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Ox40 Costimulation Enhances the Development of T Cell Responses Induced by Dendritic Cells In Vivo

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Dendritic cells (DCs) are bone marrow-derived APCs that display unique properties aimed at stimulating naive T cells. Several members of the TNF/TNFR families have been implicated in T cell functions. In this study, we examined the role that Ox40 costimulation might play on the ability of DCs to regulate CD4+ and CD8+ T cell responses in vivo. Administration of anti-mouse Ox40 mAb enhanced the Th response induced by immunization with Ag-pulsed DCs, and introduced a bias toward a Th1 immune response. However, anti-Ox40 treatment enhanced the production of Th2 cytokines in IFN-γ−/− mice after immunization with Ag-pulsed DCs, suggesting that the production of IFN-γ during the immune response could interfere with the development of Th2 lymphocytes induced by DCs. Co-administration of anti-Ox40 with DCs during Ag rechallenge enhanced both Th1 and Th2 responses induced during a primary immunization with DCs, and did not reverse an existing Th2 response. This suggests that Ox40 costimulation amplifies an ongoing immune response, regardless of Th differentiation potential. In an OVA-TCR class II-restricted adoptive transfer system, anti-Ox40 treatment greatly enhanced the level of cytokine secretion per Ag-specific CD4+ T cell induced by immunization with DCs. In an OVA-TCR class I-restricted adoptive transfer system, administration of anti-Ox40 strongly enhanced expansion, IFN-γ secretion, and cytotoxic activity of Ag-specific CD8+ T cells induced by immunization with DCs. Thus, by enhancing immune responses induced by DCs in vivo, the Ox40 pathway might be a target for immune intervention in therapeutic settings that use DCs as Ag-delivery vehicles.

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Optimal activation of naive CD4+ T cells requires at least two signals provided by the APC: the recognition of an Ag/MHC complex by the TCR, as well as the interaction of costimulatory molecules with their ligands. The widely studied CD28/B7 (CD80/CD86) interaction has been proposed to represent the predominant costimulatory signal, but a number of other costimulatory receptors have also been described (1). Among these, members of the TNF/TNFR family may possess functions distinct from those described for CD28 and B7 molecules. One TNFR family member, Ox40, has been shown to be of particular importance in T cell responses (2). Ox40 was first identified on activated rat CD4+ T cells (3), and was further described to be expressed on activated CD4+ and CD8+ murine (4, 5) and human T cells (6). Studies have shown that ligation of Ox40 on T cells costimulates proliferation as well as cytokine production in vitro (7–11). In vivo, engagement of Ox40 was shown to increase expansion and survival of naive CD4+ T cells, and to enhance memory T cell survival by inhibiting peripheral deletion (12, 13). In some experimental settings, it has been suggested that Ox40 costimulation may alter the Th1/Th2 balance and play a role in the development of Th2 cells. Indeed, it has been proposed that Ox40 costimulation induces IL-4 secretion and favors the development of Th2 responses (7, 14, 15). Also, the absence of Ox40/Ox40 ligand (Ox40L) interactions in vivo abrogated progressive leishmaniasis in susceptible BALB/c mice by suppressing T cell differentiation toward Th2 cells (16). Moreover, Ox40-deficient mice are impaired in their ability to generate a Th2 immune response and in the development of allergic inflammation in a murine model of asthma (17), suggesting that Ox40 plays a role in the development of Th2 responses. In contrast to these studies, blocking of Ox40/Ox40L interactions has been shown to ameliorate Th1-induced pathologies like experimental allergic encephalomyelitis (2) (18), inflammatory bowel disease (19), and rheumatoid arthritis (20). Also, Ox40- and Ox40L-deficient mice are defective in Th cell proliferation, and exhibit impaired IFN-γ, as well as IL-4 and IL-5 secretion, suggesting that Ox40/Ox40L interactions participate in enhancing both Th1 and Th2 responses (13, 21). Moreover, Ox40L-deficient mice demonstrated impairment in the induction of alloantigen-specific CTLs (21), but showed normal CTL responses to vesicular stomatitis virus in vivo (22).

Among APCs, which in the mouse comprise dendritic cells (DCs), B cells, and macrophages, DCs appear to have the unique capacity to activate naive T cells in vitro and in vivo. DC pulsed extracorporeally can efficiently induce Th and CTL, as well as T cell-dependent humoral responses, in vivo (23). This property correlates with the capacity of DCs to express very high levels of antigenic peptide/MHC complexes and costimulatory molecules (24–26). Upon stimulation, they also show a high propensity to travel to lymphoid tissues such as the spleen and lymph nodes, where they interact with Ag-specific T cells and stimulate their activation (23, 27).

Recently, improved isolation techniques have led to the identification of multiple DC subsets. Most investigators distinguish at least two different subclasses of mouse DCs on the basis of their relative expression of CD8α or CD11b (28, 29). In vivo experiments have shown that either subset, pulsed in vitro with Ag, can prime naive T cells, but CD8α− DCs skew the T cell response toward Th2 while CD8α+ DCs skew toward a Th1 response, in an
IL-12-dependent manner (30, 31). In the study by Pulendran et al. (32), the CD11c<sup>+</sup>CD11b<sup>−</sup> subset (containing the CD8α<sup>+</sup> cells) induced high levels of IFN-γ and IL-2, but little Th2 cytokines, whereas the CD11c<sup>+</sup>CD11b<sup>−</sup> subset induced large amounts of IL-4 and IL-10, in addition to IFN-γ and IL-2.

Given the crucial role for DCs in the regulation of T cell responses, and the potentially beneficial role of OX40 costimulation on the enhancement and the maintenance of T cell activation in vivo, we tested the effect of direct OX40 costimulation on the outcome of T cell responses induced by DCs in vivo.

**Materials and Methods**

**Mice**

All mice were used at 7–10 wk of age. Female BALB/c mice were purchased from Taconic Farms (Germantown, NY). Female BALB/c IFN-γ knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DO11.10 TCR-transgenic mice specific for chicken OVA peptide 323–339 (DO11p) in the context of I-A<sup>b</sup> (33) and OT-I CD45.1 TCR transgenic mice specific for chicken OVA peptide 357–364 (OT-Ip) in the context of H-2K<sup>β</sup> (34) were bred at Immunex (Seattle, WA). All mice were maintained at Immunex under specific pathogen-free conditions according to federal guidelines.

**Flow cytometry**

Cells were analyzed by flow cytometry with a FACSCalibur cytometer (BD Biosciences, Mountain View, CA). The cells were stained in FACS buffer (PBS containing 2% FBS, 1% normal rat serum, 1% normal hamster serum, 1% normal mouse serum, and 10 μg/ml 2,4-D (anti-rat MHC class II) mAb). All mAbs were from BD PharMingen (San Diego, CA), except the anti-clonotypic KJ1-26 mAb specific for DO11.10 T cells, which was produced and labeled at Immunex. The mAbs used were as follows: FITC-anti-mouse CD11c (HL3), FITC-anti-CD45.1 (A20), PE-anti-mouse CD8α (53-6.7), PE-anti-mouse CD25 (PC61), PE-anti-mouse CD44 (IM7), PE-anti-mouse CD62L (MEL-14), and APC-anti-mouse-CD4 (GK1.5). Propidium iodide was added in the FACS buffer, and cells were gated accordingly to eliminate dead cells and debris from analysis.

**Reagents**

Recombinant human Flt3 ligand (FL) (Chinese hamster ovary cell-derived) was produced at Immunex. Anti-OX40 mAb 53Ab was generated as followed: Splenocytes from mice immunized with murine OX40-Fc emulsified in CFA. After three boosts, rat serum was collected and tested for binding to OX40-Fc and for blocking OX40-Fc binding to OX40L expressing cells. After the rat serum achieved a blocking titer, it was fused according to standard hybridoma protocols. The fusion was screened for reactivity to OX40-Fc and for blocking OX40-Fc binding to OX40L expressing cells. Some mice were injected at the time of immunization with 100 μg control rat Ig (Sigma Aldrich) or mAb anti-OX40 M5 by the i.p. route. Draining popliteal lymph nodes were harvested 5 days after primary immunizations or 2 days after secondary immunizations.

**In vitro assays**

LN cells were restimulated in vitro in triplicate with graded doses of Ag in Click’s medium supplemented with 0.5% heat-inactivated normal mouse serum and additives (penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME, HEPEs, and t-galactamine). Triplicate culture supernatants were pooled and assayed by two-site ELISA (BD Pharmingen) for IL-2 after 24 h of incubation, and for IL-4, IL-5, IL-10, and IFN-γ after 72 h of incubation. An amplification system was used as described (35). The detection limits were 50 pg/ml for IL-2, 50 pg/ml for IL-4, 50 pg/ml for IL-5, 50 pg/ml for IL-10, and 1 ng/ml for IFN-γ.

**Measurement of CD4<sup>+</sup> T cell expansion in vivo and restimulation in vitro**

Spleen and lymph node cell suspensions were prepared from DO11.10 mice, and the percentage of T cells expressing transgenic TCR was determined by flow cytometry, using anti-CD4 and anti-clonotypic KJ1–26 mAb. The equivalent of 5 × 10<sup>5</sup> CD4<sup>+</sup> KJ1–26<sup>+</sup> T cells were injected i.v. into the lateral tail vein of BALB/c recipients. One day later, recipients were immunized with peptide-pulsed DCs in the hind footpads, popliteal LN cells were harvested at different time points and counted, and T cell expansion was measured by flow cytometry using anti-CD4 mAb and anti-clonotypic mAb KJ1–26. The expression of CD25, CD44, and CD62L on gated CD4<sup>+</sup> KJ1–26<sup>+</sup> cells on LN cells from mice immunized with Ag-pulsed DCs 4 days earlier was measured by FACS analysis. The LN cells were restimulated in vitro in triplicate with graded doses of the DO11p in Click’s medium supplemented with 0.5% heat-inactivated mouse serum and additives. Triplicate culture supernatants were pooled and assayed by ELISA for IFN-γ after 72 h of incubation. ELISPOT plates were coated overnight with 5 μg/ml anti-mouse IFN-γ mAb (BD Pharmingen) in PBS at 4°C, washed, and incubated 1–3 h at 37°C with PBS-10% FBS to block nonspecific sites. Serial dilutions of LN cells from recipient mice immunized 4 days earlier with Ag-pulsed DCs were restimulated with 2 μg/ml DO11p, or without Ag in Click’s medium, supplemented with 0.5% heat-inactivated normal mouse serum and additives for 24 h. Plates were washed and incubated overnight at 4°C with biotinylated anti-mouse IFN-γ (BD Pharmingen) in PBS-10% FBS. Plates were washed and incubated for 2 h at 37°C with a 1/100 dilution of HRP-streptavidin in PBS, washed, and the enzymatic activity was revealed after incubation in 3,3′,5,5′-tetramethylbenzidine (Sigma Fast Tablets; Sigma Aldrich). The spots were counted under a binocular microscope by two investigators independently. The data represent the mean values of spots normalized for differences in transgenic T cell numbers at culture inception. For intracellular IFN-γ staining, LN cells from mice immunized with Ag-pulsed DCs 4 days earlier were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin for 4 h in the presence of 5 μM monensin (Sigma Aldrich). After harvest, cells were stained with anti-CD4 and anti-clonotypic KJ1–26 mAb for 20 min, washed with PBS, and fixed for 20 min in Cytotix/Cytoperm buffer (BD Pharmingen). Cells were washed twice in Cytoperm buffer and staining was performed for 30 min at 4°C with PE-conjugated control Ig or anti-IFN-γ Ab (BD Pharmingen) diluted in Cytoperm buffer. After staining, cells were washed with Cytoperm buffer, resuspended in PBS, and analyzed on a FACSCalibur (BD Immunocytometry Systems, Mountain View, CA).

**Measurement of CD8<sup>+</sup> T cell expansion in vivo and restimulation in vitro**

Spleen and lymph node cell suspensions were prepared from OT-I CD4<sup>+</sup>1 mice, and the percentage of T cells expressing transgenic TCR was determined by flow cytometry using anti-CD8, anti-CD45.1, and anti-TCR V<sub>γ</sub>2 V<sub>δ</sub>2 T cells were injected i.v. into the lateral tail vein of C57Bl/6 recipients. One day later, recipients were immunized with peptide pulsed DCs in the hind footpads and popliteal LN cells were harvested at different time points, counted, and T cell expansion was measured by flow cytometry using anti-CD8 mAb and anti-CD11c mAb.
CD45.1 mAb. Direct CTL activity was measured 4 days after DC immunization in a standard 51Cr-release assay. Briefly, C1498 targets cells were pulsed for 1 h at 37°C with or without 1 μM OT-Ip in the presence of 51Cr in complete medium. Cells were washed three times and 1 × 10⁶ cells/well were added to serial dilutions of LN cells isolated from DC-immunized animals in a total volume of 200 μl. After 6 h of incubation at 37°C, 100 μl of supernatant was removed and radioactivity was counted in a gamma-counter. Spontaneous and total release were determined by adding culture medium or detergent to target cells, respectively. Percent specific 51Cr release was calculated as 100 × (experimental release – spontaneous release) / (total release – spontaneous release). For restimulation in vitro, two-fold serial dilutions of LN cells were cultured in vitro with 1 × 10⁵ C57BL/6 spleen cells pulsed with 1 μM OT-Ip in complete medium. The 3-day culture supernatants were assessed for IFN-γ production by a two-site ELISA from BD PharMingen.

Results

Ox40-mediated costimulation enhances the development of Th1 responses induced by DCs in vivo

We tested the effect of an exogenous source of Ox40 costimulation, provided in the form of a rat anti-mouse Ox40 mAb (M5, described in Materials and Methods), on the DC-induced immune response. Mouse DCs, expanded after 11 days of treatment with FL, were isolated from spleens and pulsed overnight with KLH in GM-CSF-containing medium. A total of 3 × 10⁵ DCs were subsequently injected into the hind footpads of syngeneic mice. Some mice were injected with anti-Ox40 M5 (100 μg i.p.) at the time of immunization with DCs. The popliteal lymph nodes were harvested 5 days later, and immune responsiveness was analyzed by culturing LN cells with graded doses of KLH for 3 days. Consistent with previously published results (36, 37), lymph node cells from mice immunized with KLH-pulsed splenic DCs produced IL-2, IL-4, IL-5, IL-10, and IFN-γ when restimulated with the same Ag in vitro (Fig. 1). By contrast, lymph node cells from animals primed by injection of DCs together with anti-Ox40 mAb produced higher levels of IL-2 and IFN-γ, but did not secrete detectable amounts of IL-4, IL-5, and IL-10 under the same conditions (Fig. 1). T cell priming was prevented by pretreatment with anti-CD4 mAb in vivo, demonstrating that the response observed

FIGURE 1. Ox40-mediated costimulation enhances the development of Th1 responses induced by DCs in vivo. LN cells from mice injected with KLH-pulsed DCs with anti-Ox40 mAb treatment (dotted lines and □) or with control Ig (thick lines and ■) 5 days earlier were cultured with graded doses of KLH. Cytokine production was measured as described in Materials and Methods. ○, Untreated animals.
was dependent upon the presence of CD4⁺ T lymphocytes (data not shown). Anti-Ox40 mAb treatment alone without Ag-pulsed DCs did not result in a detectable immune response (data not shown). This result suggests that costimulation through Ox40 leads to a polarized Th1 primary response induced by DCs.

**Ox40-mediated costimulation enhances the development of Th1 responses induced by both major subsets of DCs in vivo**

Two major subsets of splenic DCs have been characterized based on the expression of CD8α or CD11b (28, 29), and recent data suggest that these different subsets of Ag-pulsed DCs can differentially regulate the development of Th1- or Th2-type responses (30–32). The strong induction of a Th1-type response induced by DCs in the presence of anti-Ox40 mAb prompted us to test the effect of Ox40 costimulation on the outcome of the immune response induced by subsets of DCs. DCs were isolated from spleens of FL-treated animals, pulsed overnight with KLH in GM-CSF-containing medium, and further separated according to CD8 expression by FACS sorting. Re-analysis of the sorted cell populations showed a purity of >96% (data not shown). Ag-pulsed DCs of either subset were subsequently injected into the hind footpads of naive syngeneic recipients. Some mice were also treated by i.p. administration of anti-Ox40 mAb at the time of immunization with DCs. Consistent with previously published data (30, 31), the subclasses of DCs demonstrated a capacity to skew CD4⁺ T cell differentiation toward Th2- or Th1-like profiles; i.e., the injection of CD8α⁺ DCs resulted in the activation and expansion of T cells secreting high levels of IL-4 and IL-5, low levels of IFN-γ, and immunization with CD8α⁻ DC-sensitized cells producing IFN-γ, but little IL-4 and IL-5 (Fig. 2). Coadministration of the anti-OX40 M5 mAb together with DCs (both subsets) induced the activation and the development of cells secreting high levels of IFN-γ, and no IL-4 and IL-5. This result suggests that both subsets of DCs induce a polarized Th1-type immune response in the presence of Ox40 costimulation.

**Ox40 costimulation amplifies Th1 and Th2 cytokines in a secondary response induced by DCs**

To test whether Ox40 costimulation can directly favor the skewing to a Th1 response and shift an established Th2 response to a Th1 response, we analyzed the effect of exogenous Ox40 costimulation on secondary responses induced by DCs. To this end, a primary immune response was induced by injection of unseparated KLH-pulsed DCs. Two weeks later, when the primary response had declined to baseline levels, mice were re-immunized with Ag-pulsed DCs with or without anti-Ox40 mAb. Draining LN cells were harvested 2 days after immunization, and restimulated in vitro with graded doses of Ag, according to a protocol described by Inaba et al. (38). Results in Fig. 3 indicate that mice immunized with DCs and rechallenged with unseparated DCs developed a mixed secondary cytokine secretion, consistent with previous reports (30). The distinct kinetics of primary vs secondary responses provides evidence that the T cell response of animals injected twice with DCs is an anamnestic response (38, 39). Indeed, a single injection of Ag-pulsed DCs 2 days before in vitro restimulation induced no detectable cytokines compared with two injections of DCs, which results in strong cytokine secretion by lymph node cells under the same conditions (Fig. 3). The coadministration of anti-Ox40 mAb together with DCs during the rechallenge enhanced both Th1 and Th2 responses that were induced during the primary immunization by DCs (Fig. 3). This suggests that Ox40 costimulation does not necessarily skew the immune response to a Th1 pattern, but amplifies any ongoing immune response.

**Ox40 costimulation enhances a Th2 response induced by DCs in IFN-γ-deficient animals**

The strong bias toward a Th1 response that was induced by Ox40 costimulation in the primary immunization with both CD8α⁻ and CD8α⁺ DCs may have been due to an effect of IFN-γ. To test this, KLH-pulsed, wild-type (WT) DCs were injected, with or without anti-Ox40 M5 mAb, into IFN-γ-deficient, syngeneic-recipient animals. Five days later, LN cells from these mice were restimulated in vitro with KLH, and cytokine secretion was measured. Fig. 4 shows that KLH-pulsed DCs induced higher secretion of IL-4 and IL-5 when injected into IFN-γ-deficient mice, compared with injection into WT recipients, and Ox40 costimulation further enhanced the production of IL-4 and IL-5. This result suggests that the production of IFN-γ during the immune response could interfere with the development of Th2-type lymphocytes induced by DCs.

![FIGURE 2](http://www.jimmunol.org/). In vivo anti-Ox40 mAb treatment enhances the development of Th1 responses induced by both major subsets of DCs. LN from mice injected with KLH-pulsed CD8α⁻ (squares) or CD8α⁺ (circles), DCs with anti-Ox40 mAb treatment (dotted lines and open symbols), or with control Ig (thick lines and closed symbols) 5 days earlier were cultured with graded doses of KLH. Cytokine production was measured as described in Materials and Methods.
Ox40 costimulation amplifies both Th1 and Th2 responses after immunization with KLH emulsified in adjuvant

We next tested the effect of exogenous Ox40 costimulation on the development of Th responses induced by Ag emulsified in adjuvants. Mice were immunized subcutaneously in the hind footpads with KLH in Ribi or Alum, with or without anti-Ox40 M5 mAb. The draining lymph node cells were harvested 5 days after immunization, restimulated in vitro with KLH, and cytokine production profiles were analyzed. The data in Fig. 5 show that the adjuvants induced distinct cytokine profiles, with Ribi and Alum preferentially driving Th1- and Th2-like responses, respectively. Ox40 costimulation strongly enhanced the cytokine response induced by each adjuvant. This supports the notion that signaling through Ox40 enhances ongoing immune responses without skewing toward a particular Th profile.

Ox40 costimulation increases the number of activated CD4+ T cells in vivo in a model using transferred TCR transgenic T cells

To gain further insight into the mechanism by which Ox40 costimulation influences T cell responses, we used an adoptive transfer system that allowed us to monitor the behavior of Ag-specific TCR transgenic T cells in vivo after immunization with DCs with or without direct Ox40 costimulation. To this end, 5 × 10⁵ naive T cells from DO11.10 MHC class II-restricted, OVA-specific transgenic mice were transferred into naive, unirradiated BALB/c recipients. Their presence was detected with the anti-clonotypic mAb KJ1.26. Mice were immunized s.c. in the hind footpads with DCs pulsed in vitro with the I-Ad-restricted OVA peptide, DO11p, with or without anti-Ox40 M5 mAb. The ensuing T cell expansion in draining lymph nodes was monitored by flow cytometry. The
transferred DCs primed T cell expansion in an Ag-specific manner, with similar kinetics, in the presence or absence of anti-Ox40 M5 mAb (Fig. 6A). The magnitude of expansion was significantly higher at day 4 \((p < 0.05)\), when mice received DCs and direct Ox40 costimulation. By day 14, the absolute numbers of transgenic T cells in the draining lymph nodes returned to baseline levels in both groups. Furthermore, at day 4, the percentage of T cells presenting an activated phenotype, as assessed by increased expression of IL-2Rα (CD25), CD44, and decreased expression of CD62L, was higher in the group treated with anti-Ox40 M5 mAb, compared with control-Ig treated mice (Fig. 6B). At day 4 after priming, total popliteal LN cells were restimulated in vitro with various concentrations of OVA peptide to assess cytokine production by Ag-specific T cells primed in vivo with DCs. Measurement of cytokine production in the cultures of LN revealed that T cells primed in vivo by pulsed-DCs together with anti-Ox40 Mab exhibited a \(>100\)-fold increase in the Ag dose-dependent sensitivity of IFN-γ production, compared with T cells primed by DCs only (Fig. 6C). In addition, Ag-specific IFN-γ-secreting CD4\(^+\) T cells numbers at day 4 were quantified by ELISPOT. The data are presented relative to the number of Ag-specific cells per well. Fig. 6D shows that mice immunized with DCs and anti-Ox40 mAb had 10-fold more IFN-γ-secreting, Ag-specific CD4\(^+\) T cells compared with mice immunized with DCs only. Moreover, the size of the spots was bigger in the group of mice immunized with DCs and anti-Ox40 mAb (Fig. 6E), suggesting that the level of secretion per T cell was higher. IFN-γ production on a per cell basis was directly assessed by intracellular staining (Fig. 6F). Four days after priming, total popliteal LN cells were restimulated in vitro with PMA and ionomycin for 4 h. Measurement of intracellular IFN-γ revealed that a higher percentage of Ag-specific T cells primed in vivo by pulsed-DCs together with anti-Ox40 mAb, secreted IFN-γ, and the level of cytokine secretion was higher on a per cell basis, compared with T cells primed by DCs only. Taken together, these data suggest that both the number of activated T cells and the level of cytokine secretion per T cell were higher when mice were immunized with DCs and anti-Ox40, compared with mice immunized with DCs only.

**Anti-Ox40 treatment in vivo increases DC-induced CD8\(^+\) T cell accumulation and activation in a model using adoptively transferred TCR transgenic T cells**

To determine the effect of anti-Ox40 treatment on the activation of CD8\(^+\) T cells induced by DCs in vivo, we used an adoptive transfer system in which \(1 \times 10^6\) naive T cells from OT-I CD45.1 MHC class I-restricted, OVA-specific transgenic mice were transferred into naive, unirradiated congenic C57BL/6 CD45.2 recipients. Mice were immunized s.c. in the hind footpads with DCs pulsed in vitro with the H-2K\(^b\)-restricted OVA peptide, OT-Ip, with or without anti-Ox40 M5 mAb. Accumulation of transgenic CD8\(^+\) T cells in the draining lymph nodes was monitored by flow cytometry. The data in Fig. 7A show that Ag-pulsed DCs were able to drive the expansion of CD8\(^+\) T cells in vivo, which peaked at day 4 and returned to baseline levels at day 14. The magnitude of expansion was significantly higher at the peak of the response \((p < 0.05)\) when mice received DCs and direct Ox40 costimulation (Fig. 7A). The development of effector functions by CD8\(^+\) T cells was also measured at the peak of the response. Lymph-node cells from adoptively transferred recipients were recovered 4 days after immunization with Ag-pulsed DCs, and cytotoxic activity was assayed directly on peptide-coated target cells. As seen in Fig. 7B, DCs alone were able to prime for a low level of cytotoxicity. In contrast, in vivo treatment with anti-Ox40 M5 resulted in a strong increase in specific cytotoxicity. Lymph node cells from mice immunized with DCs were also restimulated in vitro with syngeneic

**FIGURE 5.** Ox40 costimulation amplifies both Th1 and Th2 responses after immunization with KLH emulsified in adjuvant. LN from mice immunized with KLH emulsified in either Ribi or Alum with anti-Ox40 mAb (dotted lines and □), or with control Ig (thick lines and ■) 5 days earlier were cultured with graded doses of KLH. Cytokine production was measured as described in Materials and Methods.
spleen cells pulsed with the OT-I peptide and the production of IFN-γ was assessed from the 3-day culture supernatants (Fig. 7C). In vivo priming with Ag-pulsed DCs induced IFN-γ secretion by the LN cells upon in vitro restimulation. Anti-Ox40 M5 administration at the time of priming strongly increased this production.

Discussion

The induction of strong primary immune responses is dependent upon DCs, which have the unique ability to express high levels of antigenic and costimulatory signals, and sensitize naive T cells. In this report, we show that DC-induced CD4+ and CD8+ T cell responses in vivo are strongly amplified when Ox40 signaling is provided through an anti-Ox40 mAb.

Previously, it had been proposed that the Ox40-Ox40L pair of costimulatory molecules plays an important role in the development of Th2 responses. For instance, results from in vitro studies suggested that Ox40 stimulation is necessary to induce IL-4, but not IFN-γ, secretion by DC-activated human T cells, and promotes the activation of Th2-type immune responses (7). Strong expression of Ox40 on T cells was also found in a Th2-type cytokine environment in a model of Th2-mediated, systemic autoimmunity (40). However, Chen et al. (22) demonstrated that DCs from Ox40L KO mice show a reduced capacity to induce IL-2, IL-4, and IFN-γ production by allogeneic CD4+ T cells. Similarly, Ox40L KO mice exhibited impaired T cell priming and Th cytokine production when immunized with KLH in adjuvant (21). In another study, Ox40-deficient animals exhibited reduced primary Th1 and Th2 responses to KLH (13). Blocking of Ox40L-Ox40 interactions in several models of autoimmunity involving Th1-type cells ameliorates the diseases (18–20), and reduces the levels of Th1-related cytokines such as IFN-γ, IL-12, and TNF-α, suggesting that Ox40 costimulation may also participate in the enhancement of Th1-type immune responses. Our results show that the activation of naive T cells induced by DCs in vivo can be dramatically enhanced through Ox40 ligation. Administration of anti-Ox40 mAb during primary immunization with either CD8α+ or CD8α− DCs induced a bias toward a Th1-type immune response. By contrast, administration of anti-OX40 mAb only during antigenic rechallenge enhanced both Th1 and Th2 responses that were established following priming with DCs (Fig. 3). Thus, Ox40 costimulation does not appear to redirect an ongoing Th2 response toward a Th1 response; rather, it will amplify the cytokine profile established during a primary response. We also show that Ox40 costimulation strongly enhances KLH-specific Th1- and Th2-type responses preferentially induced by Ribi and Alum, respectively. Together, these results do not support a role for Ox40-signaling in the differential polarization of CD4+ T cells, but do support the notion that costimulation through Ox40 is required for optimal activation or

![FIGURE 6. Ox40 costimulation increases the number of activated CD4+ T cells in vivo a...](http://www.jimmunol.org/content/ji/171/4/667/F6.large.jpg)

- **A**: Kinetics of expansion. DCs were pulsed in vitro with DO11p and injected into the hind footpads of mice that had previously received Ag-specific DO11.10 CD4+ TgT cells. Mice also received a single i.p. injection of 100 μg control Ig or anti-Ox40 M5 at the time of immunization. A, T cell expansion was measured by flow-cytometric analysis of pooled draining lymph node cells from three mice per group at different times after immunization. The figure represents the mean expansion + SEM combined for three (d10 and d14), four (d2), five (d4), and six (d3 and d5) independent experiments. □, DCs + control Ig; □, DCs + anti-Ox40 mAb. Statistical analysis was conducted using a paired t test, and the results were considered significant at p < 0.05. B, FACS analysis for CD25, CD44, and CD62L expression on gated CD4+ KJ1-26+ cells in LN cells from mice immunized with Ag-pulsed DCs, together with (thick line) or without anti-Ox40 mAb (thin line) 4 days earlier. Dotted line, isotype controls. C, IFN-γ production by LN cells restimulated in vitro for 72 h with graded concentrations of DO11p 4 days after priming with Ag-pulsed DCs, together with (dotted lines and □) or without anti-Ox40 mAb (thick line and □). D, Number of IFN-γ-producing cells measured by ELISPOT in LN cells restimulated in vitro for 24 h with 2 μg/ml DO11p 4 days after priming with Ag-pulsed DCs, together with (dotted lines and □) or without anti-Ox40 mAb (thick line and □). E, Representative ELISPOTs from LN cells restimulated in vitro for 24 h with 2 μg/ml DO11p 4 days after priming with Ag-pulsed DCs, together with (bottom) or without anti-Ox40 mAb (top). F, Intracellular staining for IFN-γ on gated CD4+ KJ1.26+ cells in LN cells restimulated in vitro for 4 h with PMA and ionomycin. Numbers refer to the percentage of cells producing IFN-γ above background level and the mean fluorescence intensity for the CD4+ KJ1.26+ population in each group.
maintenance of CD4+ T cells, and may serve as an amplification system for an ongoing immune response.

Two recent reports have highlighted a role for different subsets of DCs in the differentiation of distinct T helper populations (30, 32). In one study, CD11c+ CD11bhi DCs (containing a majority of the CD8α+ DCs) loaded in vitro with an OVA peptide, and injected into the footpads of syngeneic mice transferred with T cells from OVA-specific D011.10 TCR transgenic mice, induced high levels of IFN-γ and IL-2, but only low levels of Th2 cytokines. The CD11c+ CD11b+ subset (which is CD8α− DCs) induced large amounts of IL-4 and IL-10, in addition to IFN-γ and IL-2 (32). In another study, CD8α+ and CD8α− DCs, when pulsed ex vivo with KLH and injected into syngeneic mice, induced Th2- or Th1-type immune responses, respectively (30). Cytokines present in the microenvironment at the time of priming, as well as the relative expression of costimulatory molecules, may be the decisive elements driving the CD4+ T cell response toward a particular Th profile. Indeed, functional blockade of CD86 at the time of DC injection does not affect the priming capacity of DCs, but abrogates IL-4 secretion and leads to enhanced secretion of IFN-γ by T cells, suggesting that CD86 might play a role in the development of Th2 responses (37). Neutralization of CD80 in this system had little effect on cytokine production. In contrast, IL-12 appears to be the key factor biasing toward Th1 immune responses in vivo, as evidenced in part by the observation that injection of exogenous IL-12, along with either subset of Ag-pulsed DCs led to a polarized Th1 response (30). Alternatively, DCs from IL-12 p40 KO mice are incapable of inducing a Th1-type response after injection into a WT host (30). Here, we show that the presence of IFN-γ during priming with Ag-loaded DCs may have a negative effect on the development of IL-4 producing cells. Injection of Ag-pulsed DCs into an IFN-γ−/− host led to increased production of IL-4 compared with injection into a WT recipient. This effect was further enhanced by administration of anti-CD40 mAb, suggesting that the strong Th1 bias induced by CD40 costimulation during primary immunization with both CD8α+ and CD8α− DCs might be dependent upon the early production of IFN-γ during the immune response. Of note, when anti-CD40 was administered on day 3 rather than at the time of immunization, high levels of IFN-γ and moderate levels of IL-4 were induced upon in vitro restimulation (data not shown). In the same line of evidence, it is interesting to note that CD8α+ DCs, when pulsed ex vivo with KLH plus IFN-γ and injected into syngeneic animals, induce a Th1-type immune response as opposed to the Th2-type immune response normally observed with this DC subset (R. Maldonado-Lopez, unpublished observation). Whether or not this effect is dependent on an increased production of IL-12 by DCs after IFN-γ incubation is still a matter of speculation. Of note, several reports have indicated that IL-12 production by DCs requires prior activation with microbial agents or T cell derived factors (41–45). For instance, IFN-γ was shown to enhance production of both (p40)2 homodimeric and p75 heterodimeric forms of IL-12 (41). Thus, these results suggest that DCs drive Th2 development in a neutral environment and this may occur as the default response in the absence of IL-12 or other proinflammatory cytokines such as IFN-γ.

Our results also suggest that the capacity to skew to particular Th responses by different subsets of DCs is not fixed, and may depend upon microenvironmental factors to which DCs are exposed around the time of interaction with Ag-specific T cells.

Expression of OX40L has been reported on cells of the immune system including B and dendritic cells (21, 46–48). Stimulation of DCs via CD40 may be a critical signal for expression of OX40L (21). Because the expression of CD40L is induced on T cells shortly after activation (49), one might speculate that OX40L expression in vivo is closely regulated in time, and that OX40L only appears on the surface of DCs upon interaction with Ag-specific T cells. CD40L−/− mice present the same impairment of in vivo T cell priming in response to protein Ags as OX40L−/− deficient mice (50). Other costimulatory molecules such as CD80 and CD86 are highly expressed on mature DCs, and their blockade completely abrogates the priming capacity of DCs in vivo. As the DCs used in this study expressed high levels of CD80, CD86, and CD40 costimulatory molecules but no OX40L (data not shown) at the time of injection, our results suggest that OX40 costimulation could further enhance CD80/CD86/CD28 costimulation, but also could bypass the need for a further activation of DCs in vivo by Ag-specific CD4+ T cell response toward Th1 or Th2 is crucial for determining the nature of the immune response.
T cells through CD40. In conclusion, the close relationship between costimulatory systems such as CD80/CD86-CD28 and CD40-CD40L suggests that they may work cooperatively, but the plausibility of this scheme in the development of T cell responses will require further investigation.

The exact mechanism of action of Ox40 costimulation is still a matter of speculation. An agonistic mAb to Ox40 was shown to enhance clonal expansion and survival of Ag-specific CD4+ T cells (13), suggesting that the effect of Ox40 costimulation regulates the extent of T cell expansion and differentiation, rather than T cell activation and effector functions. This effect of Ox40 costimulation on T cell expansion was particularly striking at low doses of Ag, which produced a weak T cell response alone (13). Our results are somewhat in accordance with these data. Indeed, in an adoptive transfer system of transgenic CD4+ T cells specific for OVA, we have shown that Ox40 costimulation, together with pulsed DCs, induced a significant increase in the number of Ag-specific T cells 4 days after immunization and a 5- to 10-fold increase in the number of cells producing IFN-γ upon restimulation, compared to immunization with DC alone. Moreover, at the peak of the response, Ag-specific T cells from mice immunized with DCs and anti-Ox40 had a phenotype of activated cells (high expression of CD25 and CD44, low expression of CD62L) and secreted higher levels of IFN-γ on a per cell basis, compared to Ag-specific T cells from animals immunized with DCs alone. These results suggest that not only the number of T cells, but also the level of activation are increased through Ox40 costimulation.

In this report, we show that CD8+ T cell expansion and the development of specific cytotoxic activity and secretion of IFN-γ induced after immunization with Ag-pulsed DCs are strongly increased by treatment with anti-Ox40 mAb. The prevalent theory for the development of CTL responses induced by DCs postulates that specific helper CD4+ T cells are essential (51). These cells were originally thought to provide cytokines, such as IL-2, that facilitate activation and survival of CD8+ T cells. Recent reports have suggested an alternative model wherein CD4+ T helper cells must interact with and activate DCs, which are then capable of stimulating pre-CTLs. The pretreatment of DCs with agonistic anti-CD40 can replace CD4+ T cell help, suggesting that interactions between CD40 and CD40L are responsible for the “conditioning” of the DCs by CD4+ T cells (51–53). However, recent work by Lu et al. (54) has suggested that in addition to CD40-dependent activation of DCs, important components of CD4+ help CD8+ CTLs to comprise CD40-independent DC sensitization and direct CD4+. CD8+ T cell communication through the release of cytokines. The source of CD40-independent activation of CD8+ T cells is still a matter of debate, but the Ox40-Ox40L pair may be promoting candidates. Franco et al. (55) have also recently shown that priming and memory generation of Ag-specific CD8+ CTL does not require help when the immunogen binds MHC class I molecules with high affinity. The OVA peptide used in this study (SIINFEKL) is a high affinity peptide (34), and CD4+ cells are dispensable for the DC-induced CD8+ T cell activation in vivo in this system (T. De Smedt, J. Smith, and C. Maliszewski, manuscript in preparation). This suggests that the enhancement of CTL activity induced by DCs observed with anti-Ox40 treatment is a direct effect on CD8+ T cells.

Due to their powerful activity in the generation of immune responses, several approaches are being taken to use DCs to modulate immune responses (23, 56). However, the unique capacity of DCs to sensitize naive T cells is not constitutive, and develops through an activation process termed maturation, which is characterized by an increase in the expression of MHC molecules and costimulatory molecules such as CD80, CD86, and CD40 (23). In general, strategies that use DCs as vaccine adjuvants aim at increasing the maturation and/or activation of DCs through the use of stimuli, such as TNF-α, CD40L, and PGE2 (57). An alternative or complementary approach would be to increase expansion and effector functions of DC-activated T cells by targeting appropriate costimulatory molecules on T cells. The data that we present in this report are relevant to the enhancement of anti-tumor responses induced by treatment with anti-Ox40 mAb observed in different murine tumor models (58, 59). Given the strong effect of Ox40 costimulation that we observed, the combination of DCs with activating agents such as anti-Ox40 mAbs may prove to be more effective than either regimen alone in attempts to generate effective immune responses against tumors. This approach would also have the advantage of maintaining the specificity of the response because only recently activated Ag-specific T cells express the Ox40R. Conversely, altering the interactions between DCs and T cells by blocking the Ox40-Ox40L pathway might prove to be useful in deleterious immune responses such as autoimmunity.

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