. extended these studies into lethally irradiated murine F1 recipients of parental donor grafts, representing the only published study to date examining the effects of blocking the OX40/OX40L pathway, using an OX40L mAb, on GVHD-mediated lethality\(^25\). Since OX40L also has been reported to be expressed on activated T-cells, it is possible that the anti-OX40L mAb reduced GVHD lethality, at
least in part, by the clearance or alteration of the biological function of donor T-cells rather than simply blocking the engagement of OX40 receptor on donor T-cells with OX40L on recipient cells\textsuperscript{11}.

While the rodent studies described above provide evidence that OX40/OX40L blockade can reduce acute GVHD responses, the mechanism(s) responsible have not been fully elucidated. The present studies were undertaken to investigate the potency and mechanisms by which OX40/OX40L interactions regulate GVHD. Additionally, the potential role of this pathway on the engraftment of pan-T cell depleted (TCD) allogeneic bone marrow (BM) was examined. To strengthen our conclusions, we used complementary approaches designed to target the OX40/OX40L pathway: antagonistic and agonistic mAbs, OX40 receptor deficient (OX40\textsuperscript{-/-}) donors and OX40L deletional mutant (OX40L\textsuperscript{-/-}) recipients. Our data indicate that OX40/OX40L interactions preferentially regulate both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell-mediated alloresponses in vivo. The protective effects of OX40/OX40L blockade did not require Stat-4 or Stat-6 signaling. Despite data indicating that OX40L can be expressed on T-cells, we have no evidence that such expression can significantly regulate GVHD. These data provide additional insights as to the mechanism(s) responsible for GVHD as influenced by OX40/OX40L interactions.
Materials and Methods

Mice

B10.BR/SgSnJ (H2^k), C57BL/6 (termed B6:H2^b,CD45.2), CD28-deficient B6 (CD28^-/-), C.H2^bm1 (termed bm1; CD45.2), BALB/c severe combined immune deficient (BALB/c-SCID), BALB/c Stat-4 deficient (Stat-4^-/-), BALB/c Stat-6 deficient (Stat-6^-/-), and C.H2^bm12 (termed bm12; CD45.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-CD45.1 congenic mice were purchased from the National Institutes of Health (Bethesda, MD). OX40^-/- mice were generated as described and backcrossed 9 generations with B6 mice. OX40L^+/+ mice were generated as described and used as inbred 129/Sv mice or backcrossed 5 generations to BALB/c mice, as indicated. 129/Sv OX40L^-/- or littermate controls were used as donors in some experiments, while BALB/c OX40L^-/- or littermates were used as recipients in other experiments. Mice were bred and housed in a specific pathogen-free facility in microisolator cages. Donors and recipients were used at 8 - 10 weeks of age.

mAb preparation

Agonistic anti-OX40 (clone M5, rat IgG1, kindly provided by Dr. Kenneth Mohler, Immunex Corporation, Seattle, WA) and antagonistic anti-OX40L (clone RM134L, rat IgG2b) were generated as ascites fluid and subsequently purified. Control rat IgG was purchased from Rockland Laboratories (Gilbertsville, PA). mAb injections were given at a dose of 200 µg per injection i.p. days –1 to +5 then thrice weekly through day +28 unless otherwise indicated.

GVHD generation
Different GVHD systems were used to analyze the effects of the OX40/OX40L pathway on alloresponses in vivo. In the first type, recipients were heavily irradiated to simulate human transplantation conditions. B10.BR recipients were lethally irradiated with 8.0 Gy total body irradiation (TBI) by x-ray (39 cGy/minute) on day -1 followed on day 0 by the intravenous infusion of pan-TCD BM (0.8-2 x 10^7), accomplished by treatment with anti-Thy1.2 (clone 30-H-12) plus rabbit complement. Donor BM cells were supplemented with splenocytes, purified lymph node (LN) T-cells, or LN T-cell subpopulations from B6 wild-type, B6 OX40^-/-, 129/Sv OX40L^-/-, B6 CD28^-/-, BALB/c Stat-4^-/- or BALB/c Stat-6^-/- donors, as indicated. To measure CD4^+ T-cell GVHD responses, bm12 recipients were lethally irradiated (8.0 Gy TBI) and then infused with TCD BM and splenocytes obtained from B6 donors. To determine the effects of OX40/OX40L interactions on GVHD and graft-versus-leukemia (GVL) induced by delayed lymphocyte infusion (DLI), B6 recipients were lethally irradiated (8.0 Gy TBI), reconstituted with TCD BM, and then given donor B10.BR splenocytes on day 21 post-BMT along with either irrelevant or anti-OX40 mAb infusions as described above. Some cohorts of mice were challenged with acute myeloid leukemia cells (C1498 derived from B6 mice) as previously described. To determine the effects of host OX40L expression on GVHD lethality, BALB/c OX40L^-/- or littermate controls were lethally irradiated (6.0 Gy TBI) and reconstituted with B6 TCD BM along with purified LN T-cells (0 or 1 x 10^6) obtained from B6 or B6 CD28^-/- donors.

To more specifically determine the effect of OX40/OX40L interactions on the GVHD capacity of CD4^+ or CD8^+ T cells, we used a system in which purified T-cell subsets are given to MHC disparate, sublethally irradiated recipients. This system permits highly accurate quantification of the degree of GVHD responses as related to T-cell dose. MHC class II (bm12) or class I (bm1) disparate recipients were irradiated with 6.0 Gy TBI on day 0 from a ^{137}Cesium source at a dose rate of 85 cGy/minute. Four to six hours after TBI, purified LN CD4^+ or CD8^+ T cells from B6, B6 OX40^-/-, 129/Sv OX40L^-/- or littermate control donors were infused. To complement studies in irradiated mice, one set of studies was performed in non-conditioned BALB/c-SCID recipients.
Recipients were depleted of NK cells by anti-asialo-GM1 anti-sera (25 µl on days −4 and −2) and infused with purified T-cells obtained from 129/Sv OX40L⁺ or littermate controls.

To purify LN cells, single cell suspensions of axillary, mesenteric, and inguinal LN cells were depleted of NK cells and enriched for CD4⁺ or CD8⁺ T cells by depletions with anti-CD8 (hybridoma 2.43, rat IgG2b, provided by Dr. David Sachs, Charlestown, MA) or anti-CD4 (hybridoma GK1.5, rat IgG2b, provided by Dr. Frank Fitch, Chicago IL), respectively. Rat mAb coated T cells were passaged through a goat anti-mouse and goat anti-rat Ig coated column (Biotex, Edmonton, Canada). The final composition of T cells in the donor graft was determined by flow cytometry and was always found to be 94% T cells of the desired phenotype. Hematocrit values were obtained at periodic intervals as an indicator of the possible bone marrow destructive effects of infused T cells. For all GVHD (and engraftment) systems, mice were monitored daily for survival and clinical appearance and weighed twice weekly.

**Engraftment studies**

Bm1 or bm12 mice were irradiated with 4.5 or 5.0 Gy TBI, as indicated, by x-ray on day -1 and given B6 TCD BM (0.7-1 x 10⁷) cells on day 0. Recipients were given irrelevant, anti-OX40 or anti-OX40L mAb (200 µg/dose) i.p. daily from days −1 to +6 then twice weekly through day 14. Donor or host chimerism was monitored in peripheral blood at 6 weeks and 3-4 months post-BMT using with αCD45.2 (clone 104-2, rat IgG2a) and αCD45.1 (clone A20-1.7, rat IgG2a), both provided by Dr. U. Hammerling (New York, NY). The T cell, B cell, and granulocyte/macrophage constituency of peripheral blood cells was measured using mAb directed toward CD4 or CD8, CD19, and Mac1, respectively. All fluorochrome labeled mAb, unless indicated, were obtained from PharMingen (San Diego, CA). Cells were first incubated with 2.4G2 to block Fc receptors, and then incubated with an optimal concentration of fluorochrome-labeled mAb for 45 min at 4°C. Cells were washed 3 times and resuspended for analysis by 3-
color flow cytometry using fluorescein isothiocyanate, phycoerythrin, or biotin (along with SA-PerCP) conjugated mAb purchased from PharMingen or Becton-Dickinson (Mountain View, CA). Irrelevant mAb control values were subtracted from values obtained with relevant mAbs. All results were obtained using a FACScalibur (Becton-Dickinson). Forward and side scatter settings were gated to exclude red cells and debris and 1 x 10^4 cells were analyzed for each determination.

Statistical Analyses

Group comparisons of continuous data were made by Student's t-test. Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of chi-square. Actuarial survival and relapse rates are shown. Probability (P) values 0.05 were considered significant.
Results

OX40/OX40L interaction regulates GVHD lethality. To determine the role of OX40/OX40L interactions on regulating GVHD responses, initial studies were undertaken to determine the magnitude of effects on OX40 receptor ligation in heavily irradiated B10.BR (H2k) recipients of B6 (H2b) donor BM and supplemental splenocytes (0, 5, or 15 x 10⁶/recipient). The administration of an agonistic anti-OX40 mAb significantly increased GVHD severity as evidenced by clinical appearance and body weight loss (not shown). Based upon donor splenocyte dose titrations, anti-OX40 mAb infusion accelerated GVHD by 5-fold (Figure 1A).

To determine whether the acceleration in GVHD lethality observed with anti-OX40 mAb was dependent upon the use of high-dose lethal irradiation which induces pro-inflammatory cytokines the effect of anti-OX40 mAb was investigated in a setting of DLI frequently used as a salvage
therapy for treating leukemia patients that relapse post-BMT\textsuperscript{30}. DLI in the form of donor splenocyte infusion causes less GVHD mortality than when the same number is infused on the day of BMT\textsuperscript{28}. B6 recipients were lethally irradiated and reconstituted with B10.BR TCD BM. On day 21 post-BMT, some cohorts of mice were given a low dose of donor splenocytes (5 x 10\textsuperscript{6}) and irrelevant or anti-OX40 mAb (beginning on day 20 post-BMT)\textsuperscript{27}. All mice surviving until day 28 were challenged with AML cells\textsuperscript{27}. Mice that received no DLI cells all succumbed to AML by day 50. Mice receiving DLI and irrelevant mAb experienced GVL but eventually died of AML. In contrast, recipients given both DLI and anti-OX40 mAb all died of GVHD by day 28, prior to AML cell infusion (Figure 1B). Thus, anti-OX40 mAb given later post-BMT substantially increased GVHD mediated by low-dose DLI.

While administration of an agonistic anti-OX40 mAb clearly has a potent effect on GVHD acceleration, engagement of the OX40 receptor by mAb may overestimate the magnitude of effect.
that is physiologically conferred by the binding of OX40 to OX40L during the process of GVHD generation. Therefore, complementary studies were performed in which donor splenocytes were obtained from wild-type or OX40\(^{-/-}\) mice to examine the effects of OX40 loss-of-function on GVHD (Figure 1C). Lethally irradiated B10.BR recipients were infused with B6 TCD BM and supplemental splenocytes (0 or 15 \(\times\) 10\(^6\)). As compared to the uniform lethality of wild-type splenocytes, donor splenocytes deficient in OX40 had a significantly reduced GVHD capacity with 30% of recipients surviving long-term.

As further evidence that the OX40/OX40L pathway affects GVHD, two additional approaches were conducted that targeted the OX40L component of the pathway. In the first, lethally irradiated B10.BR recipients were infused with B6 TCD BM, supplemental splenocytes (0 or 25 \(\times\) 10\(^6\)), and either irrelevant or anti-OX40L mAb (Figure 2A). Anti-OX40L mAb treated recipients had a 35% long-term survival, as compared to 0% in the controls. In the second approach,
BALB/c OX40L⁻/⁻ or wild-type littermate controls were lethally irradiated, reconstituted with B6 TCD BM, and given purified B6 T-cells (Figure 2B). Whereas none of the wild-type recipients T-cells survived beyond 8 weeks post-BMT, 52% of OX40L⁻/⁻ recipients survived long-term (4 months post-BMT). Similar results were observed in a different MHC disparate system in which B10.BR T-cells (10⁶) caused 100% GVHD lethality in 129/Sv (H₂b) recipients within 40 days post-BMT in contrast to 40% survival in 129/Sv OX40L⁻/⁻ mice at 100 days (Figure 2C).

Collectively, these data indicated that intact OX40:OX40L interactions are required for optimal GVHD generation in lethally irradiated recipients of fully allogeneic donor grafts.
OX40/OX40L interactions have a more pronounced effect on CD4\(^+\) T-cell than on CD8\(^+\) T-cell mediated alloresponses in both GVHD and alloengraftment

During GVHD induction in lethally irradiated B10.BR recipients of B6 BM and supplemental splenocytes \((10^7)\), we have observed that the OX40 receptor is upregulated on both CD4\(^+\) T-cells (22\% positive) and CD8\(^+\) T-cells (20\% positive) isolated from thoracic duct lymphatics on day 7 post-BMT (data not shown). To determine whether precluding OX40:OX40L binding would have similar effects on CD4\(^+\) vs CD8\(^+\) T-cell mediated GVHD, experiments were performed using highly purified T-cell subsets infused into recipients with an isolated MHC class I or class II only disparity. Because residual host T-cells remain in the sublethally irradiated recipients, the infusion of agonistic mAbs could affect GVHD responses by either stimulating donor anti-host reactions
resulting in increased GVHD or host anti-donor responses resulting in less GVHD lethality. To avoid this complication, these studies were performed with OX40−/− donor T-cells.

To assess CD8+ T-cells responses, sublethally irradiated bm1 recipients were given B6 CD8+ T-cells (0.3 × 10^6) obtained from OX40+/+ or OX40−/− donors. The infusion of B6 OX40−/− CD8+ T-cells resulted 12% survival as compared to 0% survival in recipients of wild-type CD8+ T-cells which was not of sufficient magnitude to be statistically significant or to rescue the vast majority of mice from lethality (P = 0.06) (Figure 3A). Thus, OX40/OX40L interactions do not appear to be a major regulator of CD8+ T-cell-mediated GVHD lethality.
To ascertain the function of OX40/OX40L in a CD4⁺ T-cell mediated GVHD system, OX40⁺/⁺ or OX40⁻/⁻ CD4⁺ T-cells were infused into sublethally irradiated bm12 recipients. Recipients of OX40⁻/⁻ CD4⁺ T-cells (0.3 x 10⁶) had a 75% survival rate vs. 16% with wild-type cells (P = 0.0003) (Figure 3B). Anti-OX40L mAb treatment of bm12 recipients of wild-type B6 CD4⁺ T-cells provided a high degree of GVHD lethality protection. Specifically, anti-OX40L mAb treatment rescued 100% vs 25% (P = 0.002) of recipients of low-dose (0.3 x 10⁵) and 88% vs. 0% (P = 0.0006) of recipients of high-dose (10⁵) CD4⁺ T-cells (Figure 3C). In lethally irradiated bm12 recipients, agonistic anti-OX40 mAb resulted in rapid mortality induced by a non-lethal dose of purified CD4⁺ T-cells (P< 0.0001) (Figure 3D). Thus, the OX40/OX40L pathway is more critical in driving CD4⁺ than CD8⁺ T-cell alloresponses.
Figure 3C

C57BL/6 CD4+ T-cells --b1m12

PROPORTION SURVIVING

DAYS POST-TRANSFER

- Irrel. mAb (1)
- Anti-CD40L mAb (0.3; 1.0)
- Irrelevant mAb (0.3)
The data presented above clearly indicate that OX40/OX40L interactions preferentially accelerate GVHD induced by CD4⁺ donor T-cells that encounter MHC class II antigens that are distributed throughout the host microenvironment. We next sought to determine whether OX40 receptor signaling regulates host CD4⁺ or CD8⁺ T cell-mediated BM graft rejection, a situation in which BM cells alone serve as the source of alloantigen. Agonistic anti-OX40 mAb administration resulted in a marked reduction in engraftment in sublethally irradiated bm12 recipients of donor B6 TCD BM at 6 weeks (Table 1) or 4 months (data not shown) post-BMT. In contrast, a blocking anti-0X40L mAb increased alloengraftment at two different TBI doses. Consistent with the lack of pronounced effects of OX40/OX40L interactions on modifying CD8⁺ T-cell mediated GVHD, anti-OX40 mAb administration had only modest effects in influencing alloengraftment in a CD8⁺ T-cell mediated rejection system (Table 1). Moreover, anti-OX40L mAb had no evidence of engraftment promoting properties in this setting (Table 1). These data demonstrate a more
pronounced role for the OX40/OX40L pathway in regulating CD4⁺ as compared to CD8⁺ T cells alloresponses in vivo.

Table 1: The role of OX40:OX40L in the engraftment of MHC class I or II-disparate T cell-depleted donor BM grafts¹.

<table>
<thead>
<tr>
<th>Group</th>
<th>TBI</th>
<th>Day</th>
<th>Donor</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B6-CD45.1-&gt;bm12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irrelevant mAb</td>
<td>5.0</td>
<td>115</td>
<td>15</td>
<td>88 (4) 12 (4)</td>
</tr>
<tr>
<td>αOX40 mAb</td>
<td>5.0</td>
<td>115</td>
<td>15</td>
<td>16 (2)* 87 (2)*</td>
</tr>
<tr>
<td>αOX40L mAb</td>
<td>5.0</td>
<td>115</td>
<td>15</td>
<td>97 (1)* 6 (1)</td>
</tr>
<tr>
<td>Irrelevant mAb</td>
<td>4.5</td>
<td>120</td>
<td>13</td>
<td>81 (7) 20 (6)</td>
</tr>
<tr>
<td>αOX40L mAb</td>
<td>4.5</td>
<td>120</td>
<td>14</td>
<td>94 (1)^ 10 (1)^</td>
</tr>
</tbody>
</table>

| **B6-CD45.1->bm1**    |     |     |       |      |
| Irrelevant mAb        | 5.0 | 97  | 15    | 88 (6) 12 (5) |
| αOX40 mAb             | 5.0 | 97  | 15    | 68 (9)^ 33 (9)^ |
| Irrelevant mAb        | 4.5 | 100 | 15    | 79 (8) 22 (7) |
| αOX40L mAb            | 4.5 | 100 | 15    | 65 (10) 36 (10) |

* P 0.05 versus Irrel. mAb controls; ^0.05 > P 0.1 vs Irrel. mAb controls

¹ B6-CD45.1 recipients (n = 15/group) were sublethally irradiated with TBI doses, as indicated, on day -1 and then given 0.7-1 x 10⁷ TCD BM from bm1 or bm12 donors and either irrelevant IgG, anti-OX40, or anti-OX40L mAb as described in Materials and Methods. On the indicated
day post-BMT, all surviving recipients were phenotyped to determine the donor or host origin of peripheral blood mononuclear cells. Values shown are mean percentages. The standard deviation of the mean is listed in ( ).
OX40L expression on donor T-cells does not have a major effect on GVHD lethality

Although OX40L expression has been reported on T-cells\textsuperscript{11}, the function of OX40L on in vivo T-cell alloresponses has not been examined. CD4\textsuperscript{+} T-cells (10\textsuperscript{5}) from 129/Sv OX40L\textsuperscript{-/-} or wild-type littermate controls were given to sublethally irradiated bm12 recipients (Figure 4A). There was no significant difference in survival of recipients of OX40L\textsuperscript{-/-} vs WT T cells. (56\% vs 38\%; P = 0.24). This was reproduced at a higher T-cell dose (data not shown). These findings are in sharp contrast to the 88-100\% long-term survival rates obtained with anti-OX40L mAb in the B6\rightarrow bm12 system at two different CD4\textsuperscript{+} T-cell doses, indicating that the major effect of anti-OX40L mAb is not on blocking T-cell expressed OX40L from binding with OX40 receptor-expressing cells. As might be anticipated from our findings on the relative lack of importance of the OX40/OX40L pathway on CD8\textsuperscript{+} T-cell alloresponses, there were no significant differences in median survival time in sublethally irradiated bm1 recipients of 129/Sv OX40L\textsuperscript{-/-} vs WT CD8\textsuperscript{+} T-cells (data not shown). Moreover, in non-conditioned, NK cell-depleted BALB/c-SCID recipients, CD4\textsuperscript{+} T-cells obtained from OX40L\textsuperscript{-/-} and WT littermate controls had a comparable efficacy in inducing GVHD lethality (Figure 4B). Under the conditions of these various GVHD models, we were unable to uncover a major role for OX40L expression on donor T-cells in regulating in vivo GVHD.
Figure 4A

129/Sv OX40L−/− or OX40L+/+CD4+ T-cells --> bm12

PROPORTION SURVIVING

DAYS POST-TRANSFER
Figure 4B

129/Sv OX40L−/− or OX40L+/+ CD8+ T-cells --> bm1

PROPORTION SURVIVING

- OX40L+/+ (3)
- OX40L−/− (3)
- OX40L+/+ (1)
- OX40L−/− (1)
GVHD protection induced by OX40/OX40L blockade is not dependent upon Stat-4 or Stat-6 signaling

Some studies have proposed that OX40 receptor ligation preferentially supports Th2 differentiation, while other demonstrate that Th1 responses are also affected by the OX40 pathway\textsuperscript{12-16,31,32}. In some models, Stat signaling can regulate the propensity of T-cells to differentiate into Th1 or Th2 cells. To determine whether the anti-GVHD effects of OX40/OX40L blockade were dependent upon Stat signaling, studies were performed using allogeneic splenocyte donors deficient in Stat-4 (typically Th1-defective) or Stat-6 (typically Th2-defective) signaling pathways\textsuperscript{33}. B6 recipients were lethally irradiated and reconstituted with BALB/c TCD BM and supplemental splenocytes (25 x 10\textsuperscript{6}) from Stat-4\textsuperscript{-/-} or Stat-6\textsuperscript{-/-} mice as indicated (Figure 5).
Cohorts of mice were given irrelevant, anti-OX40, or anti-OX40L mAb. Anti-OX40 mAb accelerated and anti-OX40L mAb inhibited GVHD lethality in recipients of Stat 4⁻/⁻ splenocytes (Figure 5A). Anti-OX40L mAb also inhibited GVHD lethality induced by Stat-6⁻/⁻ donor splenocytes (Figure 5B). Due to the rapid lethality of Stat-6⁻/⁻ splenocytes (88% lethality by day 7 post-BMT), the effects of anti-OX40 mAb on accelerating GVHD lethality were not observed (Figure 5B). These data indicate that the inhibitory effect of OX40/OX40L blockade does not depend upon Stat-4 or Stat-6 signaling.
The effects of regulating OX40/OX40L interactions on GVHD lethality does not require CD28 signaling.

Data from our group and others have shown that CD28/B7 interactions can modify GVHD responses. To determine if the anti-GVHD effects of OX40/OX40L blockade were redundant with CD28/B7 blockade, donor T-cells were obtained from B6 CD28\(^{-/-}\) mice and infused into lethally irradiated allogeneic recipients reconstituted with TCD BM. B10.BR recipients received B6 CD28\(^{-/-}\) splenocytes (Figure 6A: 15 \(\times\) 10\(^6\); Figure 6B: 25 \(\times\) 10\(^6\)) along with either irrelevant, anti-OX40, or anti-OX40L mAb. At both splenocyte doses, anti-OX40 mAb accelerated GVHD lethality while anti-OX40L mAb almost completely inhibited GVHD lethality.
In other studies, the infusion of purified B6 CD28<sup>−/−</sup> T-cells (3 x 10<sup>6</sup>) into lethally irradiated, TCD BM-reconstituted BALB/c OX40L<sup>−/+</sup> recipients resulted in a significant survival advantage as compared to BALB/c OX40L<sup>++</sup> littermate controls (Figure 6C). Taken together, these data indicate that the effects of the OX40/OX40L pathway on GVHD lethality in heavily irradiated recipients do not require CD28 signaling.

![Figure 6A](image-url)

C57BL/6 CD28<sup>−/−</sup> --> B10.BR

BM

CD28<sup>−/−</sup>, Anti-OX40L mAb

CD28<sup>−/−</sup>, Irrel. mAb

CD28<sup>−/−</sup>, Anti-OX40 mAb

DAYS POST-BMT

PROPORTION SURVIVING
C57BL/6 CD28-/- --> BALB/c OX40L-/- or OX40L++/

BM; OX40L-/-

BM; OX40L++/

CD28-/-; OX40L-/-

CD28-/-; OX40L++/+

Figure 6C
Discussion

The present study provides definitive data indicating OX40/OX40L interactions are critical for CD4+ and less critical for CD8+ T-cell alloreponses in vivo. The beneficial effects of blockade of the OX40/OX40L pathway did not require CD28 signaling. Although some studies have shown that the OX40/OX40L pathway is a more potent regulator of Th2 than Th1 responses suggesting that this pathway may be preferentially affected by Stat-6 vs Stat-4 signaling, our studies using splenocytes obtained from Stat-4+/− or Stat-6+/− donors indicate that this is not the case.

We have conclusively demonstrated that the OX40/OX40L pathway is an important regulator of GVHD in a variety of GVHD models with different pathophysiologival mechanism(s). These data extend those of Tsukada who used a single donor-recipient strain combination [C57BL/6- >C57BL/6 x DBA/2)F1] and a single approach (anti-OX40L mAb administration) to reduce GVHD lethality25. However this strain combination typifies a Th1/Tc1-mediated GVHD response and may not be representative of other GVHD systems in which both Th1/Tc1 and Th2/Tc2 responses can contribute to lethality. Moreover, the separate contribution of CD4+ and CD8+ T-cells to GVHD lethality in their GVHD was not examined, which is relevant since GVHD in mice and humans may be dominated by either CD4+ or CD8+ T-cells. Additionally, anti-OX40L mAb may directly affect donor T-cells since OX40L has been reported to be upregulated on activated CD4+ and CD8+ T-cells.

Our studies extend the literature by demonstrating the importance of the OX40/OX40L pathway in regulating GVHD by using 4 distinct and complementary approaches: agonistic and antagonistic mAbs and genetic deletion of either the OX40 receptor or OX40L. Three GVHD systems in which recipients were lethally irradiated and given MHC-disparate donor T-cells were used to determine the effects of OX40/OX40L on GVHD induced early post-BMT by both CD4+ and CD8+ T-cells. Since the injury induced by heavy irradiation can lessen the requirement for T-
cell costimulation early post-BMT, additional studies using a DLI model were performed in which
the OX40 receptor was purposefully engaged by agonistic mAb later post-BMT, at a time when
pro-inflammatory cytokine release and irradiation-induced tissue injury have subsided. In each
experimental setting, the OX40/OX40L pathway had a major biological effect in regulating
GVHD lethality.

We show in both GVHD and alloengraftment systems that the dominant effect of the
OX40/OX40L pathway is on alloreactive CD4$^+$ and not CD8$^+$ T-cells. Although the majority of
the literature has focussed upon CD4$^+$ T-cell responses, several studies have shown that this
pathway also regulates CD8$^+$ T-cell responses. Chen et. al. observed decreased CD4$^+$ and CD8$^+$
T-cell proliferation in OX40L$^{-/-}$ mice challenged with oxazolone$^{13}$. Kjaergaard et. al. have reported
that OX40 receptor is upregulated on tumor-infiltrating CD8$^+$ T-cells$^{36}$. In addition, therapeutic
anti-tumor effects due to CD8$^+$ cells were observed in anti-OX40 mAb-treated recipients of
adoptively transferred T-cell populations. De Smedt et al. showed that anti-OX40 mAb
administration enhanced the expansion of and acquisition of CTL activity by antigen-specific
CD8$^+$ T-cells that were exposed to antigen-pulsed DCs in an adoptive transfer model$^{15}$. In
contrast, other studies have shown that CTL responses to viral challenge were intact in mice that
lack either OX40 or its ligand$^{11,13,37}$. Even though our studies indicate a more modest direct effect
of the OX40/OX40L pathway on in vivo CD8$^+$ vs. CD4$^+$ T-cell alloreponses, it is possible that
CD8$^+$ T-cells may be more vigorously affected by this pathway under other circumstances. Also, it
is important to note that although the OX40 pathway may have only a modest direct effect on
CD8$^+$ T cells, effects on CD4$^+$ T-cell help may have critical indirect consequences on CD8$^+$ T-cell
expansion or the induction of CD8$^+$ CTLs. Therefore, blockade of the OX40 pathway may have
significant impact on CD8$^+$ dependent responses by the interference of CD4$^+$ helper function to
CTLs.
Our data indicate that blockade of the beneficial effects of blocking the OX40/OX40L pathway does not require CD28 signaling. We previously have shown that these two costimulatory pathways can provide non-redundant functions as assessed in vitro since OX40 receptor expression can be induced on T-cells from CD28⁻/⁻ mice and the stimulation of CD28⁻/⁻ by activated B-cells is substantially inhibited by anti-OX40L mAb. Walker et al. demonstrated that blockade of the OX40/OX40L pathway reduces the effects of anti-CD28 in restoring the defective germinal center formation in CTLA4-Ig transgenic mice. We previously have shown that the defective signaling of OX40⁻/⁻ T-cells could not be restored by anti-CD28 mAb, indicating that these pathways are non-redundant under these in vitro conditions and Gramaglia et al. have shown that B7-1 and OX40L act synergistically to stimulate proliferation and cytokine production by naive CD4⁺ T-cells. In contrast, Ndhlovu and colleagues provide data indicating a dependency of the biological effects of the OX40/OX40L pathway on the CD28/B7 pathway in regulating EAE generation. Our current and previous studies along with those by other investigators have shown that blocking the CD28/B7 pathway alone by using CD28⁻/⁻ donor T-cells was insufficient to uniformly prevent lethal GVHD. Similarly, GVHD lethality is not uniformly prevented by OX40/OX40L blockade. The high survival rate of recipients given CD28⁻/⁻ donor T-cells and anti-OX40L mAb suggest that the co-blockade of these two pathways may be particularly advantageous in preventing GVHD.

Compelling evidence exists that OX40 signaling can support CD4⁺ T-cells to develop into Th2 cells as assessed in vitro and in vivo. However, not all studies have shown an absolute dependence of the generation of Th2 responses on the OX40/OX40L pathway. In our studies, GVHD lethality was lessened by the administration of anti-OX40L mAb to recipients of Th2- as well as Th1-defective donor splenocytes, indicating that the GVHD preventative effect of anti-OX40L mAb was not strictly due to the preferential induction of Th2 responses as has been suggested by other GVHD studies. Our studies indicate that the regulation of GVHD lethality...
by the OX40/OX40L pathway does not depend upon either Stat-4 (Th1) or Stat-6 (Th2) signaling.

Although OX40L expression has been reported on activated T-cells, we did not observe differences in alloreponses in vitro as measured using OX40L−/− T-cells in primary MLR cultures. Consistent with those in vitro findings, donor OX40L−/− T-cells were still capable of mediating lethal GVHD similar to wild-type T-cells. These data are in striking contrast to the in vivo infusion of anti-OX40L mAb, indicating that the major effects of this mAb are on recipient and not donor cells. Anti-OX40L mAb likely has its major effect by blocking the costimulation of donor T-cells by recipient cells. Candidate recipient cells would include DCs, activated B-cells, and activated endothelium. There are at least two main consequences of this blockade. The first would be to inhibit the initial expansion of alloreactive T-cells and sustained expansion and long-term survival of antigen-activated CD4+ T-cells. This could be accomplished by reducing the proportion of alloreactive CD4+ T-cells that enter cell cycle upon encountering host alloantigens or by the induction of anti-apoptotic genes including bcl-xL and bcl-2. Antagonists that impair OX40 signaling would be useful to reduce the frequency of alloreactive CD4+ T-cells and to impede the survival of alloreactive T-cells that escape this preventive strategy. Our data also indicates that co-blockade of the CD28/B7 pathway may serve to further inhibit initial expansion of alloreactive T-cells. Conversely, strategies to augment OX40 signaling may be highly advantageous in generating anti-tumor cell responses by promoting initial expansion and the generation of a larger anti-tumor memory T-cell pool. Finally, an additional advantage of blocking the OX40/OX40L pathway also may impede the homing and migration of activated donor T-cells to GVHD target organs via binding to OX40L-expressing endothelial cells or cells within critical lymphohematopoietic organs that are essential for priming alloreponses.

Although an agonistic anti-OX40 mAb was not well-tolerated when given 3 weeks post-BMT along with DLI, it is possible that anti-OX40 mAb could be used to induce anti-tumor effects.
post-BMT when combined with either no DLI or lower DLI cell doses. Such an approach may be of particular benefit in an autologous setting because signaling via OX40 can break peripheral tolerance which may be therapeutically helpful in patients with residual hematological malignancy post-BMT that may continue to be tolerized by tumor antigens.

Our studies indicate that the OX40/OX40L pathway has a broad importance in GVHD induction. Several but not all studies in humans have suggested that OX40 upregulation can precede acute GVHD generation and may be a marker of steroid-resistant acute GVHD or chronic GVHD. Regardless of whether the upregulation of OX40 receptor is of prognostic significance, interruption of the OX40/OX40L pathway early post-BMT warrants testing as an approach to prevent GVHD and allogeneic BM graft rejection.
Abbreviations Used

B6, C57BL/6; bm1, C.H2^bm1; bm12, C.H2^bm12; BMT, bone marrow transplantation; CTL, cytotoxic lymphocyte; DC, dendritic cell; DLI, donor lymphocyte infusion; GVL, graft-versus-leukemia; LN, lymph node; mAb, monoclonal antibody; SCID, severe combined immune deficiency; TBI, total body irradiation; Tc1, T-cytotoxic type 1; Tc1, T-cytotoxic type 2; Th1, T-helper type 1; Th2, T-helper type 2; WT, wild-type
Figure Legends

Figure 1. **Targeting of the OX40 receptor regulates GVHD in heavily irradiated MHC class I + II disparate recipients.** 1A. B10.BR recipients (n = 8/group) were lethally irradiated and reconstituted with B6 bone marrow (BM) alone or containing supplemental splenocytes (S) from B6 donors. The splenocyte number x 10^6 is shown in ( ). Recipients of splenocytes received either irrelevant or anti-OX40 mAb beginning on the day before splenocyte administration as described in Materials and Methods. 1B: B6 recipients (n = 10/group) were lethally irradiated and reconstituted with B10.BR BM. On day 21 post-BMT, recipients were given splenocytes (5 x 10^6) (termed delayed lymphocyte infusion: DLI) followed 1 week later by challenge with AML cells. Beginning on the day prior to DLI, cohorts of mice were given irrelevant or anti-OX40 mAb. Mice receiving AML cells were autopsied for gross evidence of AML cells. Mice are listed as death with or without AML cells present. 1C. Mice (n = 8/group/experiment) were transplanted as described in 1A except splenocytes (15 x 10^6) were obtained from either OX40^+/+ or OX40^-/- donors. Data from two replicate experiments with similar results are pooled. In each instance, targeting of the OX40 receptor had a significant impact on survival rates.

Figure 2. **Targeting of OX40L regulates GVHD lethality.** 2A: B10.BR recipients (n = 8/group/experiment) were lethally irradiated, BM reconstituted and cohorts of mice given supplemental splenocytes (25 x 10^6) as indicated. Splenocyte recipients were administered irrelevant or anti-OX40L mAb beginning 1 day prior to splenocyte infusion. Data from 2 replicate experiments with similar results are pooled; 2B. BALB/c OX40L^-/- or OX40L^+/+ littermate controls (n = 8/group/experiment), as indicated, were lethally irradiated, BM reconstituted and cohorts were given B6 purified T-cells (10^6) Data from two replicate experiments with similar results are pooled; 2C. 129/Sv OX40L^-/- or OX40L^+/+ littermate controls (n = 5/group), as
indicated, were lethally irradiated, reconstituted with B10.BR BM and cohorts were given purified B10.BR T-cells ($10^6$). In each instance, targeting of OX40L had a significant impact on GVHD lethality.

**Figure 3.** OX40/OX40L interactions are more potent regulators of CD4$^+$ as compared to CD8$^+$ T-cell mediated GVHD lethality. 3A. Sublethally irradiated bm1 recipients (n = 8/group) were given highly purified CD8$^+$ T-cells (0.3 x $10^6$) from OX40$^{+/+}$ or OX40$^{-/-}$ donors as indicated; 3B. Sublethally irradiated bm12 recipients (n = 8/group/experiment) were given highly purified CD4$^+$ T-cells (0.3 x $10^5$) from B6 OX40$^{+/+}$ or OX40$^{-/-}$ donors as indicated. Data from two replicate experiments with similar results were pooled; 3C. Sublethally irradiated bm12 recipients (n = 8/group/experiment) were given highly purified B6 CD4$^+$ T-cells [0.3 or 1 x $10^5$ as indicated in ( )]. Mice also were given either irrelevant or anti-OX40 mAb; 3D. Lethally irradiated bm12 recipients (n = 5/group) were given highly purified B6 CD4$^+$ T-cells (0.3 x $10^6$). Mice also were given either irrelevant or anti-OX40 mAb. In each instance, survival was significantly affected for CD4$^+$ (but not CD8$^+$) T-cell mediated GVHD.

**Figure 4.** OX40L expression on donor T-cells is not a potent regulator of CD4$^+$ T-cell mediated GVHD lethality. 4A. Sublethally irradiated bm12 recipients (n = 8/group/experiment) were given highly purified CD4$^+$ T-cells (10$^5$) from either 129/Sv OX40L$^{-/-}$ or OX40L$^{+/+}$ littermate controls, as indicated. Data from two replicate experiments with similar results are pooled. Groupwise comparisons revealed a P value of 0.24; 4B. Sublethally irradiated bm1 recipients (n = 5/group) were given highly purified CD8$^+$ T-cells [1 or 3 x $10^6$, as indicated in ( )] from either 129/Sv OX40L$^{-/-}$ or OX40L$^{+/+}$ littermate controls. No significant differences were noted between relevant groups; 4C. Anti-asialo-GM1 anti-sera-pretreated BALB/c-SCID mice were given purified CD4$^+$ T-cells (10$^6$) from 129/Sv OX40L$^{-/-}$ or OX40L$^{+/+}$ littermate controls (n = 5/group). Survival was not improved in recipients receiving OX40L$^{-/-}$ vs OX40L$^{+/+}$ CD4$^+$ T-cells.
Figure 5. The regulation of GVHD lethality by the OX40/OX40L pathway does not depend upon Stat-4 or Stat-6 signaling in donor T-cells. B10.BR recipients (n = 8/group) were lethally irradiated, reconstituted with B6 BM, and given either no supplemental splenocytes or splenocytes (25 x 10⁶) from BALB/c or BALB/c Stat-4⁻/⁻ (5A) or BALB/c Stat-6⁻/⁻ (5B) donors. Cohorts of mice that received splenocytes were given either irrelevant, anti-OX40, or anti-OX40L mAb, as indicated. Anti-OX40L mAb infusion significantly reduced GVHD lethality. Conversely, anti-OX40 mAb significantly increased GVHD lethality in recipients of Stat-4⁻/⁻ splenocytes. GVHD lethality was rapidly lethal in recipients of Stat-6⁻/⁻ splenocytes with or without anti-OX40 mAb.

Figure 6. Blockade of the OX40/OX40L pathway is effective in inhibiting GVHD lethality in settings in which CD28 signaling is precluded. 6A, B. B10.BR recipients (n = 8/group) were lethally irradiated, reconstituted with B6 BM, and cohorts given C28⁻/⁻ splenocytes (6A: 15 x 10⁶; 6B: 25 x 10⁶). Recipients given splenocytes also received either irrelevant, anti-OX40 or anti-OX40L mAb. Survival was significant different in comparing groups receiving irrelevant mAb vs either anti-OX40 or anti-OX40L mAb; 6C. BALB/c-OX40L⁻/⁻ or OX40L⁺/⁺ littermate controls (n = 5/group) were lethally irradiated, reconstituted with B6 WT BM and given either no T-cells or B6 CD28⁻/⁻ T-cells (3 x 10⁶). OX40L⁻/⁻ recipients had a significantly higher survival rate than OX40L⁺/⁺ recipients.
References


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Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogenic bone marrow transplant (BMT) recipients

Bruce R Blazar, Arlene H Sharpe, Andy I Chen, Angela Panoskaltsis-Mortari, Christopher Lees, Hisaya Akiba, Hideo Yagita, Nigel Killeen and Patricia A Taylor

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