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Mice Deficient in OX40 and CD30 Signals Lack Memory Antibody Responses because of Deficient CD4 T Cell Memory

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Recently, we reported that a CD4+ CD3− CD11c+ accessory cell provided OX40-dependent survival signals to follicular T cells. These accessory cells express both OX40 ligand and CD30 ligand, and the receptors, OX40 and CD30, are both expressed on Th2-primed CD4 T cells. OX40 and CD30 signals share common signaling pathways, suggesting that CD30 signals might substantially compensate in OX40-deficient mice. In this report we have dissected the signaling roles of CD30 alone and in combination with OX40. CD30-deficient mice showed an impaired capacity to sustain follicular germinal center responses, and recall memory Ab responses were substantially reduced. Deficiencies in OX40 and CD30 signals were additive; secondary Ab responses were ablated in double-deficient mice. Although the initial proliferation of OX40/CD30 double-knockout OTII transgenic T cells was comparable to that of their normal counterparts, they failed to survive in vivo, and this was associated with reduced T cell numbers associated with CD4+CD3− cells in B follicles. Finally, we show that OX40/CD30 double-knockout OTII transgenic T cells fail to survive compared with normal T cells when cocultured with CD4+CD3− cells in vitro. The Journal of Immunology, 2005, 174: 3891–3896.

T cell memory provides mammals with a sophisticated defense against previously encountered infections, yet the cellular and molecular signals that regulate this memory response are still poorly understood. Recently, we reported that T cells primed to provide help for the development of germinal centers (GC)1 interacted sequentially: first with dendritic cells in the T zone, then with CD4+CD3−CD11c+ (CD4+CD3−) cells at the B:T interface and within B follicles (1). These cells are the adult equivalent of inducer cells, which play a pivotal role in the development of lymph nodes and Peyer’s patches in ontogeny (2). We found that in adult mice these cells constitutively expressed OX40 ligand (TNFSF4) and CD30 ligand (TNFSF8) (1) and were able to demonstrate that the survival of follicular T cells was partly dependent on OX40 signals from CD4+CD3− cells. The receptors, OX40 (TNF receptor (TNFR) superfamily (TNFRSF4)) and CD30 (TNFRSF8), are genetically closely linked on human chromosome 1 and the syntenic region on mouse chromosome 3 (3). The genetic organization of this cluster of TNF family members is very similar in mice and men, suggesting that it was fixed before speciation. It is likely that the cluster, which also includes TNFR2 (TNFRSF1B), 4-1BB (TNFRSF9), herpes virus entry mediator (TNFRSF14), and glucocorticoid-induced TNFR family-related gene (TNFRSF18), arose by local duplication of an ancestral gene. Both murine (1) and human (4, 5) Th2 cells, which have been implicated in the development of T cell help for GC (6, 7), strongly express OX40 and CD30. This cluster of TNF family members share common signaling pathways through the TNF-related adaptor factor family (1, 2, 3, and 5, but not 4 or 6) (8); signals through these receptors enhance survival by up-regulation of the antiapoptotic proteins, Bcl-2 and Bcl-xL (9, 10). Others have shown that CD4 recall responses are impaired in mice deficient in OX40 signals (11–13), and we found that such mice have a modest defect in their capacity to sustain long-term Ab responses (1). Because of shared signaling pathways through OX40 and CD30, it seemed probable that CD30 signals from CD4+CD3− cells might compensate in OX40-deficient mice. In this report we have investigated the effects of CD30 alone and CD30 in concert with OX40 on the development and survival of Th2-primed CD4 cells. We found that CD30-deficient (CD30ko) mice had impaired capacity to sustain GC responses, and their secondary Ab responses were much reduced when they were reimmunized. The effects of CD30 on secondary Ab responses were even more marked when combined with OX40 deficiency. This was not due to a defect in T cell proliferation, because OX40/CD30 double-deficient (dko) transgenic cells proliferated as well as their normal counterparts, but failed to survive. This deficiency in T cell survival was associated with impaired retention in B follicles, and poor survival in vivo and in vitro when cocultured with CD4+CD3− cells. These data suggest that OX40 and CD30 signals act together to control the development of memory T cells to provide help for secondary Ab responses.

Materials and Methods

Generation of dko and OTII dko mice
Mice deficient in OX40 and CD30 were generated by crossing the two strains of single-knockout mice. F1 heterozygotes were backcrossed onto single-knockout mice and screened to identify mice that had undergone recombination to produce dko alleles. Identically recombinants were then bred together to generate homozygous...
dko mice. dko mice were subsequently bred with OTII mice to generate OTII dko mice.

Immunizations

For priming, groups of mice were immunized with alum-precipitated NP-chicken gammaglobulin (CγG; 100 μg/mouse i.p.), CγG alone (100 μg/mouse i.p.), or NP-OVA (200 μg/mouse i.p.) and killed at the indicated time points. Mice were boosted with soluble NP-CγG (100 μg/mouse i.p.) and killed 4 days later.

ELISA

Serum samples were analyzed by ELISA on NP15−BSA−, NP2−BSA−, and CγG-coated plates (1).

Immunohistology and quantitative microscopy

Frozen spleen sections were stained as previously described (14) with sheep anti-mouse IgD (The Binding Site) and rabbit IgG conjugated to NP or CγG-specific GC or plasma cells at a magnification of ×40. The total area of spleen section was measured by counting intercepts on a 100-μm² acetate overlay.

In vitro T cell survival assays

Splenocytes from CD45.1 OTII mice and CD45.2 OTII knockout (OX40ko, CD30ko, and dko) mice were mixed so that the starting ratio of normal to knockout OTII cells was 1:1 (total of 10^6 cells/ml). They were cultured for 4 days with 1 μM OVA323–339 peptide (15) under Th1 and Th2 conditions in vitro as previously described (1). After 4 days, the cells were washed and recultured with 10^5 CD4+CD3− cells from RAG-deficient mice. On day 10, the ratio of normal to knockout OTII CD4 T cells was checked by flow cytometry.

In vivo T cell survival assays

A final mixture of 1:1 normal:dko OTII cells (total of 10^7 cells/mouse) was labeled with the intracellular fluorescent dye CFSE (Molecular Probes) and transferred into RAG-deficient mice. The mice were immunized the next day with 200 μg of alum-precipitated NP-OVA (Molecular Probes) and transferred into RAG-deficient mice. The mice were immunized with alum-precipitated NP-CγG (100 μg), Spleens were taken on days 7 and 14 and stained with IgD (brown) and NP or CγG (blue). T, T zone; PC, plasma cells. Results are representative of at least four experiments.

Results

Ab and GC responses to T-dependent Ags depend on both OX40 and CD30 signals

Primary day 7 anti-NP IgG (Fig. 1) and IgM (data not shown) Ab responses after immunization with a T-dependent protein Ag (alum-precipitated NP-CγG) were indistinguishable among groups of mice that were normal, CD30-deficient (CD30ko), or deficient in both CD30 and OX40 (dko). Although total IgG Abs to the hapten, NP, were not significantly different among the three groups (Fig. 1A), differences were observed in the production of high affinity Abs (measured by their capacity to bind NP2) on day 21 (Fig. 1, B and C). CD30ko and dko mice (p = 0.05) had lower titers of high affinity Abs than normal mice.

Nascent Ag-specific GC to NP were observed in all mice on day 7 (Fig. 2, A–C), illustrating that the signals absent in CD30ko and dko mice were not required for the initiation of either extrafollicular foci of plasma cells or GC formation. By day 14, however, there were clear differences between the groups of mice. Unlike their normal counterparts (Fig. 2, D and G), CD30ko and dko mice had lost both NP-specific GC and plasma cells (Fig. 2, E and F) and failed to form GC to the carrier protein, CγG (Fig. 2, H and I).

FIGURE 1. IgG Ab titers and quantitative analysis of the GC area of primary responses. The mice were immunized with alum-precipitated NP-CγG (100 μg) in normal (blue), CD30ko (green), and dko (red) mice. A, NP15 titers; B, NP2 titers; C, ratio of NP2/NP15 (error bars show SDs for four mice per group). D, Quantification of NP-specific GC response on days 7 and 14. Results are representative of four experiments.

FIGURE 2. Immunohistology of primary responses. The mice were immunized with alum-precipitated NP-CγG (100 μg). Spleens were taken on days 7 and 14 and stained with IgD (brown) and NP or CγG (blue). T, T zone; PC, plasma cells. Results are representative of at least four experiments.
These observations were confirmed by quantitative analysis of GC areas on days 7 and 14 in normal, CD30ko, and dko mice (Fig. 1D).

Lack of T cell memory for secondary Ab responses in dko mice

Cognate interactions between B and CD4 T cells are required to sustain GC and secure the output of memory B and plasma cells. We reasoned that the impaired production of high affinity Abs in dko mice could have been due to failure of T cell survival within the GC. To test this, we examined the capacity of T cells primed in CD30ko and dko mice to provide help for a secondary memory B cell response. Normal, CD30ko, and dko mice were primed with alum-precipitated NP-CyG and boosted with soluble NP-CyG 3 wk later. After 4 days, the peak of the secondary response in normal mice, the spleens were analyzed for Ag-specific plasma cells and GC. Whereas normal mice made an excellent secondary IgG response to the hapten, NP (Figs. 3A and 4A), and to the carrier protein, CyG (Figs. 3D and 4A), the secondary response to the carrier protein, CyG, was absent in dko mice (Fig. 3, C and F, and Fig. 4A). CD30ko mice made a small, but reproducible, secondary Ab response to both CyG and the hapten, NP, compared with dko mice (Fig. 3, B and E, and Fig. 4A).

The above results in dko mice were consistent with defective T cell help for secondary Ab responses, although there could also have been a contribution from a memory B cell defect. To test this directly, we measured the capacity of T cells primed in dko and normal mice with a carrier protein to provide help to naive B cells specific for a new determinant conjugated to the carrier, the classical method to check T cell memory (16). First, mice were primed with alum-precipitated CyG, then they were boosted twice on days 21 and 35 with soluble NP-CyG, a poor immunogen for priming naive T cells. On day 39, normal mice developed high affinity Abs to NP (Fig. 4B). In contrast, no NP-specific serum Abs were elicited in dko mice, indicating a specific lack of T cell memory. The absence of help from memory T cells in dko mice was confirmed by the lack of NP-specific GC and plasma cells in the tissue (data not shown, but similar to those in Fig. 3, C and F).

Survival of dko CD4 transgenic T cells in vivo is compromised

To visualize the T cell deficit in dko mice, we crossed dko mice with the TCR transgenic line, OTII, in which CD4 T cells express a TCR specific for a peptide from OVA (15). The CD45 allotype marked normal (CD45.1) and dko (CD45.2) CD4 T cells were transferred in a 1:1 ratio into RAG-deficient recipients that were immunized 1 day later with alum-precipitated NP-OVA, a Th2 immunogen (17). In the absence of immunization, neither dko nor normal T cells underwent any significant proliferation, because there was no dilution of the membrane dye CFSE (Fig. 5A). In contrast, 3 days after immunization, the cell division of dko and normal cells was similar (Fig. 5A). However, by day 7 there were markedly more normal cells (Fig. 5B). At 2 and 3 wk postimmunization, this bias was maintained at ∼7:1. Analysis of tissue sections in the reconstituted RAG-deficient mice revealed that the ratio of normal to dko cells was much higher in the B follicles, where primed T cells interact with CD4+CD3+ cells (1), than in the T zone (Fig. 5C). This result appeared qualitatively similar to that we previously reported for OX40-deficient T cells in competition with their normal counterparts (1). The individual contributions of OX40 and CD30 to T cell rescue by CD4+CD3+ cells were next examined in an in vitro survival assay.
Rescue of OX40ko, CD30ko, and dko Th2 cells by CD4+CD3− cells

To test the relative capacities of OX40ko, CD30ko, and dko CD4 T cells to be rescued by CD4+CD3− cells, we used an in vitro assay in which gene-deficient (CD45.2) and normal (CD45.1) OTII CD4 T cells (ratio 1:1) were stimulated with peptide and cytokines (IL-4 and anti-IL-12 for Th2 or IL-12 and anti-IL-4 for Th1 conditions) for 4 days (Fig. 6). At this time there was no significant change in the ratio of normal to dko cells was checked on days 4, 7, 14, and 21. C, Ratio of normal/dko OTII cells on day 21 in the T zone, at the B:T interface, and in B follicles from the mice shown in B. Results are representative of two experiments.

IL-7 up-regulates OX40 on CD44high CD4+ memory T cells

The impaired secondary Ab responses in dko mice suggested that the survival of recirculating memory T cells (18) might depend on OX40 and CD30 expression (Fig. 7A). The cytokine, IL-7, which is produced by lymphoid stromal cells, has been implicated in the survival of IL-7R-positive (19, 20) CD4 (21–23) and CD8 (24, 25) memory T cells. We reasoned that IL-7 might exert its effects by regulating the expression of OX40 and CD30, independently of TCR stimulation, allowing memory T cells to receive survival signals from CD4+CD3− cells as they recirculated through secondary lymphoid tissue. To test this, we isolated memory T cells from RAG-deficient mice into which normal and dko OTII cells had been transferred and immunized 3 wk previously (day 21, Fig. 5B). Surviving normal and dko cells from these RAG-deficient recipients expressed comparable levels of IL-7R, and all normal and the few remaining dko T cells had the CD44high memory phenotype (data not shown). Freshly isolated splenic memory T cells expressed only low levels of CD44, show only modest expression of OX40 and no CD30 expression (Fig. 7A). The cytokine, IL-7, which is produced by lymphoid stromal cells, has been implicated in the survival of IL-7R-positive (19, 20) CD4 (21–23) and CD8 (24, 25) memory T cells. We reasoned that IL-7 might exert its effects by regulating the expression of OX40 and CD30, independently of TCR stimulation, allowing memory T cells to receive survival signals from CD4+CD3− cells as they recirculated through secondary lymphoid tissue. To test this, we isolated memory T cells from RAG-deficient mice into which normal and dko OTII cells had been transferred and immunized 3 wk previously (day 21, Fig. 5B). Surviving normal and dko cells from these RAG-deficient recipients expressed comparable levels of IL-7R, and all normal and the few remaining dko T cells had the CD44high memory phenotype (data not shown). Freshly isolated splenic memory T cells expressed only low levels of CD44, show only modest expression of OX40 and no CD30 expression (Fig. 7A). The cytokine, IL-7, which is produced by lymphoid stromal cells, has been implicated in the survival of IL-7R-positive (19, 20) CD4 (21–23) and CD8 (24, 25) memory T cells. We reasoned that IL-7 might exert its effects by regulating the expression of OX40 and CD30, independently of TCR stimulation, allowing memory T cells to receive survival signals from CD4+CD3− cells as they recirculated through secondary lymphoid tissue. To test this, we isolated memory T cells from RAG-deficient mice into which normal and dko OTII cells had been transferred and immunized 3 wk previously (day 21, Fig. 5B). Surviving normal and dko cells from these RAG-deficient recipients expressed comparable levels of IL-7R, and all normal and the few remaining dko T cells had the CD44high memory phenotype (data not shown). Freshly isolated splenic memory T cells expressed only low levels of CD44, show only modest expression of OX40 and no CD30 expression (Fig. 7A). The cytokine, IL-7, which is produced by lymphoid stromal cells, has been implicated in the survival of IL-7R-positive (19, 20) CD4 (21–23) and CD8 (24, 25) memory T cells. We reasoned that IL-7 might exert its effects by regulating the expression of OX40 and CD30, independently of TCR stimulation, allowing memory T cells to receive survival signals from CD4+CD3− cells as they recirculated through secondary lymphoid tissue. To test this, we isolated memory T cells from RAG-deficient mice into which normal and dko OTII cells had been transferred and immunized 3 wk previously (day 21, Fig. 5B). Surviving normal and dko cells from these RAG-deficient recipients expressed comparable levels of IL-7R, and all normal and the few remaining dko T cells had the CD44high memory phenotype (data not shown). Freshly isolated splenic memory T cells expressed only low levels of CD44, show only modest expression of OX40 and no CD30 expression (Fig. 7A). The cytokine, IL-7, which is produced by lymphoid stromal cells, has been implicated in the survival of IL-7R-positive (19, 20) CD4 (21–23) and CD8 (24, 25) memory T cells. We reasoned that IL-7 might exert its effects by regulating the expression of OX40 and CD30, independently of TCR stimulation, allowing memory T cells to receive survival signals from CD4+CD3− cells as they recirculated through secondary lymphoid tissue. To test this, we isolated memory T cells from RAG-deficient mice into which normal and dko OTII cells had been transferred and immunized 3 wk previously (day 21, Fig. 5B). Surviving normal and dko cells from these RAG-deficient recipients expressed comparable levels of IL-7R, and all normal and the few remaining dko T cells had the CD44high memory phenotype (data not shown). Freshly isolated splenic memory T cells expressed only low levels of CD44, show only modest expression of OX40 and no CD30 expression (Fig. 7A).
IL-7R expression by addition of IL-7 (Fig. 7B); it is therefore possible that CD30 expression is induced by IL-7, but that it is rapidly attenuated by signals from the ligand. The receptor induction shown was also observed on T cells isolated from cervical and axillary lymph nodes, sites remote from the point of immunization (data not shown). The induction was not just a feature of transgenic cells, because analysis of CD44<sup>high</sup> CD4<sup>+</sup> cells from normal mice also showed strong induction of OX40 and CD30 ligand (Fig. 7A). In contrast, OX40 is not induced on naive CD44<sup>+</sup> CD4<sup>+</sup> T cells.

**Discussion**

Signals through the IL-4R and the genetically closely linked IL-21R, both of which signal via the common cytokine receptor γ-chain, act together to foster GC development; mice deficient in both signaling pathways have impaired GC development and Ig class switching (26). Th2-primed cells have been linked with preferential migration into B follicles with GC development (6, 7) and strongly express OX40 and CD30 (1, 5). In this report we have investigated the roles of OX40 and CD30 signals in maintaining Ab responses that depend on follicular T cells. It has previously been reported that OX40-deficient mice have normal primary and virtually normal secondary B cell responses (1, 11, 12, 27, 28). We report in this study that primary Ab responses in CD30-deficient mice are also normal, but that GC responses are not sustained, and recall secondary Ab responses are impaired. Mice deficient in both CD30 and OX40 show an even more dramatic phenotype. Again, primary Ab responses are normal, but dko mice show impaired affinity maturation because of failure to sustain GCs, and dko mice fail to make recall secondary Ab responses after reimmunization.

We found that the early proliferation of T cells deficient in both CD30 and OX40 was comparable to that of normal T cells, and this correlated with their capacity to provide help for the primary B cell response. However, dko cells failed to survive compared with their normal counterparts both in vitro and in vivo under Th2 conditions of immunization. In vitro analysis of the capacity of CD4<sup>+</sup> CD3<sup>+</sup> cells to rescue OX40ko, CD30ko, and dko Th2 cells compared with their normal counterparts revealed an additive role for OX40 and CD30 in Th2 cell survival. Analysis of tissue sections showed that the failure to survive was particularly evident in B follicles, where we have previously demonstrated that follicular T cells interact with CD4<sup>+</sup>/CD3<sup>+</sup> cells. Although activated B cells can express OX40 ligand and have been implicated in sustaining GC T cells (29), CD4<sup>+</sup>/CD3<sup>+</sup> cells differ from B cells in that they express OX40 ligand and CD30 ligand in the absence of stimulation (1). The close association of GC T cells with CD4<sup>+</sup>/CD3<sup>+</sup> cells makes it likely that they continue to receive OX40 and CD30 signals from them during the course of the GC reaction. We have previously shown that coculture of Th2 effectors with CD4<sup>+</sup>/CD3<sup>+</sup> cells is associated with their survival, but not proliferation (1). In this study we report that the effects of OX40 and CD30 on Th2 survival are synergistic and suggest that this may be particularly important during the selection of rare B cell mutants bearing high affinity Ag receptors in GCs when the supply of Ag driving the B cell response is limiting.

Our studies also showed that the capacity of dko T cells to provide immediate help for secondary Ab responses was deficient in both CD30ko and dko mice. However, freshly isolated memory CD4<sup>+</sup> T cells expressed little OX40 and no CD30, and it was not clear, therefore, how they might obtain access to survival signals from CD4<sup>+</sup>/CD3<sup>+</sup> cells. One possibility was that IL-7 signals, which have been implicated in CD4 T cell survival, might regulate the expression of OX40 and CD30 (21–23). When we isolated OTII transgenic T cells from RAG-deficient recipients immunized 3 wk previously (dko and normal cells were CD44<sup>high</sup> memory phenotype), we found that both dko and normal populations expressed comparable levels of IL-7R. This indicated that OX40 and CD30 signals were not essential for the induction of IL-7R on the memory T cell population, and this was not the explanation for the failure of dko cells to survive. However, the freshly isolated CD44<sup>high</sup> CD4<sup>+</sup> memory population also expressed low levels of OX40 and no CD30. However, the addition of exogenous IL-7 up-regulated OX40 expression. Although CD30 expression was not induced, there was clear induction of CD30 ligand on the CD44<sup>high</sup> CD4<sup>+</sup> population. We observed IL-7R-mediated down-modulation by addition of IL-7, so we are investigating the possibility that memory T cells up-regulate both CD30 and its ligand, but that CD30 expression is rapidly down-modulated.

On the basis of these results, we propose the following model for maintenance of effective T cell help for Ab responses. Dendritic cell–primed Th2 effectors migrate into B follicles, where Ag-driven expression of OX40 and CD30 allows access to the survival signals through these molecules from CD4<sup>+</sup>/CD3<sup>+</sup> cells. The constitutive expression of the ligands on CD4<sup>+</sup>/CD3<sup>+</sup> cells is particularly important in the late phase of the GC reaction when Ag is limiting, but when it is crucial to select rare B cell mutants with high affinity. In addition to this Ag-driven expression, there is Ag-independent, IL-7R-mediated OX40 induction on CD4<sup>+</sup> T cells primed to provide immediate help for secondary B cell responses. We suggest that OX40 is induced on these cells by IL-7 produced by lymphoid stromal cells. Induction of OX40 by IL-7 then allows Th2-primed cells to receive continued survival signals from CD4<sup>+</sup>/CD3<sup>+</sup> cells. Maintaining a population of primed T cells adjacent to B cell areas ensures that secondary Ab responses occur with optimal efficiency.

In summary, our data reveal two important features of T cell help for Ab production. First, it is regulated by partially redundant signals through OX40 and CD30. Second, it is sustained by a chain
of events linking IL-7 signals, OX40 and CD30 expression, and signals from CD4+CD3+ cells. This information opens up new possibilities for the selective targeting of pathogenic Th2-mediated Ab responses, such as systemic lupus erythematosus; blocking the signals through OX40 and CD30 or manipulating the expression of their ligands on CD4+CD3+ cells should be effective approaches to unwanted Ab responses.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

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