Dendritic Cell Expression of OX40 Ligand Acts as a Costimulatory, Not Polarizing, Signal for Optimal Th2 Priming and Memory Induction In Vivo[1]

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Costimulatory cross-talk can occur at multiple cellular levels to potentiate expansion and polarization of Th responses. Although OX40L ligand (OX40L) is thought to play a key role in Th2 development, the critical cellular source of this molecule has yet to be identified. In this study, we demonstrate that OX40L expression by the initiating dendritic cell (DC) is a fundamental requirement for optimal induction of primary and memory Th2 responses in vivo. Analysis of the kinetics of the residual Th2 response primed by OX40L-deficient DC suggested a failure to stimulate appropriate expansion and/or survival of T cells, rather than an inability to polarize per se. The dependence upon OX40L was predominantly due to the provision of signaling through OX40 rather than retrograde signaling to the DC. Mechanistically, impaired Th2 priming in the absence of OX40L was not due to exaggerated regulation because there was no evidence of increased expansion or function of regulatory cell populations, suppression through IL-10 production, or hyporesponsiveness to secondary challenge. These data define a critical role for DC-derived OX40L in the induction and development of Th2 responses in vivo. *The Journal of Immunology,* 2007, 179: 3515–3523.

Generation of Th2 immunity is associated with $CD4^+$ T cell expansion, production of IL-4, IL-5, IL-13, and IL-10, and expression of GATA3 (1, 2). This polarization requires a series of interactions between APC, naive Th cells, and a network of supporting cells, which involve the delivery of both antigenic and costimulatory signals. Dendritic cells $(DC)^3$ are the key APC that link the innate and acquired immune response through their unique ability to efficiently capture and present Ag, interpret pathogen information, and subsequently polarize expanding Th cells (3). However, although the DC-derived costimulatory and polarizing signals involved in Th1 differentiation are increasingly well defined, much less is known about those required for the generation of Th2 responses.

The broad spectrum of receptors ascribed T cell costimulatory function is dominated by the CD28 and TNFR families (4). The TNFR family member OX40, and its TNF-related ligand OX40 ligand (OX40L), play a key role in $CD4^+$ Th-mediated immunity at multiple levels, including Th priming, effector cell function, and generation and maintenance of memory (5–8). The OX40:OX40L partnership appears particularly important for the generation of Th2 responses in vivo, whereas their requirement in Th1-mediated responses is not always paramount (5, 6). As such, mice deficient for OX40 or OX40L, or adoptively transferred with $OX40^{-/-}$ $CD4^+$ T cells, mount defective primary and/or memory Th2 responses in a number of experimental models, including contact hypersensitivity (5), sensitization with Ag in alum (9), allergic asthma (7, 10, 11), and infection with the helminth *Heligmosomoides polygyrus* (12).

Whereas the expression of OX40 appears restricted primarily to naturally occurring $CD25^+$ regulatory T cells (Tregs) and activated Th cells, the expression of OX40L is widespread. It has been described chiefly on APC, such as DC, following exposure to certain pathogens, and its expression can be further enhanced by CD40 signaling (6, 13). Studies using blocking Abs or OX40L-deficient cells have revealed a critical role for the expression of this molecule on DC for Th2 priming and polarization in vitro (5, 6, 14, 15). However, evidence for a specific requirement for OX40L expression by DC in vivo is currently circumstantial. Conclusions have been drawn using either complete $OX40L^{-/-}$ or $OX40^{-/-}$ mice or the adoptive transfer of OX40-deficient T cells into $OX40^{+/+}$ wild-type (WT) mice (5, 16), yet no study to date has definitively shown that expression of OX40L on DC is critical. Indeed, in terms of other APC, transfer of $OX40L^{+/+}$ B cells 24 h after immunization with OVA/alum is sufficient to rescue the defective Th2 response in $OX40L^{-/-}$ mice (17), and there is increasing evidence to support provision of OX40L by other cells, such as $CD3^-$ $CD4^+$ cells from LN (18), microglia (19), endothelium (20), and activated mast cells (21). Complicating matters further, OX40L can also be expressed in an Ag-dependent manner by $CD4^+$ T cells with the same kinetics as OX40 and, critically, in an OX40-independent manner (22, 23). Moreover, $CD4^+$ T cell expression of OX40L can contribute to the magnitude and longevity of Ag-specific responses both in vitro and in vivo (23). Therefore, the fundamental question of the importance of expression of OX40L by Ag-presenting DC during Th priming in vivo remains to be answered.

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[3] Abbreviations used in this paper: DC, dendritic cell; OX40L, OX40 ligand; Treg, regulatory T cell; WT, wild type; SEA, soluble egg Ag from *Schistosoma mansoni*; BM, bone marrow; LN, lymph node; dLN, draining LN; MHC II, MHC class II; Foxp3, Forkhead/winged helix transcription factor 3.

We have shown previously that exposure of DC to *Schistosoma mansoni*-soluble egg Ag (SEA) conditions them to drive Th2 responses, a process that is dependent upon DC expression of MHC class II (MHC II) (24), NF-κB (25), and CD40 (26). In this study, we have directly addressed the importance of DC expression of OX40L in Th2 induction by transferring OX40L-deficient Ag-loaded DC into OX40L$^{+/+}$ (WT) recipient mice. We formally demonstrate that expression of OX40L on the priming DC is critical for optimal induction of the Th2 response in vivo. The severely impaired priming observed after transfer of OX40L$^{-/-}$ DC was rescued by concurrent treatment of recipient mice with agonistic anti-OX40 mAb. Importantly, defective priming occurred independently of either recipient IL-10 production or the presence of CD25$^+$CD4$^+$ regulatory cells. Thus, we propose that OX40L-OX40 interaction provides a critical positive signal to T cells downstream of CD40 signaling to Th2-driving DC.

Materials and Methods

Mice and parasite material

All mice were bred and maintained at the animal facilities of the School of Biological Sciences at the University of Edinburgh according to home office guidelines. Animals used were ages 6 wk or older. Endotoxin-free SEA from *S. mansoni* was prepared in-house, as previously described (24), or was by provided by Prof. Mike Doenhoff (University of Bangor, Bangor, U.K.). *Propionibacterium acnes*, a Gram-positive bacterium, was obtained from American Type Culture Collection (catalog no. 6919).

Production of DC

DC were generated by culturing bone marrow (BM) from WT or gene-deficient mice for 10 days in the presence of rGM-CSF (20 ng/ml; Pepro-Tech) as previously described (24). Resultant cells were replated in 6-well non-tissue culture plates (Nalge Nunc International) for 18 h at 2×10^6 cells/ml in RPMI 1640 (Sigma-Aldrich) containing 10% FCS (Harlan Se-ralab), 2 mM L-glutamine (Invitrogen Life Technologies), 200 U/ml penicillin, 100 μg/streptomycin, and 5 ng/ml GM-CSF, with or without SEA (25 μg/ml). In certain experiments, cells were also cultured with purified agonistic anti-CD40 (FGK-45) or isotype control (MAC-1) mAbs (30 μg/ml; generated from hybridomas in-house), after which cells were harvested for RNA extraction. DC activation status was determined by flow cytometric analysis of the surface markers CD11c, MHC II, CD40, CD80, and CD86 (purity was typically >90% CD11c$^+$MHC II$^+$) and by ELISA detection of IL-12p40, IL-6, and TNF-α in culture supernatants.

In vivo T cell priming

Naive mice were injected i.p. with DC or SEA-stimulated DC (5×10^5 cells). After 7 days or, in some experiments, on day 2, 4, 7, or 14, recipient mice were sacrificed and their splenocytes restimulated in vitro with or without SEA (15 μg/ml) in X-VIVO 15 medium (BioWhittaker) supplemented with 2 mM L-glutamine and 50 μM 2-ME (Invitrogen Life Technologies). Culture supernatants were analyzed at 72 h for production of IL-4, IL-5, IL-10, IL-13, and IFN-γ. In some experiments, splenocytes were analyzed for the number of IL-4-producing cells at 72 h by ELISPOT. In other experiments, mice were injected in the hind footpads with DC (2.5×10^5 cells/footpad) and then given 200 μg of anti-OX40 (clone OX86) or isotype control (MAC-49) mAb i.p. on day 0 and again on day 2 to correspond with peak expression of OX40 on responding Th cells (27). Splenocytes and popliteal lymph node (LN) cells were restimulated on day 7 as detailed above, with total cell numbers calculated by hemocytometer. For depletion of CD25$^+$ cells, mice were injected i.p. with 1 mg of anti-CD25 depleting mAb (clone PC61) or isotype control mAb (MAC-49) 3 days before s.c. injection of DC. OX86, PC61, and MAC-49 mAbs were produced from hybridomas in-house.

Memory/tolerance assay

Mice were initially sensitized by i.p. injection of PBS or WT or gene-deficient DC that had been cultured with or without 25 μg/ml SEA. Eight weeks after sensitization, mice were challenged in each hind footpad with 2500 *S. mansoni* eggs in PBS or with PBS alone. After 7 days, popliteal LN and spleens were removed and cells restimulated in vitro with SEA as described above.
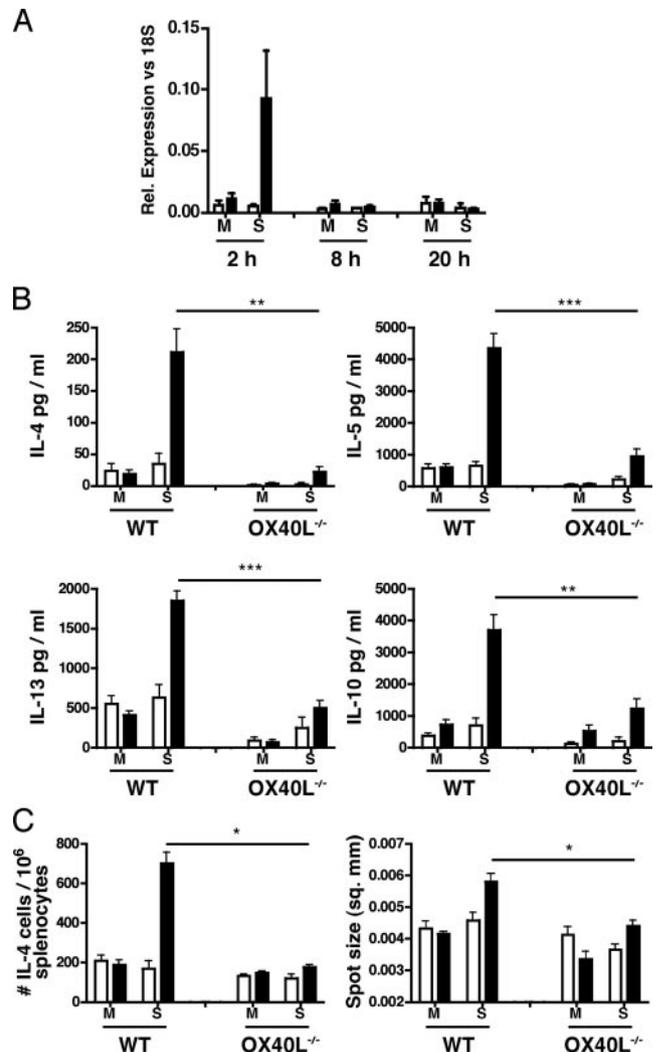

FIGURE 1. DC expression of OX40L is dependent upon signaling through CD40 and is required for optimal Th2 priming in vivo. Following in vitro conditioning with SEA (S) or medium (M) for 4 h, DC were cultured for another 2, 8, or 20 h with (■) or without (□) anti-CD40 mAb (FGK-45). DC were then harvested and expression of OX40L was measured using quantitative PCR. Expression of OX40L was normalized to 18S RNA. Data shown are mean ± SEM of expression levels measured in triplicate wells for each treatment and are representative of two experiments (A). In separate experiments, DC derived in vitro from WT or OX40L$^{-/-}$ BM were cultured overnight with medium alone or SEA before i.p. injection into WT recipient mice. Seven days later individual (B) or pooled (C) splenocytes were re-stimulated in vitro in the presence (■) or absence (□) of SEA (25 μg/ml). Culture supernatants were harvested 72 h later and tested by ELISA for IL-4, IL-5, IL-13, and IL-10 (B). ELISPOT was used to quantify IL-4-producing cells and associated spot size (C). Data are presented as mean ± SEM of five mice and are representative of five separate experiments (B) or as mean ± SEM of triplicate wells, representative of two separate experiments (C).

Flow cytometry, ELISA, and ELISPOT

For flow cytometry, cells were blocked with anti-CD16/32 mAb (produced in-house). DC were then stained with FITC-conjugated anti-MHC II (M5114; produced in-house), or PE-conjugated anti-CD40, anti-CD80, anti-CD86, allophycocyanin-conjugated anti-CD11c, and relevant isotype control mAbs (BD Pharmingen). Alternatively, blood, draining LN (dLN) and splenocyte samples were stained with anti-CD4 and anti-CD25 (mAb 7D2; BD Pharmingen) and, where indicated, fixed, permeabilized, and stained intracellularly for forkhead/winged helix transcription factor 3 (Foxp3) according to the manufacturer's instructions (eBioscience). Samples were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo
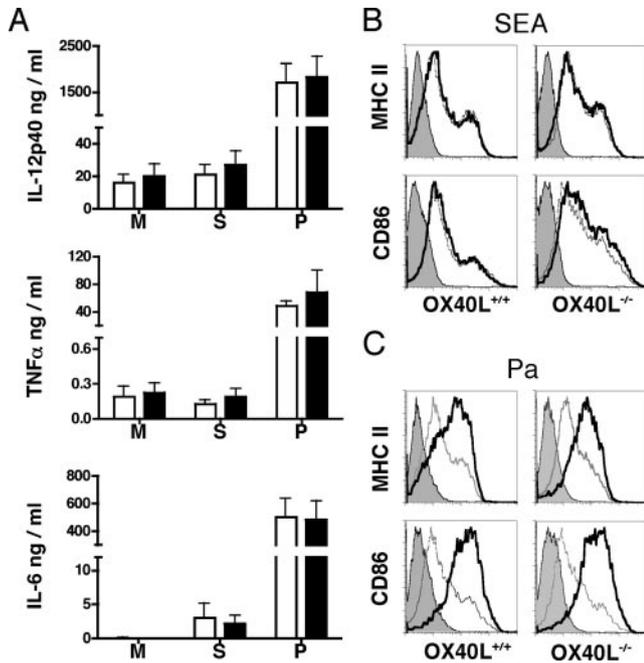
FIGURE 2. OX40L$^{-/-}$ DC respond normally to activation with Th2-driving Ag SEA or control Th1 stimuli. BM-derived DC were cultured for 18 h with medium alone (M), SEA (S), or heat-killed *P. acnes* (P). SN from WT (□) or OX40L$^{-/-}$ (■) cell cultures were tested for cytokine production by ELISA and are presented as mean ± SEM of eight experiments (*A*). Cells were assessed for surface staining of the activation markers MHC II and CD86 by flow cytometry (*B* and *C*). DC cultured with medium are represented by gray lines, whereas cells cultured with SEA (*B*) or *P. acnes* (*C*) are represented by black lines. Isotype control staining represented by shaded histograms. Flow cytograms are from one experiment representative of four (*B*) or three (*C*), respectively.

FIGURE 3. Normal kinetics but impaired expansion of primary Th2 response after priming by OX40L$^{-/-}$ DC. WT mice were injected i.p. with WT or OX40L$^{-/-}$ DC (dashed line) or SEA-treated DC (solid line). After the indicated time, splenocytes were restimulated in vitro in the presence or absence of SEA (25 μg/ml) and cytokine production was assessed by ELISA after 72 h. Cytokine production was calculated as that detected after restimulation with SEA minus background levels produced after restimulation with medium alone. Data presented are mean ± SEM of two to three mice (day 14) or three to six mice (all other time points) and are representative of three separate experiments.

software (Tree Star). Cytokine ELISAs were performed on culture supernatants using paired mAb purified in-house or purchased from BD Pharmingen, and recombinant cytokine standards were purchased from Pepro-Tech or BD Pharmingen. TNF and IL-13 were measured using the DuoSet ELISA kit and paired Abs, respectively, from R&D Systems. Plates were developed with ABTS or tetramethylbenzidine (Insight Biotechnology). The number of IL-4-secreting cells was determined by ELISPOT using HTS filter plates (Millipore) and paired mAb, as detailed for ELISA, and development with BCIP/NBT Plus (Europa Bioproducts). Plate scanning service and ImmunSpot analysis software were provided by CTL Europe.

Determination of OX40L expression by DC

Total RNA was extracted from 1×10^6 DC using TRIzol (Invitrogen Life Technologies) and cDNA was generated using a Reverse Transcriptase System with random hexamers (Promega). OX40L expression was assessed by quantitative PCR using SYBR green (Invitrogen Life Technologies), a Chromo4 detector, and Opticon Monitor software (MJ Research). Relative expression values of OX40L to 18S were calculated using the $2^{-\Delta\Delta CT}$ method. Primers used were (5′–3′): murine *OX40L* forward GG GAAGGGGTTCAACCCCTGG, reverse GAAGAGAGTTGCAGGCA GACA; and *18S* forward GTAACCCGTTGAACCCCATT, reverse CCA TCCAATCGGTAGTAGCG.

Statistical analysis

Comparisons of data were tested for significance using the Student *t* test. Alternatively, comparisons between single values obtained from pooled cells from individual mice to data obtained from multiple mice per group were tested for significance using the one-sample Student *t* test. The following symbols were used to denote significant values: *, $p \le 0.05$; **, $p \le 0.01$; and ***, $p \le 0.001$.

Results

OX40L is required for optimal Th2 priming by DC in vivo

The mechanisms used by DC to drive Th2 expansion and polarization are unclear. Exposure of DC to SEA conditions them to
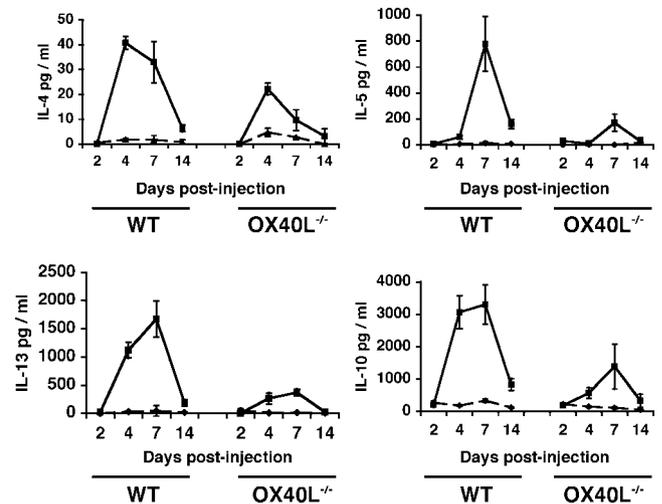
drive Th2 expansion (24). Importantly, this occurs in the absence of conventional DC maturation, demonstrated by the failure of SEA-treated DC to up-regulate expression of MHC II, CD40, CD80, CD86, or OX40L (24), yet is dependent upon their expression of CD40 (26). Furthermore, CD40-CD154 interactions are critical for the activation of DC during schistosome infection (28). Together, these findings suggest that expression of factors vital for Th2 induction by DC is likely under the control of CD40. The nature of the pathogen products to which DC are exposed dictates the effect that subsequent CD40 ligation has upon these cells (29). Although Th1-associated pathogens frequently condition DC to produce IL-12 upon CD40 ligation, the CD40-dependent events programmed by exposure of DC to Th2-driving helminth products are less clear. Using an agonistic anti-CD40 mAb (FGK-45), we observed that expression of OX40L was transiently up-regulated on SEA- but not medium-conditioned BM DC following CD40 signaling (Fig. 1*A*). To our knowledge, these data represent one of the first examples of a molecule with ascribed function in Th cell priming that is differently regulated between immature and SEA-activated DC.

Because the importance of DC expression of OX40L in the generation of Th2 responses in vivo has yet to be determined, we used a system of Th2 polarization by adoptive transfer of SEA-exposed DC (24) to restrict OX40L deficiency to those cells responsible for priming naive Th cells. High levels of SEA-specific IL-4, IL-5, IL-13, and IL-10 could be detected in the supernatants of splenocytes from WT mice injected 1 wk previously with OX40L$^{+/+}$ (WT) SEA-pulsed BM-derived DC (DC/SEA) (Fig. 1*B*). In contrast, priming by SEA-pulsed DC generated from OX40L$^{-/-}$ BM (6) led to significantly ($p < 0.01$–0.001) reduced Ag-specific Th2 cytokine production. Furthermore, considerable numbers of SEA-specific IL-4-producing cells that also individually produced greater amounts of cytokine (as judged by spot size) were detectable by ELISPOT only in splenocytes from mice injected with WT
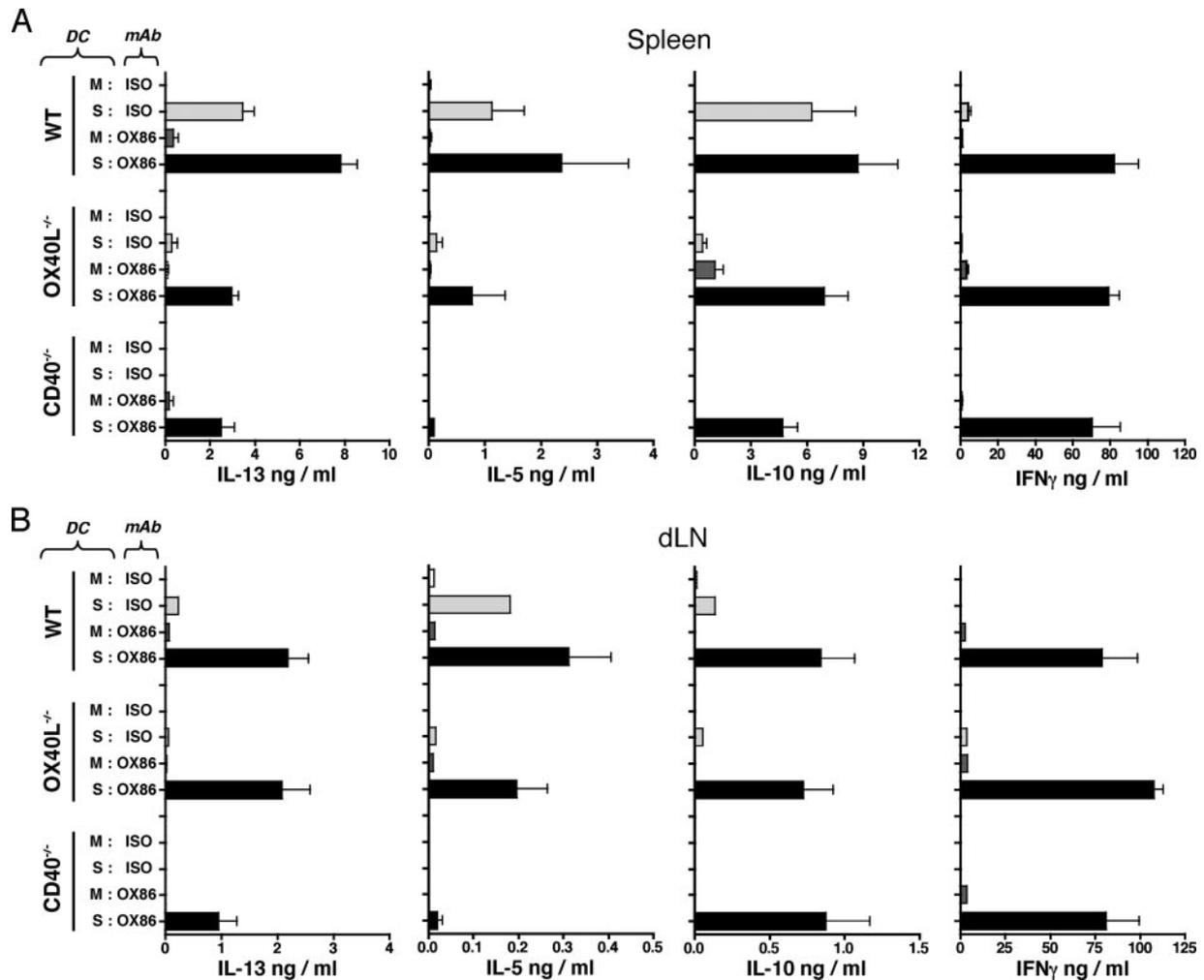
FIGURE 4. Signaling through OX40 rescues impaired cytokine responses primed by $OX40L^{-/-}$ and $CD40^{-/-}$ DC. WT mice were injected s.c. with DC (M) or DC/SEA (S) derived from WT, $OX40L^{-/-}$, or $CD40^{-/-}$ BM and then received i.p. injections of agonistic anti-OX40 mAb (OX86; 100 μg) or isotype control mAb (100 μg) at days 0 and 2. Seven days later, spleens (*A*) and dLN (*B*) were removed and cells were restimulated in vitro with SEA (25 μg/ml). Cytokine production was assessed by ELISA after 72 h and was calculated as that detected after restimulation with SEA minus background levels produced after restimulation with medium alone. For spleen, data presented are mean $\pm$ SEM of three to four mice. For dLN, cells from mice receiving DC/SEA and OX86 Ab treatment were restimulated individually, whereas cells from mice receiving all other treatments were pooled. Data are representative of two individual experiments.

DC/SEA (Fig. 1*C*). This result was independent of the route of DC administration, because similar data were obtained from splenocytes and inguinal or popliteal LN cells following s.c. injection of DC into the flank (data not shown) or footpad (see Figs. 4 and 7), respectively.

Despite their severely impaired ability to prime a Th2 response, there was no significant difference in the activation state of $OX40L^{-/-}$ DC compared with WT cells after culture with SEA or with medium, as determined by their production of IL-12p40, IL-6, and TNF-α, or surface expression of MHC II and CD86 (Fig. 2, *A* and *B*). Since SEA has little effect on conventional DC phenotypic activation, cells were also cultured with an overt maturation stimulus, heat-killed *P. acnes* bacteria, to test whether classical maturation was affected by OX40L deficiency. Both WT and $OX40L^{-/-}$ DC responded equally to activation with *P. acnes*, with up-regulation of both cytokine production and surface expression of MHC II and CD86 (Fig. 2, *A* and *C*).

Assessment of the kinetics of the Th2 response induced by WT SEA-pulsed DC showed that SEA-specific cytokine production by splenocytes was detectable by day 4 postinjection, at which point production of IL-4, IL-13, and IL-10 peaked and then remained

elevated until day 7 before returning to near background levels primed by DC alone (Fig. 3). Production of IL-5 was unusual among the cytokines measured in that its production increased significantly ($p < 0.001$) from days 4 to 7 before declining by day 14 (Fig. 3). Importantly, the response primed by $OX40L^{-/-}$ cells showed the same kinetics as that primed by their WT counterparts, but was significantly reduced at all time points (Fig. 3).

The deficiency in Th2 cytokine production primed by $OX40L^{-/-}$ DC was not due to a switch from Th2 to Th1 dominance of the T cell response, as determined by IFN-γ production (data not shown). Similarly, priming by $OX40L^{-/-}$ DC/SEA was not characterized by expansion of a nonpolarized response, because SEA-specific IL-2 production and proliferation (data not shown) was also dramatically reduced following priming by these DC. This demonstrates an overall deficiency in the ability of $OX40L^{-/-}$ DC to prime Th responses under Th2-polarizing conditions.

OX40 signaling rescues priming by $OX40L^{-/-}$ and $CD40^{-/-}$ DC

We reasoned it was likely that defective priming by $OX40L^{-/-}$ DC was due to a lack of signaling through OX40 rather than

FIGURE 5. Signaling through OX40 rescues impaired dLN cellularity primed by OX40L$^{-/-}$ and CD40$^{-/-}$ DC. WT mice were injected s.c. with PBS or DC (M) or DC/SEA (S) derived from WT, OX40L$^{-/-}$, or CD40$^{-/-}$ BM and then received i.p. injections of OX86 or isotype control mAb (100 μg) at days 0 and 2. Seven days later, dLN were removed and total cell numbers were enumerated. Data are presented as mean $\pm$ SEM of four to seven mice (*A*). Alternatively, dLN were pooled before counting, except for those from mice receiving DC/SEA and OX86 Ab treatment, which were counted individually (*n* = 3–4; *B*).
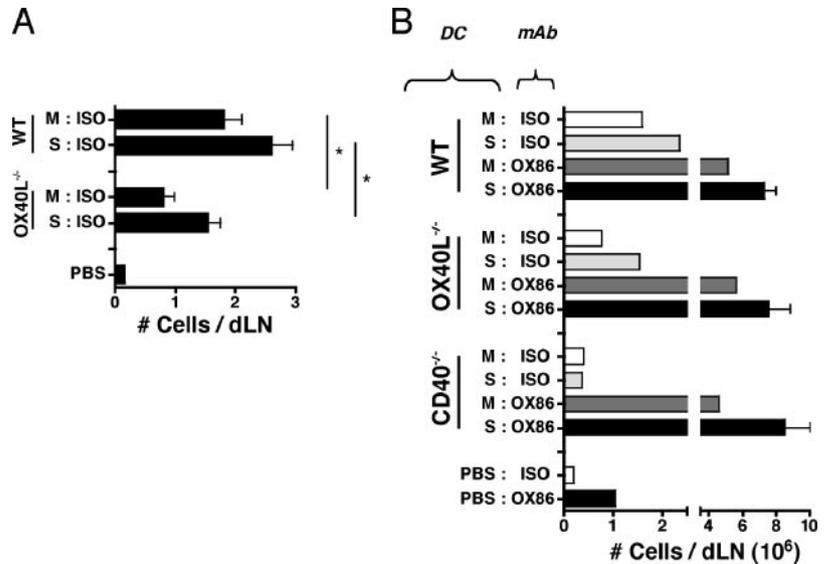
retrograde signaling through OX40L. To determine whether OX40 signaling could salvage defective Th2 priming by OX40L$^{-/-}$ DC in vivo, we transferred cells in conjunction with an agonistic anti-OX40 mAb (OX86).

Strikingly, OX86 treatment rescued defective Th responses primed by OX40L$^{-/-}$ DC/SEA in both the spleen and dLN (Fig. 4). This was observed for production of Th2 cytokines, as represented by IL-13, IL-5, and IL-10, and for production of IFN-γ and IL-2 (data not shown), with levels of cytokine elevated in all cases, although not always to the same level as that induced by WT DC/SEA in conjunction with OX86 treatment. Furthermore, OX86 treatment had an adjuvant effect on cellularity in the dLN of mice receiving WT or OX40L$^{-/-}$ cells, effectively restoring the defective dLN cellularity observed following transfer of gene-deficient DC (Fig. 5). OX86 treatment also greatly amplified splenic and LN production of IFN-γ (Fig. 4) and IL-2 (data not shown) primed by WT DC/SEA, whereas a similar effect on Th2 cytokine production was only observable for IL-13 in both the spleen and dLN. This is similar to the effect of this mAb on T cell cytokine production during polarization at high Ag doses in vitro (30).

Because we have shown previously that CD40-deficient DC are incapable of driving an SEA Th2 response (26) and that DC expression of OX40L was dependent upon CD40 signaling (Fig. 1), we tested whether OX86 mAb could also rescue priming by SEA-stimulated DC derived from CD40$^{-/-}$ BM (31). For most parameters assessed, OX86 treatment clearly restored the defective immune response primed by CD40$^{-/-}$ DC/SEA. The majority of Th2 cytokines in the spleen and dLN were restored (Fig. 4), as was production of IFN-γ (Fig. 4) and IL-2 (data not shown) and the defective cellularity in the dLN (Fig. 5). However, OX86 treatment could not restore defective production of IL-5 primed by CD40$^{-/-}$ cells in the dLN or spleen (Fig. 4*B* and data not shown).

Defective Th2 priming by OX40L$^{-/-}$ DC is not under control of endogenous IL-10

Blocking of OX40L-OX40 interactions during differentiation of human T cells by DC preferentially drives expansion of an IL-10-producing population over other Th2 cytokines (15). Furthermore, immature BM DC that lack CD40 can induce the differentiation of IL-10-producing T cells (32), which could be due to a failure of these DC to up-regulate OX40L. Although we found that

priming by OX40L-deficient DC leads to impaired Ag-specific production of IL-10 as well as other Th2 cytokines (Figs. 1 and 3), this did not exclude the possibility that there may be early induction of IL-10 in recipient mice following in vivo transfer of SEA-treated OX40L-deficient DC that acts to limit the expansion of the ensuing Th2 response. To test this possibility, we compared priming by WT and OX40L$^{-/-}$ DC/SEA in an IL-10-deficient setting (33). Impaired Th2 cytokine responses were generated by OX40L$^{-/-}$ DC irrespective of whether the recipient mice were WT or IL-10$^{-/-}$ (Fig. 6 and data not shown). A lack of recipient IL-10 led to the elevated production of SEA-specific IFN-γ primed by WT DC/SEA as previously reported (34). Similarly, priming with OX40L$^{-/-}$ DC resulted in significantly increased IFN-γ production in IL-10-deficient compared
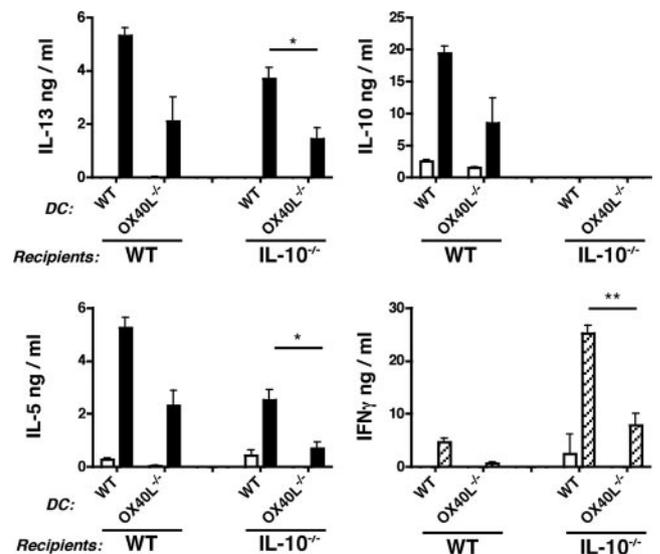

FIGURE 6. Impaired Th2 induction by OX40L$^{-/-}$ DC is not due to recipient-derived IL-10. WT and IL-10$^{-/-}$ mice were injected i.p. with WT or OX40L$^{-/-}$ derived unstimulated ($\square$) or SEA-activated ($\blacksquare$ and $\boxtimes$) DC. Seven days later, splenocytes were restimulated in vitro with SEA (25 μg/ml) for 72 h, after which cytokine production was assessed by ELISA. Cytokine production was calculated as that detected after restimulation with SEA minus background levels produced after restimulation with medium alone. Data presented are mean $\pm$ SEM of two to three mice per group and are representative of two experiments.

with WT recipients, although the levels of cytokine remained defective compared with that primed by WT DC/SEA in IL-$10^{-/-}$ mice (Fig. 6).

Priming by WT and OX40L$^{-/-}$ DC is independent of regulation by CD25$^+$ cells

Blockade of costimulatory pathways during T cell priming in vitro can lead to the generation of regulatory cells that suppress the developing Th response (35). We addressed whether failed priming by OX40L$^{-/-}$ DC might be due to Treg expansion by examining the frequency of CD25$^+$ and CD25$^-$ Foxp3$^+$ Treg subsets in the spleen and dLN of DC recipients. Little difference was observed in the proportion of CD4$^+$ cells that were Foxp3$^+$CD25$^+$ or Foxp3$^+$CD25$^-$ following injection of PBS or WT or OX40L$^{-/-}$ DC (7, A–C). However, the balance of the Treg:Th ratio may not be a critical factor for regulatory cells to have dominance in a costimulatory molecule-deficient setting, since signaling through OX40 that is expressed constitutively on some CD4$^+$CD25$^+$ T cells can directly reduce their immunosuppressive function (36, 37). Therefore, we addressed whether impaired Th2 induction in the absence of DC OX40L may be the result of uncontrolled suppression by CD4$^+$CD25$^+$ cells by depleting CD25$^+$ cells before DC transfer. Administration of PC61 mAb effectively depleted CD25-expressing CD4$^+$ cells before transfer of DC (Fig. 7D). CD25$^+$Foxp3$^+$ Tregs remained depleted for the duration of the experiment while there was no concurrent increase in the Foxp3$^+$CD25$^-$ population (Fig. 7E and data not shown). Despite this, CD25 depletion had no significant effect on WT induction of SEA-specific Th2 cytokine, IFN-γ, or IL-2 production in the dLN or spleen (Fig. 7F and data not shown). Furthermore, PC61 treatment failed to restore the defective SEA-specific response driven by OX40L$^{-/-}$ DC/SEA (Fig. 7F).

Priming in the absence of OX40L or CD40 leads to defective Th2 memory but not to a state of Ag tolerance

Th1 priming in the absence of costimulation can lead to Ag-specific tolerance (35, 38) that can be directly attributed to a lack of signaling through OX40 (39). To examine whether the same is true in a Th2 setting, we tested whether defective priming by OX40L$^{-/-}$ or CD40$^{-/-}$ DC/SEA resulted in hyporesponsiveness to subsequent challenge using an assay of Th2 memory in which mice were initially sensitized by i.p. injection with SEA-treated or medium-conditioned DC and then challenged 8 wk later by footpad injection with schistosome eggs. Interestingly IL-5, and, to a lesser extent, IL-13 were the only indicators of Th2 memory following challenge of mice primed with WT DC/SEA (Fig. 8), with only low levels of IL-4 production across all groups (data not shown) and no significant difference in IL-10 (Fig. 8), IL-2, or IFN-γ production (data not shown). Priming with OX40L$^{-/-}$ and CD40$^{-/-}$ DC/SEA resulted in significantly reduced levels of SEA-specific IL-5 production following egg challenge compared with priming with WT DC/SEA, demonstrating impaired memory (Fig. 8). Similar data were obtained using cells isolated from the draining popliteal LN (data not shown). However, initial priming with gene-deficient DC did not induce antigenic hyporesponsiveness, because sensitization with OX40L$^{-/-}$ or CD40$^{-/-}$ DC/SEA did not result in reduced levels of SEA-specific Th2 cytokines upon egg challenge compared with their respective unstimulated control DC (Fig. 8). Failed induction of Th2 memory in the absence of subsequent antigenic hyporesponsiveness also resulted from transfer of costimulatory deficient DC when the antigenic challenge was SEA rather than eggs (data not shown).
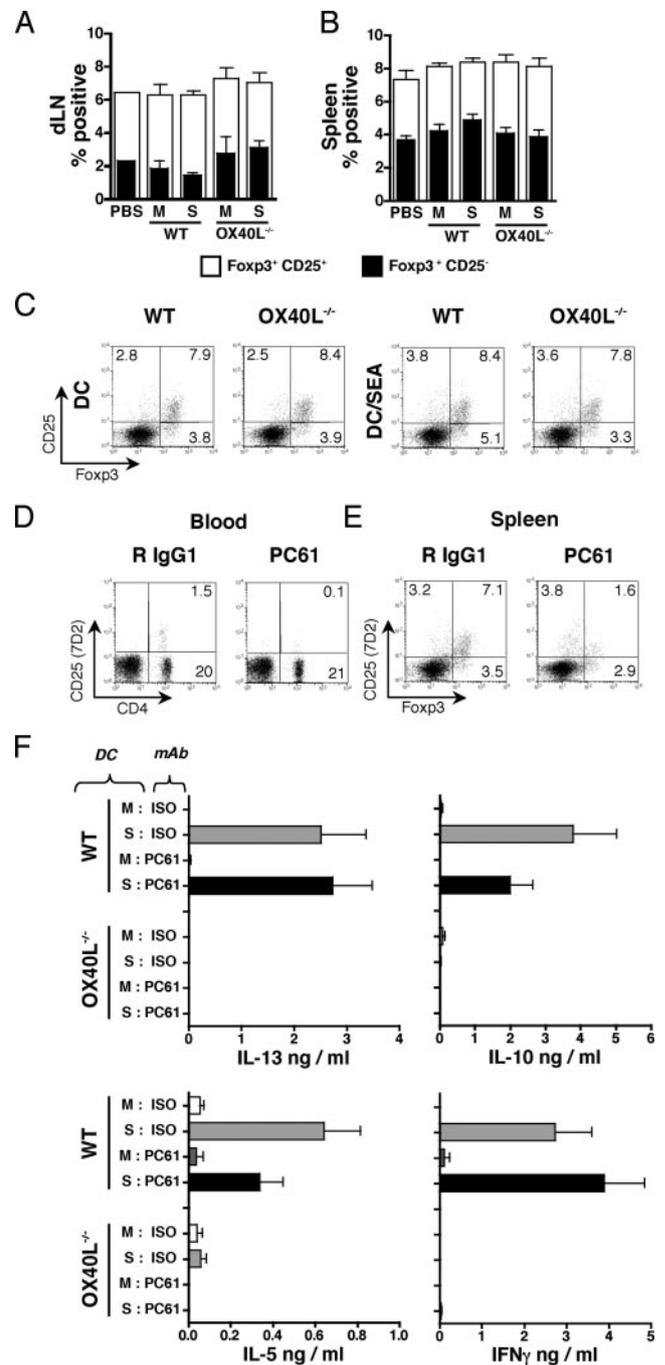

FIGURE 7. Depletion of CD25$^+$ cells does not affect priming by WT or OX40L$^{-/-}$ DC. WT mice were injected i.p. with anti-CD25 mAb (PC61; 1 mg) or control mAb (1 mg). Three days later, mice were injected s.c. with PBS or DC (M) or DC/SEA (S) derived from WT or OX40L$^{-/-}$ BM. Seven days after DC transfer CD4$^+$ populations in the spleen and dLN of mice injected with control mAb were analyzed ex vivo for their expression of Foxp3 and CD25. Plots from representative mice (C) or combined data from multiple animals showing the percentage of CD4$^+$ cells that were Foxp3$^+$CD25$^+$ (□) or Foxp3$^+$CD25$^-$ (■) in the dLN (A) or spleen (B). Mice were tail bled and checked for depletion of CD4$^+$CD25$^+$ cells (using mAb 7D4) (D) or CD4$^+$ splenocytes were checked for Foxp3 and CD25 expression (E) immediately before or 7 days after administration of DC, respectively. Plots from representative animals are shown. Seven days after DC transfer, dLN cells were restimulated in vitro with SEA (25 μg/ml) for 72 h and cytokines were detected by ELISA. Cytokine production was calculated as that detected after restimulation with SEA minus background levels produced after restimulation with medium alone (F). Data presented are mean ± SEM of four to six mice per group except for dLN cells from control mice injected with PBS that were pooled due to their limited number. Data are representative of two to three experiments.
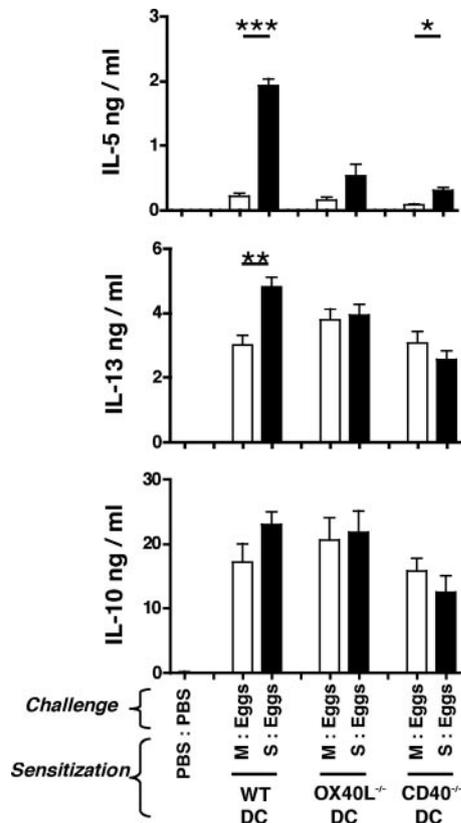
FIGURE 8. Priming by OX40L$^{-/-}$ and CD40$^{-/-}$ DC results in impaired Th2 memory response development. To test for recall responsiveness 2500 *S. mansoni* eggs were injected into the hind footpads of mice 8 wk after initial i.p. injection of WT, OX40L$^{-/-}$, or CD40$^{-/-}$ DC (□) or DC/SEA (■). Naive controls were initially injected i.p. with PBS and then injected in the footpad with PBS (▦). Seven days later, splenocytes were restimulated in vitro with SEA (25 μg/ml). Data are mean ± SEM for five to seven mice per group and are presented as SEA-specific cytokine production with background (medium) values subtracted. Data are representative of three separate experiments.

Discussion

We have previously described a model of T cell priming in which transfer in vivo of DC activated with the Th2-driving pathogen product SEA results in the generation of a SEA-specific Th2 response in a nontransgenic naturally occurring polyclonal population of T cells (24, 34). We now reveal a fundamental requirement for OX40L expression by the Ag-bearing DC to generate proficient primary and memory Th2 responses in vivo. Compared with the response elicited by WT cells, OX40L$^{-/-}$ DC displayed a severely impaired ability to prime for Ag-specific Th2 cells and Th2 cytokine production. This defect was neither transient (Fig. 3) nor due to a switch to a Th1 (Fig. 4) or a nonpolarized response (data not shown). Together with the reduced cell numbers evident in the dLN following priming by OX40L$^{-/-}$ DC (Fig. 5), our data demonstrate that an absence of OX40L on Th2 priming DC does not lead to a failure to polarize the response, rather a failure to efficiently prime and/or maintain that response.

The role of OX40L in the polarization of Th cells is unclear. It is reported to be both critical and dispensable for a Th1 response (5) but appears to have a universally essential function in Th2 development. When considering the function of OX40L in polarization in our system, it is important to consider that Th2 priming by DC/SEA is an active not a passive or "default" process. This has been most clearly demonstrated by the fact that DC coactivated

with SEA and Th1-driving bacteria retain the ability to prime SEA-specific Th2 responses while also priming bacteria-specific Th1 responses (40). Although it has been suggested that DC OX40L may play a Th2-specific role in directing polarization in vitro (14), our data indicate that OX40L on DC during Th2 priming in vivo acts not as a polarizing signal, but as an essential costimulatory signal required for normal immune response development per se., with SEA-specific production of non-Th2 cytokines such as IFN-γ and IL-2 equally impaired following priming by OX40L$^{-/-}$ DC. However, treatment with an anti-OX40 agonist mAb affected the character of the Th response induced by OX40L$^{+/+}$ DC, leading to exaggerated production of SEA-specific IFN-γ in both the spleen and dLN compared with a more modest enhancing effect on Th2 cytokines. Despite enhancing IFN-γ production in Th1-biased settings (9), this mAb has previously been reported to amplify Th2 cytokine without any corresponding increase in IFN-γ in a Th2-biased system in vivo (39). However, the study in which this was reported used a default Th2 model resulting from failed Th1 priming due to an absence of CD40 signaling. In contrast, ours is the first study to use this mAb in a nondefault Th2 setting in vivo. The IFN-γ-enhancing effect of anti-OX40 mAb we have observed highlights further that SEA contains components that have the capacity to drive Th1 responses, a feature that is probably true of most Th2-dominated settings.

It is clear that the role of DC OX40L involves, primarily, signaling to host cells through OX40, since defective priming by OX40L$^{-/-}$ DC was in the main part rescued by agonistic anti-OX40 mAb treatment (Figs. 4 and 5). However, the incomplete restoration of all cytokine production using this approach could reflect a minor requirement for retrograde signaling through OX40L, as proposed by several in vitro studies (13, 41). We also demonstrate that the absolute requirement for CD40 signaling to SEA-treated DC during Th2 priming is predominantly due to the up-regulation of OX40L on these cells and not, for example, as a failure to migrate to the dLN (42). Crucially, OX40 signaling induced by anti-OX40 mAb could rescue defective priming by CD40$^{-/-}$ DC (Figs. 4 and 5). Further support for this is provided by the fact that OX40L expression by SEA-treated DC was induced by CD40 ligation (Fig. 1). Although DC expression of OX40L is clearly important for appropriate Th2 induction, there is likely a requirement for additional "downstream" events following CD40 signaling in this process, since defective priming by OX40L$^{-/-}$ DC contrasts the absolute failure of priming by CD40$^{-/-}$ DC. Furthermore, treatment with anti-OX40 mAb failed to fully restore some aspects of the defective Th2 response resulting from transfer of CD40$^{-/-}$ DC that it could restore for priming by OX40L$^{-/-}$ cells, in particular IL-5 production.

It has been suggested that priming in a costimulation-deficient environment can lead to active suppression of developing Th responses by the induction of regulatory networks (35). We saw no evidence for this in our system. IL-10 was not responsible for impaired priming by OX40L$^{-/-}$ DC since Th responses remained markedly impaired in IL-10$^{-/-}$ recipient mice (Fig. 6). We also did not observe any exaggerated expansion or recruitment of Foxp3$^+$CD25$^+$ T cells following transfer of OX40L$^{-/-}$ DC (Fig. 7). Indeed, if Th response expansion was actively inhibited, subsequent responses to Ag challenge might also be expected to be impaired compared with challenge of naive animals. However, we demonstrated that defective priming by OX40L$^{-/-}$ DC did not lead to a state of Ag hyporesponsiveness (Fig. 8). This lack of tolerance induction was not due to the rapid replacement of the SEA-specific T cell pool or a reduction in active suppression in the 8-wk period between priming and challenge, as similar data were obtained when mice were challenged 1 wk postpriming (data not

shown). Finally, despite several recent reports suggesting that co-stimulatory interactions could limit regulation of Th responses by Tregs (36, 37, 43), we observed that depletion of CD25$^+$ cells before DC transfer did not affect defective priming by OX40L$^{-/-}$ (Fig. 7) or CD40$^{-/-}$ DC (data not shown), thus excluding any possibility of DC directly inactivating these cells via OX40:OX40L (36, 37) or becoming resistant to regulation through CD40 signaling (43), at least before an overriding requirement for OX40 signaling to another cellular compartment. Thus, we favor the hypothesis that the absence of OX40L leads to a passive defect in priming, such as a failure to provide sustained proliferation, survival, and polarization of expanding Th cells via activation of protein kinase B (44), up-regulation of survivin, Bcl-x$_L$ and Bcl-2 expression (45, 46), and direct transcription of IL-4 (47).

We recently demonstrated that during priming by DC/SEA the source of the IL-10 that suppresses IFN-γ production and allows full development of the Th2 response was neither B nor T cells (34). Our current data strengthen this observation, since depletion of CD25$^+$CD4$^+$ cells did not result in emergence of exaggerated SEA-specific IFN-γ (Fig. 7), contrasting what was seen in IL-10$^{-/-}$ recipients (Fig. 6). However, given the recent reports demonstrating the suppressive function of natural Tregs on both Th1 and Th2 cytokine production in models of schistosome and other helminth infections and schistosome egg-induced responses (48–50) using PC61 mAb-mediated depletion, it is surprising that CD25$^+$ cell depletion affected neither Th1 or Th2 induction by SEA-treated DC. The discrepancies between these models of Th2 priming likely reflect that the induction of regulatory responses following schistosome egg injection or infection is a more complex event than Th2 priming by Ag-exposed DC alone, involving the innate effect of egg Ags on accessory cells such as macrophages (51) and basophils (52).

The OX40:OX40L partnership is important for the generation of Th2 memory (7, 9, 12). Although DC expression of OX40L has been implicated in maintenance of Th2 memory in vitro (8), we now reveal its critical role in the generation of Th2 memory in vivo. This supersedes a requirement for OX40L upon other cells that have been shown to be important for efficient recall responsiveness (7). In addition, we extend our original observation of the absolute importance of CD40 expression on DC for primary Th2 responses (26) to include its requirement in the generation of Th2 memory. It is noteworthy, however, that priming by CD40$^{-/-}$ DC led to a detectable, albeit defective, IL-5 recall memory response. Because no primary response is detectable after transfer of CD40$^{-/-}$ DC, it is possible that a CD40-independent pathway to Th2 memory exists, which does not require the development of a primary effector response, as has previously been proposed in a Th1 setting (53).

Priming of Th cell responses against pathogens in vivo likely involves a complex network of interactions between multiple cell types. We show here that expression of OX40L on DC is up-regulated downstream of CD40 signaling and is critical for optimal Th2 priming in vivo. Expression of OX40L on host cells is unable to compensate for its absence on the priming DC, and thus proposed T cell-T cell (23), T cell-B cell (17) or T cell-CD4$^+$CD3$^-$ cell (18, 54) interactions must be of secondary importance in this process. Furthermore, we have established that the interaction between DC and CD25$^+$ Tregs via OX40L and OX40 is not of great importance during the development of Th2 responses and that priming in the absence of DC-expressed OX40L does not result in active suppression of the developing Th2 responses through IL-10 production. These data help clarify the role and importance of OX40L during DC-driven Th2 induction to pathogen-derived Ag

in vivo and establish a hierarchy of importance between CD40-CD154 and OX40-OX40L partnerships.

Acknowledgments

Disclosures

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