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J Immunol 2000; 165:3043-3050; ;
doi: 10.4049/jimmunol.165.6.3043
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The OX40 Costimulatory Receptor Determines the Development of CD4 Memory by Regulating Primary Clonal Expansion¹

Irene Gramaglia,* Amha Jember,* Susanne D. Pippig,[†] Andrew D. Weinberg,[‡] Nigel Killeen,[†] and Michael Croft^{2*}

The costimulatory receptor OX40 has recently been shown to be involved in primary CD4 responses to several defined Ags. However, to date there has been little information regarding the mechanism of action of OX40, such as whether it regulates T cell numbers, reactivity, or both, and whether it contributes to induction of long-term T cell responses. With an agonist Ab to OX40, and by tracking Ag-specific TCR transgenic T cells *in vivo*, we show that ligation of OX40 induces clonal expansion and survival of CD4 cells during primary responses, and results in the accumulation of greater numbers of memory cells with time. Significantly, OX40-deficient T cells, from mice generated by gene targeting, secrete IL-2 and proliferate normally during the initial period of activation, but cannot sustain this during the latter phases of the primary response, exhibiting decreased survival over time. Mice lacking OX40 develop only low frequencies of Ag-specific CD4 cells late in primary responses *in vivo* and generate dramatically lower frequencies of surviving memory cells. These results demonstrate that OX40-OX40L interactions control primary T cell expansion and the ability to retain high numbers of Ag-specific T cells. In this way, OX40 signals promote survival of greater numbers of T cells with time and control the size of the memory T cell pool. *The Journal of Immunology*, 2000, 165: 3043–3050.

The ability to expand a small number of Ag-specific T cells is pivotal to a successful immune response, and, in many cases, may determine the extent and longevity of immune memory. However, the critical components that regulate clonal expansion and the generation of memory T cells are not clear.

The importance of several costimulatory interactions for CD4 T cell responses is well documented (1). Many studies have shown that CD28/B7 and CD40L/CD40 are essential for the initial phases of the naive T cell response (2–5). CD28 may function by enhancing transcription of IL-2 and altering expression of the bcl family of molecules. In contrast, CD40L interaction with CD40 may function largely at the level of the APC, regulating the expression of costimulatory molecules such as B7-1/-2, and the production of cytokines such as IL-1 and IL-6.

In addition to CD28 and CD40, a number of other costimulatory receptors have been described (1, 6). One in particular, the TNFR family member OX40 (CD134), may possess functions distinct from those described for CD28 and CD40. OX40 was originally described with an Ab that bound to rat CD4 T cells (7, 8), and unlike CD28, is not constitutively expressed, but found on T cells 1–2 days after activation (9–12). Several groups have shown that

OX40L expressed on APC can provide costimulation to CD4 cells (9, 12–14). OX40L is present on activated APC (13, 15–19), activated endothelium (20, 21), and activated T cells (22). OX40-positive T cells have been visualized *in situ* in lymph nodes during the peak of primary T cell responses (23), and at the site of inflammation during the active phases of experimental allergic encephalomyelitis (EAE),³ graft-vs-host disease (GVHD), and rheumatoid arthritis, and on tumor infiltrating lymphocytes (24–29).

Although the exact function of OX40 has not yet been clarified, several recent reports highlighted the importance of this molecule in T cell responses. Blocking OX40 reduced gut inflammation in colitis (30), suppressed paralysis in EAE (19), and prevented hyperplasia in GVHD (31). An initial study of OX40-deficient animals showed that they mounted normal responses to *Leishmania* and *Nippostrongylus* *in vivo*, but T cells from these mice proliferated poorly *in vitro* (22). Additional studies of OX40-deficient mice also showed that they generated normal CD8 responses to lymphocytic choriomeningitis virus (LCMV) and influenza virus, but exhibited reduced primary CD4 responses to these infections, characterized by lower numbers of IFN- γ -secreting cells and fewer T cells infiltrating the lungs of infected animals (32). OX40L-deficient mice were also found to be defective in primary contact hypersensitivity responses to oxazolone and DNBS in one study, with reduced proliferation and IFN- γ secretion seen after *in vitro* stimulation (33), and in a separate study, these mice also showed reduced primary Th1 and Th2 responses to keyhole limpet hemocyanin (KLH), the defect in response being suggested to be related to poor initial T cell priming (34).

Although the studies with OX40 and OX40L knockout mice demonstrated the critical role of these molecules in CD4 responses,

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Received for publication April 17, 2000. Accepted for publication June 28, 2000.

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¹ This work was supported by National Institutes of Health Grant AI42944 (to M.C.), a Grant-in-Aid from the American Heart Association (to N.K.) (96-221), and a fellowship from the Deutsche Forschungsgemeinschaft (to S.D.P.). This is publication no. 349 from the La Jolla Institute for Allergy and Immunology.

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³ Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; GVHD, graft-vs-host disease; LCMV, lymphocytic choriomeningitis virus; KLH, keyhole limpet hemocyanin; wt, wild type; MCC, moth cytochrome *c*; DNBS, dinitrobenzenesulfonic acid; GC, germinal center.

they did not distinguish between an effect on regulating T cell numbers or an effect on regulating T cell activity and differentiation, nor whether the major deficit was related to a lack of OX40 signals to the T cell or a lack of OX40L signals affecting APC function. In addition, there was little data to suggest whether targeting OX40 would affect the development of functional T cell memory, as the primary responses in these studies were only partially impaired.

In the present study, we have addressed the mechanism of action of OX40 and whether this molecule is critical to long-term T cell responses and memory. We had previously proposed that costimulation through OX40 may regulate the ability of CD4 T cells to expand and survive, based on experiments *in vitro* that showed greatly enhanced T cell proliferation to OX40L expressing APCs in a situation where cell division was normally limited (12, 35). In this report, we present data that support this and show that OX40 regulates the extent of T cell expansion in the primary T cell response and this translates into an ability to persist as a population over time. An agonist Ab to OX40 promoted greater numbers of CD4 T cells to accumulate after the peak of a normal primary response and to survive with time as memory cells. Moreover, CD4 cells from OX40-deficient mice could not sustain IL-2 production and a proliferative response as time progressed, resulting in reduced survival. OX40-deficient mice could not generate normal numbers of Ag-specific T cells in the later stages of a primary response and this led to severely impaired development of memory, again characterized by much lower frequencies of CD4 cells surviving over time. These data support the conclusion that OX40-OX40L regulates the number of T cells that can be generated in a primary response and that persist as memory cells.

Materials and Methods

Mice

OX40-deficient mice were generated at University of California, San Francisco (UCSF) (22). These were backcrossed four times onto C57BL/6 at UCSF, and a further three times at La Jolla Institute for Allergy and Immunology (LIAI). Wild-type (wt) BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or used as OX40^{+/+} littermates from crossing heterozygous mice. AND TCR transgenic mice were bred on a B10.BR background (36) and wt B10.BR mice were purchased from The Jackson Laboratory.

Adoptive transfers and immunizations

Chimeric mice were produced by injecting $2-3 \times 10^6$ V β 3/V α 11-positive CD4 cells from AND transgenic mice *i.v.* into unirradiated wt B10.BR recipients. After 2 days, these were immunized *s.c.* in the tail base with peptide 88–103 of moth cytochrome *c* (MCC) (synthesized at LIAI) emulsified in CFA (Fischer Scientific, Pittsburgh, PA). For experiments with BL/6 mice, KLH (Calbiochem, La Jolla, CA) was used *s.c.* emulsified in CFA, or *i.p.* precipitated with alum and mixed with 10^9 *Bordetella pertussis* organisms (Michigan Public Health Department, Lansing, MI). Anti-mouse OX40 (rat IgG1) was generated from the hybridoma OX-86 (11) obtained from the European Cell Culture Collection (Wiltshire, U.K.). This or an isotype-matched control rat IgG1 (PharMingen, San Diego, CA) was injected *i.p.* in PBS 2 days after Ag.

Tracking Ag-specific transgenic T cells

Expansion of MCC-reactive transgenic T cells was assessed in adoptive recipients using a method similar to that developed by Jenkins and colleagues (37), by staining with PE-labeled anti-V β 3, FITC-labeled anti-V α 11, and cychrome-labeled anti-CD4 (PharMingen). Controls were with labeled anti-rat isotype-matched Abs (PharMingen) and analyses conducted on a Becton Dickinson (Mountain View, CA) FACScan with CellQuest software.

Cell cultures

In vitro cultures were with lymph node or splenic populations depleted of CD8 T cells using anti-CD8 (3.155, *in-house*) and rabbit complement (Accurate Scientific, Westbury, NY), or with purified CD4 cells isolated using

complement and Abs to CD8 (3.155), heat stable Ag (JIID), and class II MHC (M5/114 and CA-4.A12). Irradiated splenocytes from wt mice were added as APCs, either unactivated, or preactivated overnight with LPS and dextran sulfate (DXS; both at 10 μ g/ml). Ag was added directly into culture or prepulsed onto the APCs (100 μ g/ml). Cultures were set up in 0.2-ml volumes in 96-well plates (Costar, Cambridge, MA) in triplicate. With stimulation from anti-CD3 (2C11, *in-house*), Ab was added into culture (soluble), or immobilized on plastic by incubating 50 μ l in PBS in 96-well plates for 2 h at 37°C.

Proliferation

Cell division was assessed by addition of 1 mCi tritiated thymidine (ICN Biomedicals, Irvine, CA) to 0.2-ml cultures for ~18 h. Response was assessed between 72–90 h, 96–114 h, or 120–138 h.

Cytokine secretion

Duplicate supernatants were recovered 20–24 h after T cell stimulation and assayed after pooling. IL-2 production was determined by titrating supernatants onto NK.3 cells, in duplicate, in the presence of anti-IL-4 (11B11) and measuring proliferation 48 h later (36). IL-4, IL-5, and IFN- γ were measured by ELISA as before (36).

Limiting dilution assay

CD8-depleted splenocytes (corrected for CD4 numbers by FACS) were added to 96-well plates in replicates of 36 in the presence of irradiated APCs (2×10^5 /well) from wt mice, prepulsed overnight with 100 μ g/ml KLH. CD4 cells were plated in 1.5-fold dilutions down to 300 cells per well with at least 11 dilutions used per T cell group. Supernatants were harvested at 24 h and used neat in the NK bioassay to assess IL-2. Wells giving cpm values greater than 2 SDs above the mean of wells containing only T cells or APCs were considered positive. The fraction of negative wells were plotted as a log against the number of CD4 cells, and frequencies calculated using the 37% negative point (38).

Results

An agonist Ab to OX40 enhances primary CD4 responses by promoting clonal expansion and survival

To determine the mode of action of OX40, we initially assessed the T cell response to MCC in an adoptive transfer system, tracking TCR transgenic T cells in mice given an agonist Ab to OX40. We have shown the agonist activity of this Ab *in vitro*, and have not noted any antagonist ability (41). V β 3/V α 11 CD4 cells from AND TCR transgenic mice were adoptively transferred into syngeneic recipients and these were subsequently immunized with two doses (50 or 5 μ g) of MCC in CFA *s.c.* (Fig. 1). OX40 expression is induced 1–2 days after immunization *in vivo*, and down-regulated 3–4 days later (our unpublished observations). To coincide with the induction of OX40, the Ab was given on day 2 and T cell responses assessed on day 4 and 8 in comparison to mice receiving a control Ab.

In the controls, a high concentration of Ag (50 μ g) resulted in expansion of CD4 cells in draining lymph nodes and spleen, peaking at day 4 when analyzed either as a percent of transgenic cells or total number of cells present in each organ (Fig. 1, *a* and *b*, *top panels*). Anti-OX40 enhanced T cell expansion compared with controls resulting in 2- to 3-fold increases in T cell number at 4 days in the draining lymph nodes, and at 8 days in the spleen. A greater effect was seen with a suboptimal dose of Ag (5 μ g), which only produced a weak T cell response alone. With anti-OX40, the percentage of Ag-specific T cells increased from 2% to 12% in draining lymph nodes on day 4, and to 5% in spleen on day 8 (Fig. 1, *a*, *bottom panels*). When converted to total numbers of T cells, a 34-fold increase was observed in the draining lymph nodes at day 4, and a 10-fold increase in the spleen at day 8 (Fig. 1, *b*, *bottom panels*). Nondraining lymph nodes either showed no difference or a slight increase in numbers of transgenic T cells after anti-OX40 treatment (data not shown). These results demonstrate that OX40 signals promote increased accumulation of CD4 T cells at the sites of Ag exposure, which resulted from clonal expansion and survival

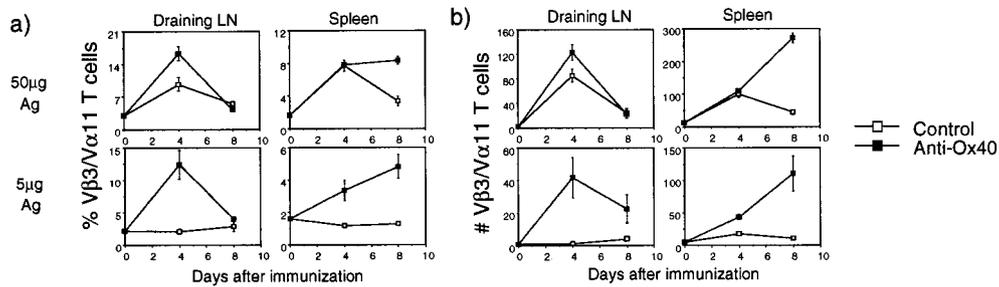


FIGURE 1. Anti-OX40 promotes increased expansion of MCC-specific CD4 T cells. A total of 2×10^6 Vβ3/Vα11 CD4 cells from H2^k AND TCR transgenic mice were transferred i.v. into groups of three syngeneic B10.BR mice, followed by immunization with 50 or 5 μg of MCC peptide s.c. in CFA at the base of the tail 2 days later (day 0). Mice were treated with 75 μg of control rat IgG1 (open symbols) or anti-OX40 (mAb, OX86; filled symbols) given i.p. on day 2. Transgenic T cells were visualized in the draining lymph nodes (periaortic plus inguinal) and spleen by staining for Vβ3, Vα11, and CD4. All results represent mean values ± SEM from three individual mice per time point per group. Similar responses were seen in three separate experiments. *a*, Percentage (%) of Vβ3/Vα11 T cells within the CD4 population. *b*, Total number of Vβ3/Vα11 CD4 T cells ($\times 10^4$).

of these cells, rather than simply altering recruitment of T cells to the site of response.

Anti-OX40 also up-regulated the secretion of IL-2, IFN-γ, and IL-5, by 2- to 3-fold over controls, depending on the cytokine and the organ studied, when assessed in bulk cultures (Fig. 2*a*, top panels). Two previous reports had suggested that OX40 ligation preferentially augmented Th2 cytokines (39, 40), although a recent study in OX40L knockout mice showed defective Th1 and Th2 responses (34). We did not see a bias to one particular subset *in vivo* with the agonist anti-OX40, and we have seen similar results *in vitro* (41). The effect on cytokine secretion was more dramatically illustrated when responses were recalculated to take into account the total number of Ag-specific T cells (Fig. 2*a*, bottom panels). In this case, cytokine responses were augmented 4- to 6-fold. Lastly, cytokine secretion per *in vitro* culture was normalized for the number of Vβ3/Vα11 cells plated to assess whether anti-OX40 increased the secretion ability of each individual T cell generated. By this calculation, there was no detectable effect on the functional capabilities of each individual effector T cell, as measured by IL-2 production (Fig. 2*b*), or IFN-γ and IL-5 (data not shown). This suggests that although OX40 signals result in more effector cells being generated and surviving, and hence more cytokine-secreting cells, each individual effector cell does not increase their output of cytokines.

OX40 signals promote the generation of memory T cells

To determine the impact on memory, adoptive transfer experiments were conducted assessing responses 5 wk after immuniza-

tion with 50 or 5 μg MCC in CFA (Fig. 3). In control mice, the total number of Ag-specific cells was significantly elevated at this time compared with an unimmunized mouse, indicating memory cells had developed, with anywhere from 5- to 50-fold more cells present depending on the initial dose of Ag (e.g., in one experiment, on average 1.1×10^4 Vβ3/Vα11 cells were found in unimmunized animals, whereas 30×10^4 cells were found 35 days after Ag immunization). Treatment with anti-OX40 resulted in elevated percentages (Fig. 3*a*) and total numbers of Vβ3/Vα11 CD4 cells (Fig. 3*b*) in the spleen and pooled periaortic and inguinal lymph nodes compared with control animals, and this effect was seen at both Ag doses. A larger increase in the number of memory T cells was observed with 5 μg Ag, correlating with the greater action of anti-OX40 on the primary response (Figs. 1 and 2). Frequencies were 4- to 8-fold higher than controls in the spleen, and 2- to 4-fold in the lymph nodes. Analysis of other lymph nodes again showed similar numbers of T cells compared with controls, demonstrating that recruitment could not account for the elevated frequencies (our unpublished data).

IL-2, the major cytokine produced by memory T cells (42), was also increased after anti-OX40 treatment (Fig. 3*c*). Bulk cultures showed up to 12-fold increases in IL-2 (Fig. 3*c*, left panel), and this was reflected when values were normalized based on total T cell numbers with 4- to 8-fold more IL-2 from anti-OX40 treated animals (Fig. 3*c*, middle). Again, when evaluated as IL-2 production per transgenic cell, there was little difference between control and anti-OX40 groups (Fig. 3*c*, right). We found little evidence of other cytokines secreted from the memory cells (IL-4, IFN-γ, not

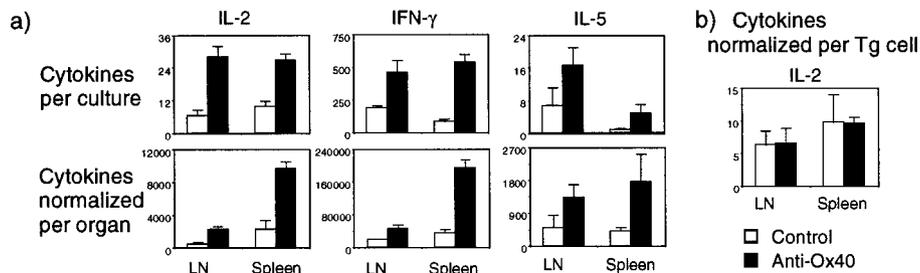


FIGURE 2. Anti-OX40 promotes increased numbers of MCC-specific cytokine-secreting CD4 T cells. Experiments were set up as in Fig. 1. with Vβ3/Vα11 CD4 cells transferred i.v. into groups of three syngeneic B10.BR mice, followed by immunization with MCC peptide s.c. in CFA at the base of the tail 2 days later (day 0). Mice were treated with 75 μg of control rat IgG1 (open symbols) or anti-OX40 (mAb, OX86; filled symbols) given i.p. on day 2. Similar responses were seen in 3 separate experiments. *a*, Secretion of IL-2, IFN-γ, and IL-5, after restimulation *in vitro* with MCC for 24 h. Cytokine data are from mice immunized with 50 μg MCC and taken on day 4 for lymph node cultures and day 8 for spleen. Upper panels are cytokines per culture (ng/ml), plating 4×10^5 cells per well. Lower panels are cytokine secretion, reflecting the total number of transgenic T cells in each lymph node or spleen. *b*, Secretion of IL-2 in ng/ml normalized for differences in transgenic T cell number, and reflecting the relative amount made by individual Ag-reactive T cells. Similar data were derived with IFN-γ and IL-5 (not shown).

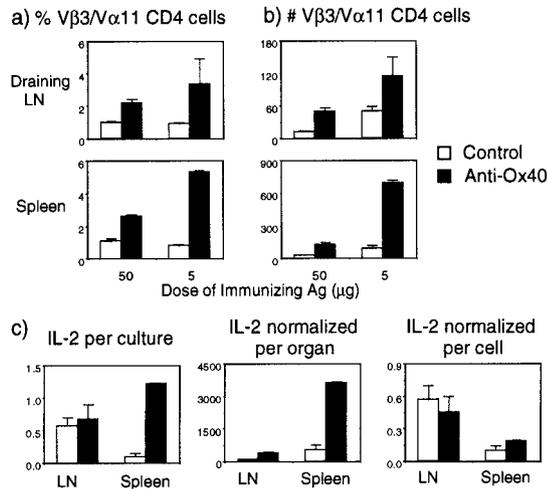


FIGURE 3. Anti-OX40 enhances memory T cell development. Recipients of adoptively transferred Vβ3/Vα11 transgenic CD4 cells were immunized as in Fig. 1 with MCC (50 and 5 μg). Two days later, 75 μg of anti-OX40 (■) or control IgG (□) was given i.p. Memory development was assessed at 5 wk. All results represent mean values ± SEM from three individual mice per group. Similar responses were seen in two separate experiments. *a*, Percentage (%) of Vβ3/Vα11 T cells within the CD4 population. *b*, Total number of Vβ3/Vα11 CD4 T cells ($\times 10^{-4}$). *c*, Secretion of IL-2 after restimulation in vitro with 2 μg/ml peptide. IL-2 data are from mice immunized with 5 mg MCC. Similar data were seen from mice immunized with 50 μg (not shown). *Left graph* is IL-2 per culture (ng/ml), plating 4×10^5 cells per well. *Middle graph* is IL-2 adjusted from data in the *left graph*, reflecting the total number of transgenic T cells. *Right graph* is IL-2 (ng/ml) normalized for differences in transgenic T cell number put in culture.

shown), implying that OX40 signals had not resulted in the preferential development of Th1- or Th2-like memory.

Therefore, these results demonstrate that OX40 signals can enhance development of CD4 memory. Again, the major action is to determine the number of memory cells that survive over time, rather than increase their activity on a per cell basis.

CD4 cells from OX40-deficient animals are impaired in their ability to sustain a proliferative response and to survive over time

Next, we analyzed the responses of CD4 T cells from mice made deficient in OX40 (22). We initially assessed responses of CD8-depleted populations in vitro. APCs were not removed so they could provide a source of OX40L. In some cases, activated syngeneic APCs from wt mice were additionally added to further provide OX40L costimulation. Equivalent results were obtained with OX40^{-/-} APCs, showing we were analyzing a selective defect in OX40, and that any reduced responses were due to lack of costimulation from OX40L.

Significantly, little difference was observed in proliferation after 3 days with a low concentration of plate-bound anti-CD3, whereas after 5 days OX40^{-/-} T cells were proliferating at levels greater than 50% lower than those of wt T cells (Fig. 4*a*, *top graphs*). Similar results were obtained with the weaker stimulus of soluble anti-CD3, except slightly reduced proliferation was evident at 3 days (~60% of wt), but again much less at 5 days (~30% of wt). When analyzing T cell survival, comparable results to proliferation were seen in that nearly identical numbers of cells were recovered from OX40^{-/-} cultures after 4 days with 2 μg/ml plate-bound anti-CD3 (Fig. 4*b*, *middle graphs*). Subsequently, survival of T

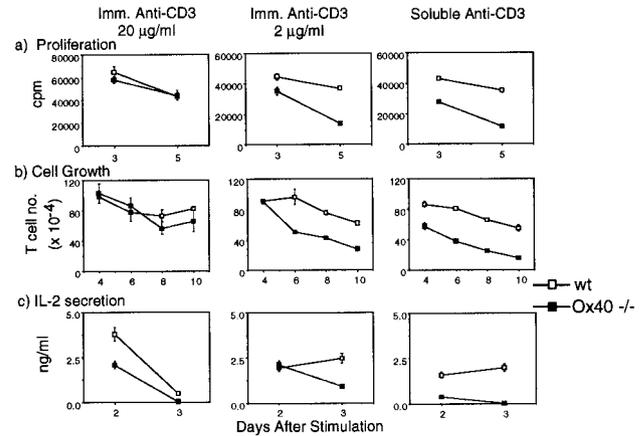


FIGURE 4. CD4 cells from OX40-deficient mice are impaired in response to mitogens in vitro. Splenocytes from H2^b OX40^{-/-} mice (■) and syngeneic wt OX40^{+/+} mice (□) were depleted of CD8 T cells and stimulated in vitro at 1×10^6 /ml in either 200 μl cultures in triplicate for proliferation and IL-2, or in 2 ml cultures in duplicate (for cell recovery). TCR signals were provided by immobilized anti-CD3 precoated at 20 μg/ml (*left panels*) or 2 μg/ml (*middle panels*), or by soluble anti-CD3 adding into culture at 20 μg/ml (*right panels*). Results are mean values ± SEM from duplicate or triplicate cultures. *a*, Proliferation (cpm) at 3 days and 5 days. *b*, T cell recovery ($\times 10^{-4}$) after 4, 6, 8, and 10 days of stimulation. *c*, IL-2 production (ng/ml) after 2 and 3 days. Background response in the absence of stimulation was less than 1000 cpm for proliferation, and with no detectable IL-2 (<0.02 ng/ml). Similar results were seen in two other experiments and when additional APC from wt mice, preactivated with LPS/DXS, were added into culture to provide further costimulation (not shown).

cells was markedly reduced in the OX40^{-/-} cultures (averaging ~50% of wt), although the rate of death/survival over time, shown by the slope of the curves, was fairly equivalent to that seen in wt cultures. With soluble anti-CD3, somewhat reduced T cell survival was evident by 4 days (~65% of wt) and numbers declined with increasing time (~30% of wt by day 10), in this case the rate of death/survival being greater in the absence of OX40. We have recently reproduced the latter effect with T cells from OX40^{-/-} mice bred to AND transgenics and responding to peptide in vitro (P. R. Rogers and M.C., unpublished observations), suggesting that OX40 may regulate both clonal expansion and the ability of the expanded T cells to survive.

IL-2 production correlated with proliferation and cell growth. OX40^{-/-} T cells produced less IL-2 (10–40% of wt) with stimulation from a low concentration of immobilized anti-CD3 and soluble anti-CD3 at late times, whereas IL-2 production was more comparable to wt cultures when analyzed at earlier times (Fig. 4*c*, *bottom graphs*). Because OX40-negative T cells were proliferating equivalently or less than wt T cells, it is unlikely that the lower IL-2 levels reflected increasing consumption by the responding cells.

Therefore, these results complement our in vivo data with anti-OX40 and show that OX40 signals regulate the ability of CD4 cells to sustain cell division during the later phases of the primary T cell response, and are required for continued survival of high numbers of cells over time. Interestingly, with a high dose of immobilized anti-CD3 (Fig. 4, *left graphs*), OX40-deficient T cells behaved identically to wt T cells with respect to proliferation, IL-2, and cell survival, suggesting that OX40 signals can be bypassed if there is a sufficiently strong antigenic/TCR stimulus.

Primary and memory CD4 responses are impaired in OX40-deficient mice

Functional responses of OX40^{-/-} mice were assessed using KLH given i.p. in alum/*B. pertussis*. The primary effector response was analyzed 7 days after immunization, and development of memory at 35 days (Fig. 5). At 7 days, OX40^{-/-} CD4 cells proliferated at levels significantly below those of wt T cells when restimulated in vitro with KLH (25–35% of wt), and produced lower levels of all cytokines measured (<30% of wt), including IL-2, IFN- γ , IL-5, and IL-4 (Fig. 5*a*). These results are similar to previous data obtained with primary KLH responses in OX40L-deficient mice (34). Importantly, we also demonstrated the novel finding that the extent of memory was lower in the OX40^{-/-} mice with proliferation reduced by 70–80%, and IL-2 reduced by 80%, when assessed 35 days after immunization (Fig. 5*b*). Again, IL-2 was the major cytokine detected in memory T cell cultures, and if other cytokines were seen, these were universally reduced in the OX40^{-/-} cultures, suggesting no preferential control of a particular T cell subset (not shown). These data directly correlate with, and complement, our earlier results with the agonist Ab to OX40 and confirm that OX40 signals are integral to the effective development of a memory CD4 response.

An OX40 deficiency results in generation of reduced frequencies of primary effector and memory CD4 T cells

Lastly, we assessed the frequency of KLH-specific T cells that developed in OX40 knockout mice. Frequencies of primary effector cells and memory cells were measured by limiting dilution 5–7 days and 35 days after immunization, respectively. As a measure of the T cell frequency, we assessed IL-2 production, firstly because this cytokine is produced by both subsets, and secondly because the bioassay for IL-2 is the most sensitive way of determining T cell responsiveness that we have.

Fig. 6 shows the primary effector and memory responses of three individual mice per group. In all cases, the frequency of

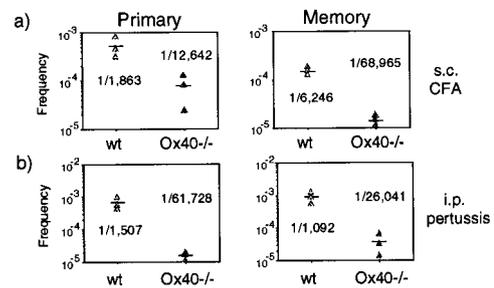


FIGURE 6. Frequencies of primary effector and memory KLH-specific T cells are reduced in OX40 knockout mice. OX40^{-/-} mice (\blacktriangle) and wt mice (\triangle) were primed in groups of three with 100 μ g KLH given either s.c. in CFA (*a*) or i.p. in alum/*pertussis* (*b*). T cell frequencies were determined by limiting dilution assay as described in experimental procedures. The frequency of primary effector T cells was determined on day 5 with CFA and day 7 with alum/*pertussis*, and the frequency of memory T cells was determined on day 35 in both cases. Frequencies with CFA for both primary and memory were performed on mice immunized at the same time. Memory frequencies with alum/*pertussis* were derived from separate immunizations.

KLH-specific T cells was substantially reduced in the OX40-deficient animals, regardless of the time of assay or method of immunization. With CFA immunization, there was on average a 7-fold difference in the frequency in the primary response, and an 11-fold difference once memory had developed. With alum/*B. pertussis*, a 40-fold lower frequency of KLH-responsive T cells was detected in the OX40^{-/-} mice during the primary response, and a 24-fold lower frequency at the memory stage. Thus, OX40 signals regulate the number of Ag-specific CD4 cells that develop in the primary response, and the number that survive over time as memory cells.

Discussion

Recent studies of OX40 and OX40L knockout mice have clearly implicated these molecules in regulating primary CD4 T cell responses (22, 32–34). However, the only indication of the mechanism of action of OX40-OX40L was reduced recall proliferative responses in vitro, which could have been due to either a defect in generating Ag-specific T cells, or a defect in the activity of individual T cells. In this report, we have shown that OX40 signals sustain clonal expansion of T cells during primary Ag-specific responses and are instrumental in determining the number of cells that survive over time as a memory population. In addition, OX40 signals indirectly regulate the functional capacity of the T cell response in that overall higher levels of cytokines are produced from primary effector and memory populations generated by engagement of this molecule, although OX40 signals do not lead to the generation of individual T cells, which respond better. Similarly, a deficiency in OX40 leads to both reduced primary Th1 and Th2 responses and reduced memory responses, but this can be attributed to fewer Ag-specific T cells generated rather than a reduction in the activity of a similar number of T cells. The results provide new insight into the action of OX40 as a major regulator of T cell growth and survival.

The importance of OX40 in primary CD4 but not CD8 responses was shown in initial studies of OX40 and OX40L knockout mice (22, 32–34), highlighting the requirement for this molecule in T cell priming. Our previous results demonstrated that OX40L transfected APCs dramatically enhanced CD4 proliferation in vitro, several days after initial stimulation, in a situation where cell division had begun to diminish (12), implying that a major action was to prolong clonal expansion. This was similarly

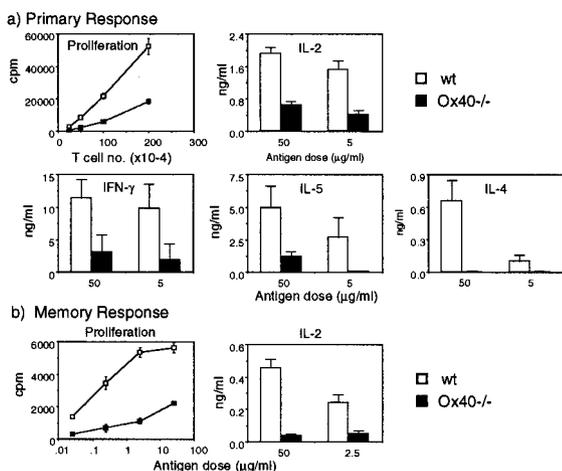


FIGURE 5. Primary effector and memory responses to KLH are impaired in OX40 knockout mice. Groups of three OX40^{-/-} mice (\blacksquare) and three wt OX40^{+/+} mice (\square) were primed i.p. with 100 μ g KLH in alum/*pertussis*. On day 7 (*a*, primary effector response) and on day 35 (*b*, memory response), CD8-depleted spleen cells were cultured in vitro in triplicate at either different T cell concentrations or with varying doses of KLH in the presence of 2×10^5 syngeneic wt APC. Proliferation was measured after 96 h, and secretion of IL-2 (primary effector and memory), or IFN- γ , IL-5, and IL-4 (primary effector only) was assessed at 24 h. Results are the mean responses \pm SEM of three individual mice, and are representative of three separate experiments.

implied in the study of OX40^{-/-} mice responding to LCMV, where a 3- to 4-fold reduction in the number of IFN- γ -secreting cells was detected by FACS analyses 15 days after infection (32). The experiments here with an agonist Ab to OX40, and with OX40-deficient T cells, conclusively show that clonal expansion in the primary CD4 response is regulated by OX40 ligation. As well as regulating T cell expansion, our data also show that OX40 can impact on T cell survival. Thus, frequencies of Ag-specific cells were dramatically lower 35 days after priming OX40^{-/-} animals, and they were significantly higher after treatment with anti-OX40. Overall, the data therefore suggest that a major action of OX40 is to regulate the number of T cells that are expanded in primary responses and thus survive through to memory. These results appear to be consistent with recent data assessing the impact of OX40-OX40L interactions on germinal center (GC) reactions. In one study, transgenic expression of OX40L on dendritic cells resulted in greater numbers of CD4 cells in B cell follicles (43), and in a second study, an inhibitory OX40.Fc fusion protein reduced GC size, and T cell numbers in GCs (44).

Although we have focused on the effects of OX40 signaling to the T cell, it is possible that defects in the OX40 knockout animals were also related to a lack of OX40L signaling to various APCs. Previous studies showed that both primary and secondary IgG responses could be inhibited with a polyclonal antiserum to OX40 (23), implying that OX40 and/or OX40L signals regulated B cell responses, but not distinguishing this from an effect on inhibiting T cell expansion. Although we did not assess B cell responses in the studies here, separate analyses of OX40 knockout animals did not reveal any major defects in the B cell response to several Ags including TNP-KLH, NP-CGG, *Leishmania*, and *Nippostrongylus* (22), or VSV, LCMV, and influenza (32). These results are therefore at variance with the earlier studies with the antiserum (23), and suggest that OX40/OX40L does not have a dominant role in B cell responses in vivo. It was also recently proposed, from in vitro experiments with an agonist Ab, that OX40L could signal dendritic cells to promote secretion of inflammatory cytokines such as IL-1, TNF, and IL-6 (17). Our results in the OX40 knockout animals could then be interpreted as a requirement for OX40L to enhance dendritic cell action, which in turn would have regulated the T cell response, rather than a direct effect of OX40 signaling to the T cell. This then would be comparable to the CD40-CD40L interaction which many people regard as largely affecting the APC rather than a requirement for CD40L signals for the T cell. However, the fact that similar conclusions were derived with anti-OX40 in vivo, and that anti-OX40 also promotes T cell growth and survival in vitro (41), suggests that the main deficiency in T cell responses in OX40 knockout mice were related to a lack of signals to the T cell. It remains to be determined whether OX40L signals are critical to APC function.

Whether OX40 signals directly regulate T cell survival is not clear. OX40 can bind TRAFs 2, 3, and 5, and this results in NF- κ B activation (45, 46), consistent with the notion of a survival effect. Our data demonstrate a major difference in the frequencies of memory cells if OX40 is ligated and if OX40 is lacking. However, because frequencies were also different during the peak of the primary response, and the relative difference between the frequency from the primary effector to the memory stage was similar regardless of the presence or absence of OX40 signals (see Fig. 6), it could be argued that OX40 does not intrinsically provide a survival advantage on an individual cell basis, only at the population level. Thus, OX40 may largely regulate clonal expansion in the primary response, and because of the increased numbers at this stage, more cells will persist through to memory by default. However, other recent data, under potentially tolerizing conditions with soluble

superantigen, have shown a dramatic difference in T cell survival over time when LPS is combined with the agonist OX40 Ab (47), a phenomenon presumably brought about by suppressing activation induced cell death. OX40 may therefore synergize with, or regulate, signals from other surface molecules or cytokines, rather than being a bona fide survival factor. Regardless, from the data with anti-OX40 and OX40 knockout animals, it is clear that OX40 signals can have a major impact on the frequency of T cells that form the memory pool. As such, these studies suggest that agonist reagents to OX40 may be useful therapeutically in enhancing T cell memory, and antagonist reagents may limit long-term detrimental immune responses such as those which occur in many autoimmune states.

The data from OX40 and OX40L knockout animals predict that OX40 signals will be required during development of functional T cell populations in most immune responses. As mentioned before, inhibition of OX40-OX40L interaction reduced inflammatory responses in proteolipid protein-induced EAE, trinitrobenzene sulfonic acid-induced colitis, and in GVHD (19, 30, 31), and more recently an anti-OX40L Ab suppressed the Th2 response in *Leishmania*-infected BALB/c mice (48). Preliminary results with myelin oligodendrocyte glycoprotein in OX40-deficient mice also show reduced severity and incidence of EAE (A.D.W., unpublished data). However, H2^b OX40 knockout mice are resistant to *Leishmania major*, and can mount normal responses to *Nippostrongylus brasiliensis* (22). Because of the latter results, it is therefore not clear what ultimately governs the use of OX40. It was originally suggested that OX40 may only control Th2 responses (39, 49), which could have partially explained these results. However, the data in OX40-deficient mice with LCMV and influenza (32), and with KLH (this paper), and in OX40L-deficient mice with DNBS (33) and KLH (34), clearly show that IFN- γ is also regulated by these molecules. A more attractive idea, therefore, is that the use of OX40 is dependent upon the magnitude of the antigenic insult. Significantly, in vitro, we saw normal responses of OX40^{-/-} T cells to a high dose of immobilized anti-CD3, but defective responses when the apparent strength of T cell signaling was reduced (Fig. 4). Thus, much like CD28 (50, 51), a requirement for OX40 may be bypassed if sufficient signals can be provided by Ag to the TCR, or via other costimulatory receptors.

In conclusion, the results of our study show that OX40-OX40L interactions regulate clonal expansion of primary CD4 T cells. Because of the ability to control T cell numbers in the primary response, OX40 signals therefore determine the extent of memory that develops. We propose the following scenario. Initial activation of a naive CD4 T cell is controlled solely by Ag/TCR signals. These result in CD40L expression, which in turn promotes APC activation and induction of B7-1 and/or B7-2. Signals through CD28 regulate the early events in the T cell, and up-regulate IL-2 production, and drive cell cycle progression and initial expansion. One to two days into the response, OX40 is induced on the T cell and OX40L on APCs. These secondary interactions then promote sustained IL-2 production, continued T cell expansion, and suppression of cell death, and result in the high frequencies of T cells characteristic of primary responses and memory. Although we stress the importance of OX40 in this paper, it is likely that OX40 is not the only molecule which functions in this regard. Two other ligand-receptor pairs of the TNFR-TNF family may also perform similar functions, namely 4-1BB-4-1BBL (52-54) and CD27-CD70 (14, 55, 56). There is insufficient data at present to compare the merits of one to the other, but it could be envisioned that different antigenic stimuli may invoke their relative involvement to different degrees, or alternatively that there may be preferential use depending on whether the responding cell is a CD4 or a CD8 T

cell. Together, these molecules may determine the vigor of the primary T cell response and consequently have a critical role in establishing effective T cell memory.

Acknowledgments

We thank Mohammed Ullah for assistance in preparation of peptides used in these studies.

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