Immunity, Vol. 15, 445–455, September, 2001, Copyright ©2001 by Cell Press

OX40 Promotes Bcl-xL and Bcl-2 Expression and Is Essential for Long-Term Survival of CD4 T Cells

Paul R. Rogers,[1,4] Jianxun Song,[1,4] Irene Gramaglia,[1] Nigel Killeen,[2] and Michael Croft[1,3]

[1]Division of Immunochemistry
La Jolla Institute for Allergy and Immunology
San Diego, California 92121

[2]Department of Microbiology and Immunology
University of California, San Francisco
San Francisco, California 94143

Summary

It is important to understand which molecules are essential for long-lived immunity. We show that OX40 (CD134) is required with CD28 for the survival of CD4 T cells following antigen-driven expansion. In contrast to *CD28*$^{-/-}$ T cells, which show defects early, *OX40*$^{-/-}$ T cells are relatively unimpaired in IL-2 production, cell division, and expansion. However, *OX40*$^{-/-}$ T cells fail to maintain high levels of Bcl-xL and Bcl-2 4–8 days after activation, and undergo apoptosis. Conversely, OX40 stimulation promotes Bcl-xL and Bcl-2 and suppresses apoptosis. Moreover, retroviral transduction of *OX40*$^{-/-}$ T cells with Bcl-xL or Bcl-2 reverses their survival defect. Thus, a temporal relationship exists between CD28 and OX40, with OX40 being a critical regulator of antigen-driven T cell survival.

Introduction

At least two signals are needed for optimal activation of T cells (Lafferty and Woolnough, 1977). The first is delivered by TCR interaction with MHC and peptide, and the second involves ligation of costimulatory receptors that are needed for enhancing cytokine production, augmenting cell proliferation, and promoting cell survival. Without second signals, T cells can become anergic or undergo apoptosis. The best characterized second signal is the interaction of CD28 with CD80 and CD86 (Linsley and Ledbetter, 1993). CD28 can enhance T cell proliferation (June et al., 1987), cytokine secretion (Jenkins et al., 1991), and expression of antiapoptotic proteins such as Bcl-xL (Boise et al., 1995). In vivo studies have supported a dominant role for CD28 in many responses including contact hypersensitivity, asthma, colitis, EAE, and some viral and certain parasitic infections.

Although CD28 provides a critical signal for naïve T cell activation, additional costimulatory interactions have been described which may represent essential components of a long-lived T cell response (Croft and Dubey, 1997; Watts and DeBenedette, 1999). In particular, recent data on OX40 (CD134) and its ligand (CD134L), members of the TNFR/TNF superfamily, suggest that they play a complementary role to CD28. Unlike CD28, which is constitutively expressed on resting T cells, OX40 is only expressed 1 to 2 days after activation (Al-Shamkhani et al., 1996; Calderhead et al., 1993; Gramaglia et al., 1998; Paterson et al., 1987). OX40L is also not expressed on resting APC but is induced several days after activation, by CD40L-CD40 interactions or nonspecific inflammatory stimuli (Godfrey et al., 1994; Ohshima et al., 1997; Stuber et al., 1995). OX40^{+} cells are found in the T cell zones of lymphoid organs following priming with antigen (Stuber and Strober, 1996), and at peripheral inflammatory sites (Weinberg et al., 1994, 2000).

Ligation of OX40 on CD4 T cells by agonist reagents can increase clonal expansion and cytokine production (Flynn et al., 1998; Gramaglia et al., 1998, 2000; Maxwell et al., 2000), enhance memory T cell development (Gramaglia et al., 2000; Maxwell et al., 2000) and antitumor immunity (Weinberg et al., 2000), and prevent and reverse the tolerance process (Bansal-Pakala et al., 2001). There is now a body of evidence which demonstrates the natural use of OX40 in several types of T cell response, with recent data from knockout mice showing decreased CD4 response to nominal antigen and in various models of disease, all scenarios where CD28 has been shown to play a role (Chen et al., 1999; Gramaglia et al., 2000; Jember et al., 2001; Kopf et al., 1999; Murata et al., 2000).

Thus, OX40 is equally as important to a T cell response as CD28. However, there has been little direct data to indicate the mode of action of OX40. We recently showed that the frequency of antigen-specific memory T cells was dramatically reduced in OX40-deficient mice after priming (Gramaglia et al., 2000), suggesting that OX40 may function to regulate T cell survival. In this study, we now present direct evidence for this. We demonstrate that OX40 is integral to a response initiated by CD28, and that it acts sequentially in a late capacity to suppress apoptosis of primary CD4 T cells. Moreover, we show that the antiapoptotic proteins Bcl-xL and Bcl-2 which are initially regulated by CD28, are also targets of OX40 action. Thus, CD28 and OX40 contribute synergistic signals provided in a temporal fashion, with OX40 representing an essential late signal that is required to promote long-term CD4 T cell survival.

Results

Maximal Expression of OX40 and OX40L Are Dependent on CD28 Signals

In order to characterize the role of OX40 in relation to CD28, we bred *OX40*$^{-/-}$ and *CD28*$^{-/-}$ mice onto the AND (Vβ3/Vα11) TCR transgenic background. Resting wild-type (wt) T cells expressed CD28 but not OX40 (Figure 1A). OX40 was expressed 1 day after activation with moth cytochrome c (MCC), and peaked after 3 days (Figure 1B). *CD28*$^{-/-}$ T cells expressed OX40 after activation but expression was delayed, and absolute levels were 4-fold lower than on wt T cells. OX40L was expressed on APC between 2 and 4 days with wt T cells (Figure 1C), with most dendritic cells and ~50% B cells being positive (data not shown). OX40L was minimally induced in cultures with *CD28*$^{-/-}$ T cells. Therefore,

[3]Correspondence: mick_croft@liai.org

[4]These authors contributed equally to this work.

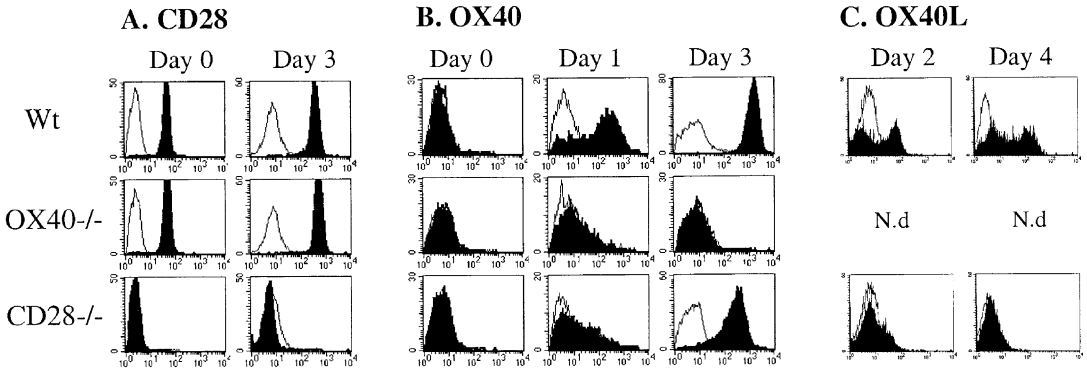

Figure 1. CD28 Is Required for Rapid and Maximal Expression of OX40 and OX40L

CD4 T cells from control AND mice (wt), OX40-deficient AND mice (*OX40*$^{-/-}$), and CD28-deficient AND mice (*CD28*$^{-/-}$) were obtained ex vivo (day 0) or were cultured for varying lengths of time with T-depleted spleen APCs and peptide (1 μM).

(A) Expression of CD28 on CD4 cells.

(B) Expression of OX40 on CD4 cells.

(C) Expression of OX40L on non-CD4 cells. OX40L was not found on resting APC. N.d., not done.

CD28 is not required for OX40 expression, but is needed to obtain early and maximal expression, and CD28 indirectly regulates the induction of OX40L on APC.

OX40, Unlike CD28, Is Minimally Involved in the Initial Naïve T Cell Response

To determine whether OX40 functions during the early naïve T cell response, *OX40*$^{-/-}$ T cells were stimulated with peptide presented on splenic APC. Whereas *CD28*$^{-/-}$ T cells were dramatically impaired in their ability to produce IL-2 (5% of wt), *OX40*$^{-/-}$ T cells showed only a moderately decreased ability to secrete IL-2 (<40% of wt; Figure 2A).

In contrast to *CD28*$^{-/-}$ T cells, which showed substantially reduced proliferation at 3 days, *OX40*$^{-/-}$ T cells proliferated similarly to wt T cells (Figure 2B). By CFSE analysis, after 2 days, there was no difference in the number of divided *OX40*$^{-/-}$ cells versus wt cells (Figure 2C). Again, this was in contrast to *CD28*$^{-/-}$ T cells, which lagged behind controls in the number that divided and the extent of division. Collectively, this shows that CD28 controls much of the early T cell response with little participation of OX40.

OX40 Controls Late Cell Division and Prolongs T Cell Turnover

The requirement for OX40 for continued T cell expansion was determined. After 5 days, *OX40*$^{-/-}$ T cells had undergone fewer divisions (Figure 2E). Most *OX40*$^{-/-}$ T cells divided, but with a lag in the rate of division. As expected, *CD28*$^{-/-}$ T cells were more dramatically impaired. Proliferation after 5 and 6 days was reduced in *OX40*$^{-/-}$ T cells under those antigenic conditions (1 and 10 μM) where proliferation was still evident in control wt populations (Figure 2D). Thus, OX40 prolongs T cell division initially induced by CD28.

OX40 Controls T Cell Survival by Suppressing Apoptosis

Previously, we showed in vivo that the frequency of antigen-specific primary effector T cells and memory T cells generated after priming with KLH was significantly reduced in *OX40*$^{-/-}$ mice (Gramaglia et al., 2000). This suggested that OX40 may control T cell survival, but did not distinguish between an antiapoptotic effect as opposed to a decreased ability of T cells to expand in numbers during the initial phase of the primary response. This was determined here by monitoring T cell survival and death over time (Figure 3). With high-dose peptide, part of the long-term viability of wt T cells could have been due to continued cell division, as proliferation was still evident at day 5 and day 6 (Figure 2D). With lower peptide doses, the stability in T cell numbers solely reflected the capacity to survive, as no proliferation was seen after 5 days.

OX40$^{-/-}$ T cells were dramatically impaired in their ability to survive over time with 60%–80% fewer T cells being viable 12 days after activation, numbers almost identical to those in the absence of CD28 (Figures 3A and 3B). Little difference was observed in the number of cells that were initially generated without OX40, whereas there were 50% fewer in the absence of CD28 (Figure 3B, day 4). The majority of cell death occurred between day 4 and day 8 in *OX40*$^{-/-}$ T cells (Figure 3C), and this was confirmed by demonstrating a markedly greater number of annexin^{+} T cells in *OX40*$^{-/-}$ cultures (Figure 3D, left). The majority of apoptotic *OX40*$^{-/-}$ T cells had undergone four divisions, whereas most apoptotic wt T cells had undergone five or six divisions (Figure 3D, middle). This suggests that part of the reduced ability of *OX40*$^{-/-}$ T cells to divide at late times was due to cell death. Two main pathways of death were described a number of years ago (Strasser et al., 1995), one involving signals from TNFR family members possessing death domains (active cell death) and the second governed by a number of proteins of the Bcl-2 family (passive death). Both pathways led to activation of the caspase cascade. Demonstrating that defective survival of *OX40*$^{-/-}$ T cells did involve caspase proteins, the death of *OX40*$^{-/-}$ T cells was efficiently blocked by peptide inhibitors of several caspases (Figure 3D, right).

These results show that a temporal relationship exists, with CD28 signals acting initially to provide expansion

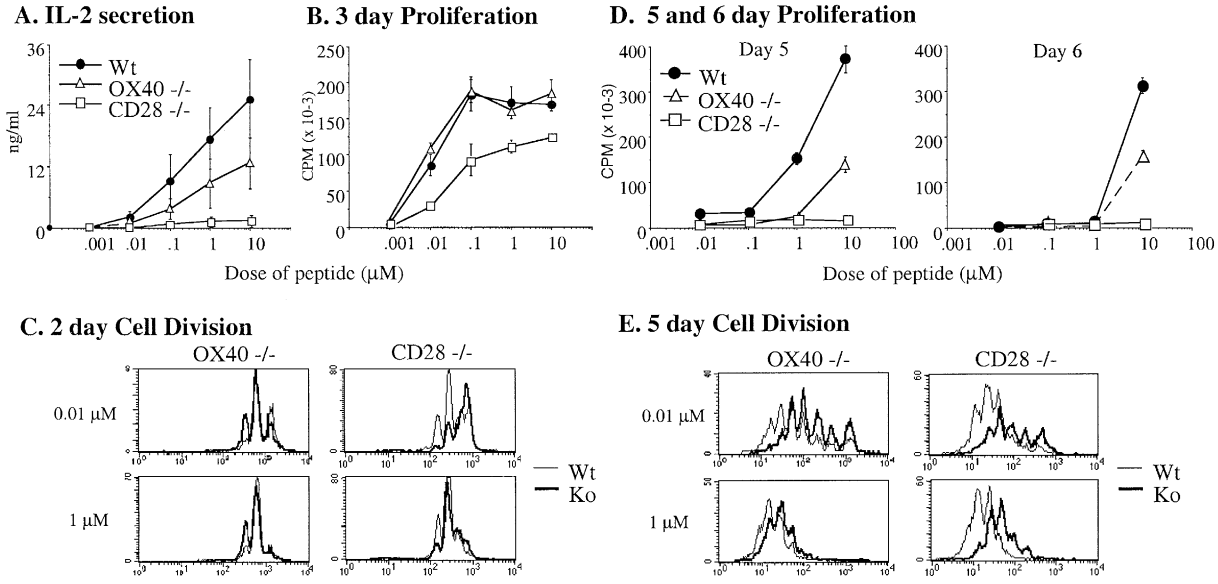

Figure 2. IL-2 Secretion, Early Proliferation, and Cell Division of OX40-Deficient T Cells Is Only Minimally Impaired Compared to CD28-Deficient T Cells

Naïve CD4 T cells from wt AND mice (closed circles), OX40-deficient AND mice (open triangles), and CD28-deficient AND mice (open squares) were stimulated with mitomycin C-treated T-depleted APCs and varying doses of peptide.

(A) IL-2 production at 40 hr. Data are the average and SD from three experiments.

(B) Proliferation at 72 hr. Data are representative of three experiments.

(C) Cell division of CFSE-labeled live CD4 cells 2 days after antigen stimulation. The thick line represents knockout T cells, while the thin line represents wt T cells. *OX40*$^{-/-}$ and *CD28*$^{-/-}$ data are from different experiments, and are representative of three experiments.

(D) Proliferation after 5 days (left) and 6 days (right). Similar data were seen in three experiments.

(E) Cell division of CFSE-labeled live CD4 cells 5 days after antigen stimulation. *OX40*$^{-/-}$ and *CD28*$^{-/-}$ data are from different experiments, and are representative of three experiments.

and life signals, followed by OX40 signals which prolong division at late times and allow survival in the absence of division by preventing caspase activation.

OX40$^{-/-}$ T Cells Cannot Maintain High-Level Expression of the Antiapoptotic Proteins Bcl-xL and Bcl-2

Previous studies have suggested that T cell death in the late primary response to foreign antigen is brought about by apoptotic mechanisms that do not involve death receptors such as Fas (Strasser et al., 1991, 1995; Van Parijs et al., 1998). Because our system faithfully mimics expansion and contraction of CD4 T cells responding to antigen encountered in an inflammatory environment in vivo (Gramaglia et al., 2000; Kearney et al., 1994; Van Parijs et al., 1998), it was likely that the death of *OX40*$^{-/-}$ T cells was brought about by passive rather than active mechanisms. As previous studies of CD28 showed an action on Bcl-xL and Bcl-2 (Boise et al., 1995; Mueller et al., 1996; Sperling et al., 1996), their levels were determined in responding *OX40*$^{-/-}$ T cells (Figure 4).

T cells lacking CD28 or OX40 did not vary appreciably from wt T cells with respect to Bcl-2 and Bcl-xL early in response at day 2 (data not shown). However, both *OX40*$^{-/-}$ and *CD28*$^{-/-}$ T cells exhibited significantly lower levels of Bcl-xL and Bcl-2 at day 4, a time point that precedes extensive cell death. Levels in *CD28*$^{-/-}$ T cells were less than in *OX40*$^{-/-}$ T cells (Figures 4A–4D). The more pronounced phenotype of *CD28*$^{-/-}$ T cells most likely reflects the combined lack of both CD28 and OX40 signals due to the lower expression of OX40 on *CD28*$^{-/-}$ T cells and minimal OX40L on APCs in these cultures (Figure 1).

After 8 days, the levels of Bcl-xL were significantly reduced in all populations, with wt as well as *OX40*$^{-/-}$ and *CD28*$^{-/-}$ T cells expressing lesser amounts than at day 4 (Figures 4A–4C). However, Bcl-xL was still lower in the absence of OX40 or CD28 signals. Bcl-2 was maintained at high levels in wt T cells at day 8 but was significantly reduced in the absence of OX40 or CD28 (Figures 4A, 4B, and 4D). In contrast, neither CD28 nor OX40 affected the levels of the proapoptotic molecule Bax (Figures 4A, 4B, and 4E).

To investigate whether reduced production of IL-2 was responsible for the death of *OX40*$^{-/-}$ T cells, cultures were supplemented with exogenous IL-2 such that levels reflected those seen from wt T cells (Figure 4F). A positive effect was seen on T cell proliferation after 5 days (Figure 4F, left), but exogenous IL-2 did not rescue survival of *OX40*$^{-/-}$ T cells (Figure 4F, middle), nor did it upregulate the levels of Bcl-xL or Bcl-2 (Figure 4F, right).

Agonist Antibody to OX40 Can Directly Augment Expression of Bcl-xL and Bcl-2 and Suppress Apoptosis

To directly show that OX40 can maintain expression of Bcl-xL and Bcl-2, and to determine the requirement for CD28, an agonist antibody to OX40 was used to stimu-

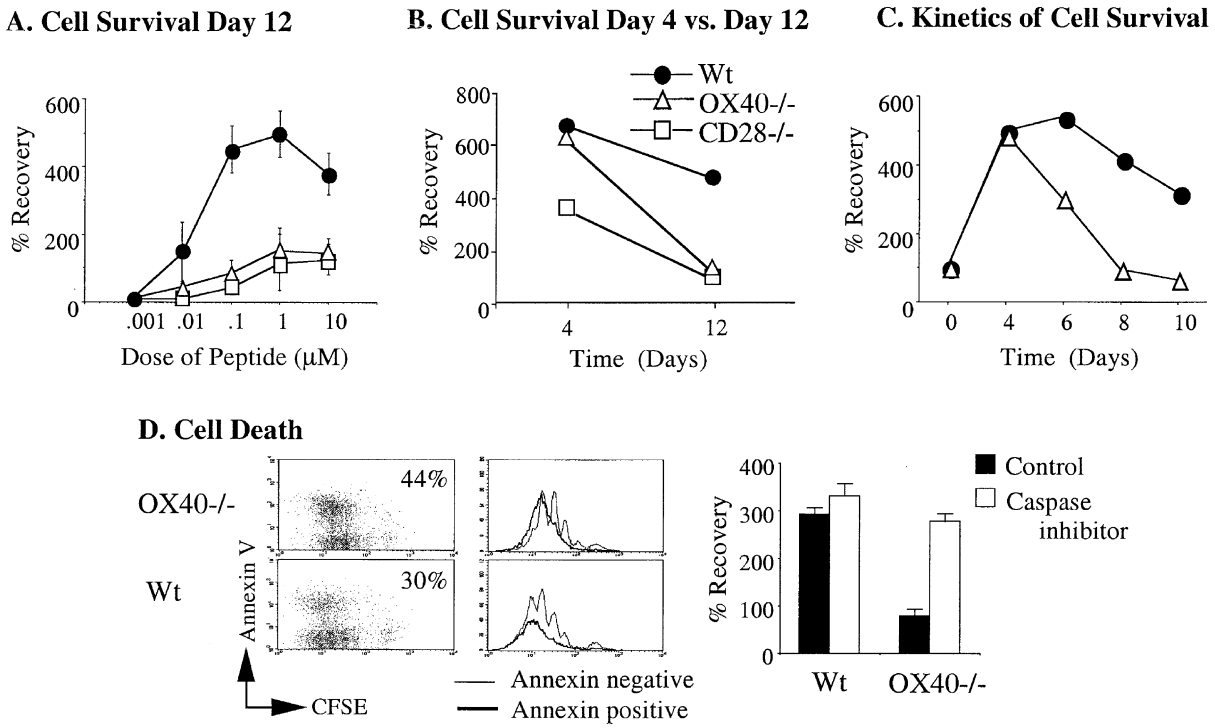

Figure 3. Long-Term T Cell Survival Is Severely Reduced in the Absence of OX40

5×10^5 *OX40*$^{-/-}$ (open triangles), *CD28*$^{-/-}$ (open squares), and wt (closed circles) AND T cells were stimulated with varying doses of peptide for 4–12 days as in Figure 2. Live CD4 cells were counted by trypan blue exclusion and the percent survival was calculated based on the input number of cells.

(A) T cell survival on day 12 from three experiments.

(B) A representative experiment out of three, comparing T cell survival on day 4 and day 12 after stimulation with 0.1 μM peptide.

(C) A representative experiment out of three, assessing the kinetics of T cell survival after stimulation with 0.1 μM peptide.

(D) Left panel shows annexin V staining as a function of cell division with CFSE-labeled T cells. Results show CD4 gated populations at day 6 with 0.1 μM peptide. Numbers represent % annexin V$^+$ T cells and are representative of two experiments. Right panel shows T cell survival after 8 days in the presence of control peptide or peptide inhibitors of caspase 3, caspase 9, or a global caspase inhibitor given on days 3, 5, and 7. Data are means of responses from all individual inhibitors from two experiments.

late *CD28*$^{-/-}$ T cells (Figure 5A). Anti-OX40 resulted in elevated Bcl-xL and Bcl-2 levels at late times (2- to 4-fold above controls). OX40 signals were similar in activity to CD28 signals, which also augmented both Bcl-xL and Bcl-2 in *OX40*$^{-/-}$ T cells. Anti-OX40 only minimally increased IL-2 from naïve T cells (<20% with wt or *CD28*$^{-/-}$ cells; Rogers and Croft, 2000 and data not shown), and had no effect on the number of T cells generated over 4 days (Figure 5B). However, anti-OX40 enhanced survival (Figure 5B) and suppressed apoptosis (Figure 5C) at late times, correlating directly with the positive effects on Bcl-xL and Bcl-2 expression.

Retroviral Transduction of Bcl-xL or Bcl-2 Prevents Defective Survival of *OX40*$^{-/-}$ T Cells

In order to directly demonstrate that defective survival of *OX40*$^{-/-}$ T cells was associated with reduced levels of the prosurvival members of the Bcl-2 protein family, a bicistronic retrovirus vector was used to transduce *OX40*$^{-/-}$ T cells with Bcl-xL or Bcl-2 and green fluorescent protein (GFP; Dahl et al., 2000; Ouyang et al., 1998).

CD4 cells from *OX40*$^{-/-}$ mice were transduced on day 2 and day 3 after activation with vector containing human Bcl-xL (MSCV-Bcl-xL) or Bcl-2 (MSCV-Bcl-2), both of which are functional in mouse cells. Death is accompanied by the loss of GFP, allowing the survival of transduced T cells to be followed by flow cytometry. Figure 6 shows a representative experiment comparing the transition of T cell populations from day 4 immediately after transduction and before extensive cell death (Figure 6A) through to day 12 (Figure 6C). Transduction with the respective vectors led to high-level expression of Bcl-xL and Bcl-2 in *OX40*$^{-/-}$ T cells (Figure 6B). After 12 days, only 7% of Vβ3$^+$ T cells were alive in MSCV cultures as assessed by F/SSC analysis, versus 54% in MSCV-Bcl-xL and 25% in MSCV-Bcl-2 cultures (Figure 6C, left). Both Bcl-xL and Bcl-2 suppressed apoptosis as measured by annexin V staining of Vβ3$^+$ T cells (Figure 6C, middle). Moreover, if gene transduction provided a survival advantage, it would be expected that the proportion of GFP$^+$ cells within the live gate would increase. This was the case with the % GFP remaining constant with control vector (54% versus 53% on day 4), but increasing to 93% and 77% in MSCV-Bcl-xL and MSCV-Bcl-2 cultures, respectively (Figure 6C, right).

Calculation of actual numbers of GFP-expressing T cells directly showed that transduction of Bcl-xL or Bcl-2 restored the ability of *OX40*$^{-/-}$ T cells to survive over

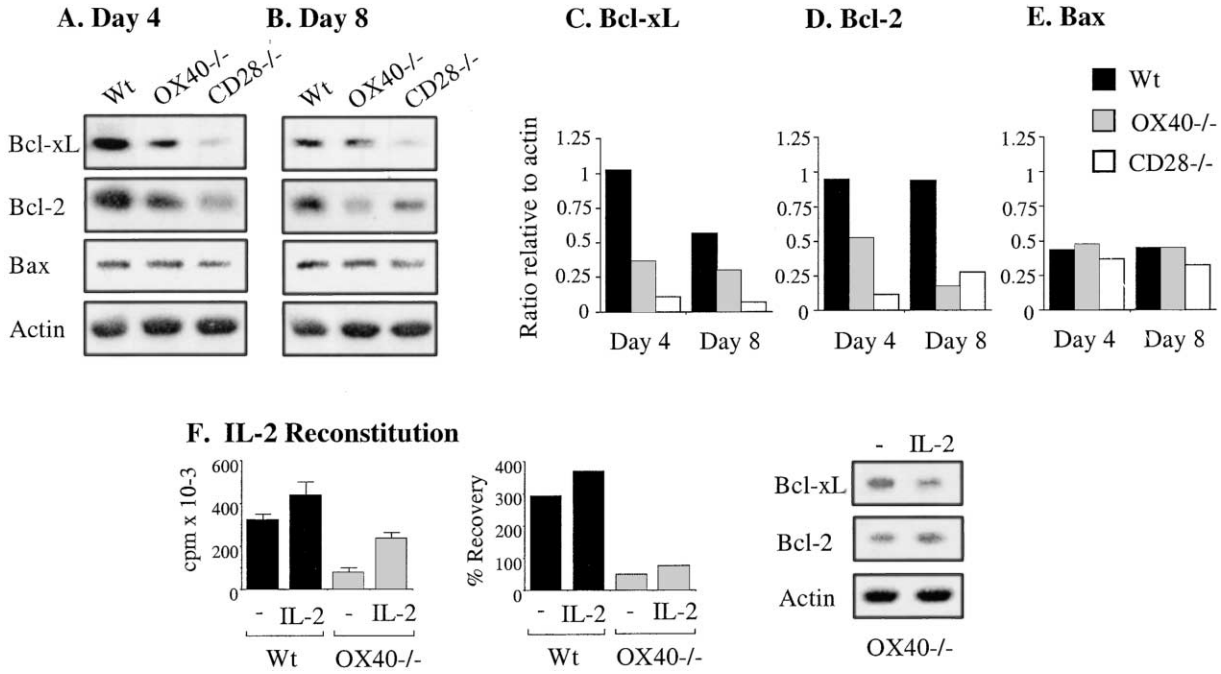

Figure 4. Expression of Bcl-xL and Bcl-2 Is Not Maintained over Time in the Absence of OX40

Wt, *OX40*$^{-/-}$, and *CD28*$^{-/-}$ AND T cells were cultured as in Figure 2 with APCs and 1 μM peptide for 4 or 8 days. Bcl-xL, Bcl-2, Bax, and actin levels were measured by Western blotting of equal amounts of protein lysate taken from viable CD4 cells at day 4 (A) or day 8 (B). Graphs show the relative expression of Bcl-xL (C), Bcl-2 (D), and Bax (E) compared to actin, expressed as a ratio after quantitation by densitometry. Data shows a representative experiment out of four. Bcl-xL and Bcl-2 levels on day 4 and day 8 ranged from 30%–60% of wt for *OX40*$^{-/-}$ T cells and 15%–40% of wt for *CD28*$^{-/-}$ T cells. T cell proliferation after 5 days (left), T cell survival after 12 days (middle), and Bcl-xL and Bcl-2 levels after 8 days (right) from cultures of wt or *OX40*$^{-/-}$ T cells stimulated in the presence or absence of exogenous IL-2 (15 ng/ml) added on day 1 (F). Data are representative of three experiments.

time (Figure 7). Differences were observed between the action of Bcl-xL and Bcl-2, in that Bcl-xL resulted in approximately 2-fold expansion in T cell numbers between day 4 and day 8, whereas Bcl-2 simply allowed the T cells to survive without an appreciable change in number (Figure 7B). This suggests that both Bcl-xL and Bcl-2 contribute to OX40-induced survival but that there may be differential roles in proliferating versus nonproliferating T cells, with Bcl-xL being more important early in response, allowing continued expansion of those T cells still undergoing cell division.

Collectively, our results provide compelling evidence that a major target of OX40 is the antiapoptotic proteins of the Bcl-2 family, and that the death of OX40-deficient T cells is due to an inability to maintain high levels of these proteins over time.

Discussion

OX40 has now been shown to play a role in many CD4 responses in vivo, including regulating memory T cell development. We as well as others have suggested that a major action of OX40 is to control the overall level of clonal expansion in the primary immune response, but direct data on the mechanism of action of OX40 has been lacking. In this report, we demonstrate that OX40 does not act in the initial phases of the response, but that it acts sequentially after CD28 to regulate late T cell turnover at the peak of the expansion phase and to regulate the subsequent survival of T cells when antigen becomes limiting. We show that OX40 is required to maintain high-level expression of the antiapoptotic proteins Bcl-xL and Bcl-2, suggesting that this is a key event which controls the number of memory T cells that are generated. A temporal sequence can be proposed, with CD28 providing signal 2 to promote T cell expansion and survival initiated by antigen and signal 1, and OX40 providing an additional late-acting signal which is required to prolong expansion and further suppress cell death, allowing a proportion of the expanded T cells to survive. This study suggests an updated view of the two-signal model of T cell activation to incorporate multiple sequential signals, and that long-term immunity can only result when secondary costimulatory signals are provided several days after the initial activation event.

Although previous reports of OX40 have highlighted the importance of this molecule, there has been little indication of its mode of action and relationship to other molecules. Our recent study in *OX40*$^{-/-}$ mice showed that the precursor frequency of effector T cells in the late primary response to KLH was reduced 10- to 40-fold compared to wt mice, as was the frequency of memory T cells present 5 weeks after priming (Gramaglia et al., 2000). This also correlated with data which showed a 4- to 5-fold reduction in the number of IFN-γ-secreting CD4 cells after LCMV infection of *OX40*$^{-/-}$ mice (Kopf et al., 1999). Both reports implied that OX40 signals regulate the number of antigen-specific T cells that are

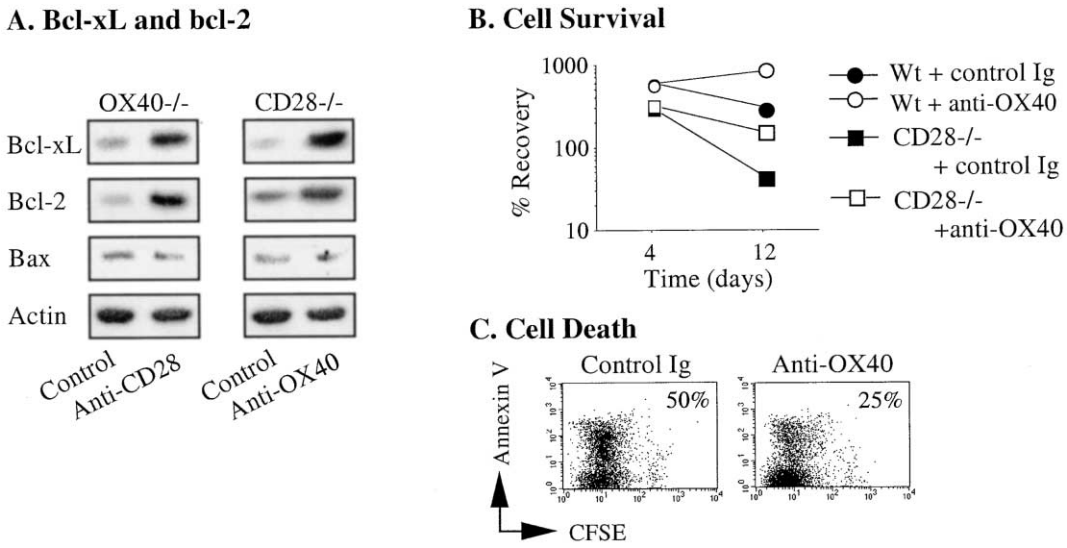

Figure 5. OX40 Ligation Enhances Bcl-xL and Bcl-2 Expression and T Cell Survival, and Suppresses Apoptosis

(A) CD4 T cells from *OX40*$^{-/-}$ and *CD28*$^{-/-}$ AND mice were stimulated with 1 μM peptide for 8 days in the presence of control IgG, with agonist anti-OX40 added on day 0 and day 2 or agonist anti-CD28 added on day 0. Bcl-xL, Bcl-2, Bax, and actin expression were determined by Western blotting as in Figure 4. Data are representative of three experiments.

(B) Survival (% recovery compared to the input number) of CD4 T cells from wt (circles) and *CD28*$^{-/-}$ (squares) AND mice stimulated with 1 μM peptide for 4 and 12 days, in the presence of control IgG (closed symbols) or agonist anti-OX40 (open symbols) added on day 0 and day 2. Data are the average of three separate experiments with standard errors being less than 15% of the means.

(C) Cell death (annexin V staining) of wt T cells labeled with CFSE and stimulated as above for 8 days in the presence of anti-OX40 or control IgG. Numbers represent the % annexin V^{+} T cells. Similar data were seen in one repeat experiment.

generated and/or the number that survive. Other data directly and indirectly supports this. In mice made transgenic for OX40L, five times as many CD4 cells were found in the B cell areas after antigen priming (Brocker et al., 1999), and in mice receiving a blocking antibody to OX40L, four times fewer T cells were found in the B cell areas (Walker et al., 1999). In addition, an agonist antibody to OX40, injected in two separate studies into recipients of adoptively transferred TCR transgenic T cells primed with either soluble antigen or antigen in adjuvant, resulted in 4- to 17-fold more cells accumulating in the primary response and the development of 4- to 15-fold more memory cells (Gramaglia et al., 2000; Maxwell et al., 2000). The data presented in this paper complement these reports, and now conclusively show that OX40 acts to suppress T cell death, with the effects being evident at and after the peak of the primary proliferative and expansion phase.

The mechanism of apoptotic death that occurs late in a primary T cell response is controversial. While some studies support a role for death receptors, others do not. It is likely that the mechanism may vary, and that the death of responding T cells in the presence of costimulation is fundamentally different from the death of T cells in the absence of costimulation. Death of T cells by neglect (antigen or costimulation) is mediated by caspases and adaptors that are distinct from those employed by death receptors (Lenardo et al., 1999; Rathmell and Thompson, 1999; Strasser et al., 1995). This type of death is controlled by the interplay between the proapoptotic members of the Bcl-2 family such as Bax, Bad, and Bim and the antiapoptotic members such as Bcl-xL and Bcl-2. The ratio of antagonists to agonists appears to determine whether apoptosis results, and is mediated in part by competitive dimerization between pairs of molecules (Kroemer, 1997). Thus, the relative levels of expression of one or more of these molecules may dramatically affect the propensity of a cell to survive. Our data suggest that death in the absence of OX40 is brought about by passive mechanisms and that the antiapoptotic proteins Bcl-xL and Bcl-2 are central to OX40-mediated survival. In line with this, inhibitory reagents of Fas and TNFR (data not shown), as well as exogenous IL-2, did not prevent the defect in survival of *OX40*$^{-/-}$ T cells. Bcl-xL is massively induced in naïve T cells 2 days after activation, peaks at day 4, and subsequently reverts back to baseline levels as T cells return to rest. In contrast, Bcl-2 is more moderately elevated with slower kinetics and then maintained over time (Figure 4 and data not shown). Several studies have implied that Bcl-2 and Bcl-xL are analogous and perform the same function (Chao et al., 1995; Petschner et al., 1998). As a variation of this idea, it was suggested that Bcl-2 may control the survival of resting T cells, whereas Bcl-xL may control the survival of activated effector-type T cells (Behrens and Mueller, 1997). Thus, maintaining Bcl-xL levels might be more important for T cell survival during the peak of the expansion phase around day 4 to day 6, whereas maintaining Bcl-2 levels might be more important for the survival of T cells as they revert back to a more resting state around day 8 and beyond. The finding that the overall levels of Bcl-2 were higher than Bcl-xL in wt T cells recovered after 8 days supports this notion (Figure 4), as does the fact that retroviral transduction of Bcl-xL augmented T cell expansion between 4 and 8 days, whereas Bcl-2 purely maintained T cell numbers (Figure 7).

Because the phenotype of *CD28*$^{-/-}$ T cells with re-

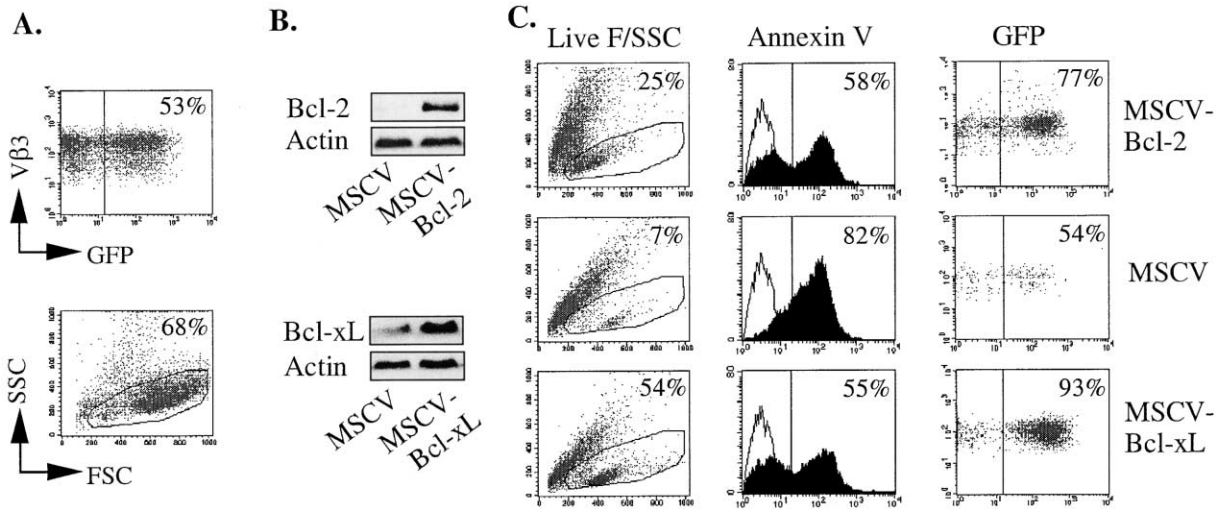

Figure 6. Retroviral Expression of Bcl-xL and Bcl-2 Suppresses Apoptosis of *OX40*$^{-/-}$ T Cells

CD4 cells from *OX40*$^{-/-}$ AND mice were stimulated with 1 μM peptide and APCs. On day 2 and day 3, T cells were transduced with retroviral vectors expressing GFP alone (MSCV) or GFP with Bcl-2 (MSCV-Bcl-2) or Bcl-xL (MSCV-Bcl-xL). T cells were recultured and analyzed for survival and death by flow cytometry.

(A) Vβ3/GFP and FSC/SSC profiles of gated Vβ3$^+$ T cells in a representative control culture on day 4. Numbers represent % GFP$^+$ cells and % Vβ3$^+$ cells within the live gate. Similar profiles were seen in Bcl-2- and Bcl-xL-transduced cultures.

(B) Human Bcl-2 (top) and human/mouse Bcl-xL (bottom) expression in live T cells from control and Bcl-2- or Bcl-xL-transduced cultures, respectively assessed on day 8.

(C) FSC/SSC (left) and annexin V (middle) profiles of gated Vβ3$^+$ T cells on day 12 and day 8, respectively. GFP/Vβ3 (right) profiles of cells within the live F/SSC gate on day 12. Numbers represent % cells in live gate, % annexin$^+$ cells, and % GFP$^+$ cells. Data are representative of two to three separate experiments.

spect to Bcl-xL and Bcl-2 was similar but more dramatic than that of *OX40*$^{-/-}$ T cells this suggests that *CD28*$^{-/-}$ T cells were in fact behaving as T cells this lacking both CD28 and OX40. OX40 expression was slow and submaximal in the absence of CD28, and little OX40L was induced on APCs. The rationale for the latter is that OX40L expression is regulated by CD40 signals (Ohshima et al., 1997), and only low levels of CD40L are induced on T cells in the absence of CD28-B7 interactions (Jaiswal et al., 1996). Thus, the combined effect of the absence of CD28 most likely translates during a physiological response in only weak provision of OX40 signals. This again highlights the strong cooperation between these molecules in providing synergistic signals to suppress death over time. It is clear from the data with *CD28*$^{-/-}$ T cells that the expansion phase of the primary T cell response is largely regulated by CD28, partially through production of IL-2. OX40 may function to promote continued cell division via increasing the output of IL-2 and maintaining high levels of Bcl-xL. OX40 may then provide IL-2-independent survival signals at late times to further suppress cell death that is

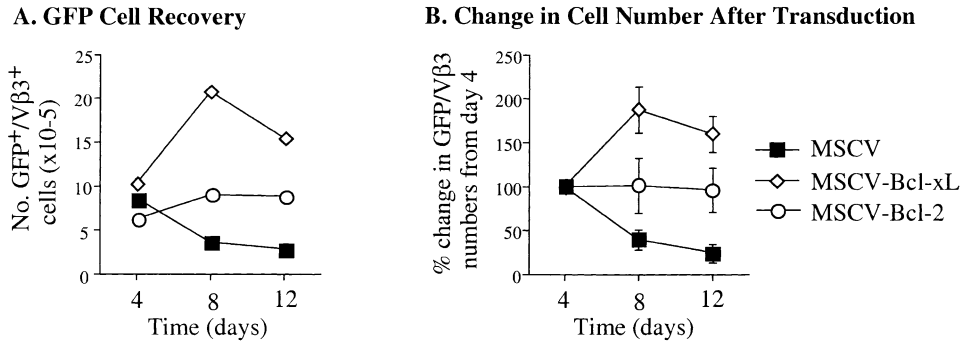

Figure 7. Retroviral Expression of Bcl-xL and Bcl-2 Corrects the Defect in Survival of *OX40*$^{-/-}$ T Cells

5 × 10^5 CD4 T cells from *OX40*$^{-/-}$ AND mice were stimulated with 1 μM peptide and APCs. On day 2 and day 3, T cells were transduced as in Figure 6 with control retroviral vector (MSCV; closed squares) or vectors expressing Bcl-xL (MSCV-Bcl-xL; open triangles) or Bcl-2 (MSCV-Bcl-2; open circles). T cells were recultured and analyzed on days 4, 8, and 12 for survival by trypan blue exclusion and flow cytometry.

(A) Total numbers of GFP$^+$ Vβ3$^+$ T cells recovered over time from a representative experiment.

(B) GFP$^+$ Vβ3$^+$ T cell recovery normalized to take into account differences in transduction efficiency between cultures. Cell numbers present on day 4 were assigned a value of 100%. Cell numbers surviving on day 8 and day 12 were used to calculate % recovery relative to day 4. Data represent mean % change ± SD from two to three separate experiments.

initially regulated by CD28, possibly via Bcl-xL, but most likely primarily through maintaining Bcl-2 levels.

We can only speculate at the present time how OX40 regulates Bcl-xL and Bcl-2. The one signaling pathway described for OX40 to date is that involving activation of NF-κB (Arch and Thompson, 1998; Kawamata et al., 1998). Binding experiments have shown that OX40 signals could be mediated by either TRAF2 or TRAF5 (Arch and Thompson, 1998; Kawamata et al., 1998), adaptor proteins that are implicated in NF-κB activation by other members of the TNFR family. NF-κB has long been thought to regulate cell survival (Liu et al., 1996), and direct links have now been described between this molecule and the prosurvival Bcl-2 family proteins (Andjelic et al., 2000; Grumont et al., 1999; Lee et al., 1999). It is therefore likely that NF-κB is the central mediator of the effects of OX40 on T cell survival. Interestingly, CD28 signaling can also result in NF-κB activation (Harhaj and Sun, 1998; Lin et al., 1999). A common NF-κB pathway regulating the antiapoptotic members of the Bcl-2 protein family provides a rationale for the synergistic temporal relationship between CD28 and OX40.

Cytokine withdrawal has been proposed to be a major mechanism of passive apoptosis of T cells. However, the majority of data that relate to this are derived from IL-2-dependent T cell lines (Deng and Podack, 1993; Vasilakos et al., 1995) or non-T cells responding to cytokines such as IL-3 (del Peso et al., 1997). IL-2 has in some cases been reported to enhance expression of Bcl-2, but IL-2 may also function to promote apoptosis, through relieving a block on Fas-mediated death (Refaeli et al., 1998). As we saw no effect of exogenous IL-2, our data on OX40, combined with previous data and that here on CD28, suggest that passive apoptosis may be equally governed by a lack of direct costimulatory signals as by a lack of cytokine receptor signals. Apoptosis associated with cytokine withdrawal can involve Bad (del Peso et al., 1997), although recent data suggest that Bim may be the physiological regulator of passive apoptosis in T cells (Bouillet et al., 1999; Puthalakath et al., 1999). Both molecules can competitively dimerize with Bcl-xL and Bcl-2 and inhibit their antiapoptotic action (Puthalakath et al., 1999; Yang et al., 1995). Bad is inactivated by PI-3-kinase and Akt (del Peso et al., 1997), and PI-3-kinase has been implicated in downregulating Bim (Shinjyo et al., 2001). Thus, it is possible that Bim or Bad are also direct targets of costimulatory signals. PI-3-kinase and Akt have been associated with CD28 signaling (Parry et al., 1997), and several studies of TNF/TNFR family members including TNF, TRANCE, and CD40 (Andjelic et al., 2000; Ozes et al., 1999; Pastorino et al., 1999; Wong et al., 1999) have also demonstrated the use of PI-3-kinase and Akt. It remains to be determined whether the antiapoptotic effects of OX40 and CD28 are simply due to elevating and maintaining the levels of Bcl-xL and Bcl-2, or whether they also involve concomitant antagonism of Bim or Bad, and whether all are controlled by a common PI-3-kinase/Akt/NF-κB pathway.

In summary, we provide evidence that OX40 represents a crucial costimulatory molecule for CD4 T cells because it induces survival signals which prevent excessive T cell death. The effect of these signals is observed after the phase of clonal expansion in the primary response, and therefore is critical to the efficient generation of memory. We show that a costimulatory receptor other than CD28 can regulate antiapoptotic proteins such as Bcl-xL and Bcl-2, and suggest that through this action, OX40 plays a vital role in shaping the overall magnitude and longevity of the CD4 response. These data provide a model whereby antigen-specific expansion, survival, and differentiation are driven by the concerted and sequential action of several costimulatory receptor-ligand pairs of the Ig and TNFR superfamilies. They suggest that a nontolerizing, long-lasting T cell response requires multiple interactions to be provided over a period of many days, most likely brought about during numerous encounters of the T cell with antigen-presenting cells. It is tempting to speculate that these secondary cosignals are principally transmitted by the TNFR/TNF family of molecules because of their inducible expression on T cells and APC several days after initial antigen encounter, and that molecules such as 4-1BB, CD27, and HVEM will play analogous roles to that shown here for OX40.

Experimental Procedures

Mice

AND TCR transgenic mice bearing T cells reactive with peptides of moth cytochrome c (MCC) and expressing the Vβ3/Vα11 TCR were bred on a B10.BR background (Croft et al., 1992). CD28-deficient mice (*CD28*$^{-/-}$) were obtained from Jackson Laboratories and OX40-deficient mice (*OX40*$^{-/-}$) were generated as described (Pippig et al., 1999). Both strains were backcrossed at least five generations to AND mice. Thymic selection was normal in the resultant mice, with similar numbers of peripheral CD4 cells in both wt and knockout animals. All CD4 cells expressed the transgenic TCR.

Peptides and Antibodies

T102S, a variant superagonist of MCC (amino acids 88–103; ANERA DLIAYKQASK), was synthesized at LIAI (Rogers and Croft, 2000). Anti-OX40 (OX86) was obtained from the European cell culture collection (Wiltshire, UK). Anti-CD28 (37.51) was obtained from Dr. J.P. Allison (University of California, Berkeley).

T Cells and APC

CD4$^+$ T cells were purified from spleen and lymph nodes of TCR transgenic mice by nylon wool depletion, followed by Ab and complement treatment as described (Croft et al., 1992). The resulting cells were >90% CD4$^+$, and >95% of these cells expressed the Vβ3/Vα11 TCR. APC were isolated from spleens of B10.BR mice by depleting T cells. APC were treated with mitomycin c (100 μg/ml) for 30 min at 37°C before use, which results in their death after ~72–96 hr.

Cell Cultures

Cultures were set up in 48-well plates containing 1 ml of RPMI 1640 (Irvine Scientific) supplemented with 10% fetal calf serum (Omega Scientific). Naïve CD4 cells were plated at 5×10^5/ml with 2×10^6/ml APCs and various concentrations of T102S peptide. In some experiments, stimulatory antibodies to CD28 (4 μg/ml) or OX40 (10 μg/ml) were added at 0 hr, and 0 and 48 hr, respectively. Both were compared to control rat and hamster IgG. A global caspase inhibitor (Boc-D(OMe)-FMK; Alexis Corporation), a caspase 3, or a caspase 9 inhibitor (Ac-DEVD-CHO and Z-LE(OMe)HD(OMe)-FMK, respectively) were added individually at 20 μM in 0.05% DMSO. Peptide Ac-Asp-Glu-Val-Asp-OH was added in DMSO as a control.

Cytokine Secretion, Proliferation, Cell Division, Death

IL-2 was measured by ELISA using the Abs JES6-1A12 and biotin-JES6-5H4 (BD PharMingen). Proliferation was measured in triplicate by the incorporation of [^{3}H]thymidine (1 μCi/well; ICN Pharmaceuticals) during the last 12 hr of culture. To follow cell division, T cells

were labeled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE, C-1157; Molecular Probes) and analyzed by flow cytometry. Apoptosis was assessed using annexin V conjugated to PE (BD Pharmingen).

Western Blots

T cells were spun through histopaque (Sigma), and lysed in ice-cold TNE lysing buffer (1% NP-40, 20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin and leupeptin) for 30 min. Insoluble material was removed and lysates were stored at −80°C. Protein content was determined by bicinchoninic acid protein assay kit (Pierce). Equal amounts (50 μg) were loaded onto 12.5% acrylamide gels (SDS-PAGE), transferred onto Immobolin-P membrane (Millipore), and immunoblotted with various antibodies. Binding of polyclonal rabbit antibodies to mouse/human Bcl-xL (Transduction Laboratories), mouse Bcl-2, and mouse Bax (BD Pharmingen) were detected with peroxidase-conjugated donkey anti-rabbit Ig (Amersham Life Sciences). Binding of monoclonal mouse antibodies to human Bcl-2 (BD Pharmingen) and mouse actin (ICN Biomedical) were detected with peroxidase-conjugated goat anti-mouse Ig (Southern Biotech). Blots were developed with ECL immunodetection system (Sigma) and Kodak film. Band intensities were quantitated using a computing densitometer with NIH Image 1.61 software.

Retroviral Transduction

The murine stem cell virus (MSCV) 2.2 vector containing cDNA for Bcl-xL was a generous gift of Dr. Abul Abbas (University of California, San Francisco; Dahl et al., 2000). This vector was originally modified to contain an internal ribosome entry site and the cDNA of green fluorescent protein (Ouyang et al., 1998). Subsequently, the cDNA for human Bcl-xL (Boise et al., 1993) was cloned into an XhoI site (Dahl et al., 2000). cDNA for human Bcl-2 was kindly provided by Dr. Doug Green (LIAI) and subcloned into the empty vector at BglII and XhoI sites. The virus was produced by chloroquine-mediated calcium phosphate transfection of the Phoenix E ecotropic packaging cell line (Costa et al., 2000). Supernatants were collected after 3 days, titered by measuring GFP expression, and used directly for transducing T cells. 5×10^5 T cells were stimulated with peptide and APCs in 48-well plates as described. After 2 days, the supernatant was replaced with 1 ml viral supernatant containing 5 μg/ml Polybrene, and the cells were spun for 3 hr at 32°C and then incubated at 32°C for 8 hr. This procedure was repeated the following day. Viral supernatant was removed and replaced with fresh medium, and T cells were recultured for up to 9 more days. Expression of GFP was determined by flow cytometry gating on Vβ3-positive cells.

Acknowledgments

This work was funded by NIH grant AI42944 to Michael Croft. This is manuscript #395 from the La Jolla Institute for Allergy and Immunology. The authors would like to thank Dr. Abul Abbas for the generous gift of the MSCV vector containing Bcl-xL, and acknowledge Dr. Bill Sha from whom the MSCV vector originated. The authors would also like to thank Dr. Doug Green for providing cDNA for Bcl-2 and Dr. Jianyong Huang for assistance with Western blotting and retroviral transduction.

Received October 27, 2000; revised July 20, 2001.

References

Al-Shamkhani, A., Birkeland, M.L., Puklavec, M., Brown, M.H., James, W., and Barclay, A.N. (1996). OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand. Eur. J. Immunol. *26*, 1695–1699.

Andjelic, S., Hsia, C., Suzuki, H., Kadowaki, T., Koyasu, S., and Liou, H.C. (2000). Phosphatidylinositol 3-kinase and NF-κ B/Rel are at the divergence of CD40-mediated proliferation and survival pathways. J. Immunol. *165*, 3860–3867.

Arch, R.H., and Thompson, C.B. (1998). 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor κB. Mol. Cell. Biol. *18*, 558–565.

Bansal-Pakala, P., Jember, A.G.-H., and Croft, M. (2001). Signaling through OX40 (CD134) breaks peripheral T cell tolerance. Nat. Med. *7*, 907–912.

Behrens, T.W., and Mueller, D.L. (1997). Bcl-x and the regulation of survival in the immune system. Immunol. Res. *16*, 149–160.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597–608.

Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., and Thompson, C.B. (1995). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL. Immunity *3*, 87–98.

Bouillet, P., Metcalf, D., Huang, D.C., Tarlinton, D.M., Kay, T.W., Kontgen, F., Adams, J.M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735–1738.

Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundalia, C., and Lane, P. (1999). CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. Eur. J. Immunol. *29*, 1610–1616.

Calderhead, D.M., Buhlmann, J.E., van den Eertwegh, A.J., Claassen, E., Noelle, R.J., and Fell, H.P. (1993). Cloning of mouse Ox40: a T cell activation marker that may mediate T-B cell interactions. J. Immunol. *151*, 5261–5271.

Chao, D.T., Linette, G.P., Boise, L.H., White, L.S., Thompson, C.B., and Korsmeyer, S.J. (1995). Bcl-XL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. *182*, 821–828.

Chen, A.I., McAdam, A.J., Buhlmann, J.E., Scott, S., Lupher, M.L., Jr., Greenfield, E.A., Baum, P.R., Fanslow, W.C., Calderhead, D.M., Freeman, G.J., and Sharpe, A.H. (1999). Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. Immunity *11*, 689–698.

Costa, G.L., Benson, J.M., Seroogy, C.M., Achacoso, P., Fathman, C.G., and Nolan, G.P. (2000). Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune disease. J. Immunol. *164*, 3581–3590.

Croft, M., and Dubey, C. (1997). Accessory molecule and costimulation requirements for CD4 T cell response. Crit. Rev. Immunol. *17*, 89–118.

Croft, M., Duncan, D.D., and Swain, S.L. (1992). Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. J. Exp. Med. *176*, 1431–1437.

Dahl, A.M., Klein, C., Andres, P.G., London, C.A., Lodge, M.P., Mulligan, R.C., and Abbas, A.K. (2000). Expression of bcl-X(L) restores cell survival, but not proliferation or effector differentiation, in CD28-deficient T lymphocytes. J. Exp. Med. *191*, 2031–2038.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science *278*, 687–689.

Deng, G., and Podack, E.R. (1993). Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2. Proc. Natl. Acad. Sci. USA *90*, 2189–2193.

Flynn, S., Toellner, K.M., Raykundalia, C., Goodall, M., and Lane, P. (1998). CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, blr-1. J. Exp. Med. *188*, 297–304.

Godfrey, W.R., Fagnoni, F.F., Harara, M.A., Buck, D., and Engleman, E.G. (1994). Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. J. Exp. Med. *180*, 757–762.

Gramaglia, I., Weinberg, A.D., Lemon, M., and Croft, M. (1998). OX40

ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J. Immunol. *161*, 6510–6517.

Gramaglia, I., Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., and Croft, M. (2000). The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J. Immunol. *165*, 3043–3050.

Grumont, R.J., Rourke, I.J., and Gerondakis, S. (1999). Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev. *13*, 400–411.

Harhaj, E.W., and Sun, S.C. (1998). IκB kinases serve as a target of CD28 signaling. J. Biol. Chem. *273*, 25185–25190.

Jaiswal, A.I., Dubey, C., Swain, S.L., and Croft, M. (1996). Regulation of CD40-ligand expression on naive CD4 T cells: a role for T cell receptor but not costimulatory signals. Int. Immunol. *8*, 275–285.

Jember, A.G., Zuberi, R., Liu, F.T., and Croft, M. (2001). Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. J. Exp. Med. *193*, 387–392.

Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B. (1991). CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J. Immunol. *147*, 2461–2466.

June, C.H., Ledbetter, J.A., Gillespie, M.M., Lindsten, T., and Thompson, C.B. (1987). T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Mol. Cell. Biol. *7*, 4472–4481.

Kawamata, S., Hori, T., Imura, A., Takaori-Kondo, A., and Uchiyama, T. (1998). Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF-κB activation. J. Biol. Chem. *273*, 5808–5814.

Kearney, E.R., Pape, K.A., Loh, D.Y., and Jenkins, M.K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity *1*, 327–339.

Kopf, M., Ruedl, C., Schmitz, N., Gallimore, A., Lefrang, K., Ecabert, B., Odermatt, B., and Bachmann, M.F. (1999). OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL responses after virus infection. Immunity *11*, 699–708.

Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. *3*, 614–620.

Lafferty, K.J., and Woolnough, J. (1977). The origin and mechanism of the allograft reaction. Immunol. Rev. *35*, 231–262.

Lee, H.H., Dadgostar, H., Cheng, Q., Shu, J., and Cheng, G. (1999). NF-κB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc. Natl. Acad. Sci. USA *96*, 9136–9141.

Lenardo, M., Chan, K.M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. (1999). Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. Annu. Rev. Immunol. *17*, 221–253.

Lin, X., Cunningham, E.T., Jr., Mu, Y., Geleziunas, R., and Greene, W.C. (1999). The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-κB acting through the NF-κB-inducing kinase and IκB kinases. Immunity *10*, 271–280.

Linsley, P.S., and Ledbetter, J.A. (1993). The role of the CD28 receptor during T cell responses to antigen. Annu. Rev. Immunol. *11*, 191–212.

Liu, Z.G., Hsu, H., Goeddel, D.V., and Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell *87*, 565–576.

Maxwell, J., Weinberg, A.D., Prell, R.A., and Vella, A.T. (2000). Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. J. Immunol. *164*, 107–112.

Mueller, D.L., Seiffert, S., Fang, W., and Behrens, T.W. (1996). Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells. J. Immunol. *156*, 1764–1771.

Murata, K., Ishii, N., Takano, H., Miura, S., Ndhlovu, L.C., Nose, M., Noda, T., and Sugamura, K. (2000). Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. J. Exp. Med. *191*, 365–374.

Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. (1997). Expression and function of OX40 ligand on human dendritic cells. J. Immunol. *159*, 3838–3848.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., and Murphy, K.M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity *9*, 745–755.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., and Donner, D.B. (1999). NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature *401*, 82–85.

Parry, R.V., Reif, K., Smith, G., Sansom, D.M., Hemmings, B.A., and Ward, S.G. (1997). Ligation of the T cell co-stimulatory receptor CD28 activates the serine-threonine protein kinase protein kinase B. Eur. J. Immunol. *27*, 2495–2501.

Pastorino, J.G., Tafani, M., and Farber, J.L. (1999). Tumor necrosis factor induces phosphorylation and translocation of BAD through a phosphatidylinositide-3-OH kinase-dependent pathway. J. Biol. Chem. *274*, 19411–19416.

Paterson, D.J., Jefferies, W.A., Green, J.R., Brandon, M.R., Corthesy, P., Puklavec, M., and Williams, A.F. (1987). Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. Mol. Immunol. *24*, 1281–1290.

Petschner, F., Zimmerman, C., Strasser, A., Grillot, D., Nunez, G., and Pircher, H. (1998). Constitutive expression of Bcl-xL or Bcl-2 prevents peptide antigen-induced T cell deletion but does not influence T cell homeostasis after a viral infection. Eur. J. Immunol. *28*, 560–569.

Pippig, S.D., Pena-Rossi, C., Long, J., Godfrey, W.R., Fowell, D.J., Reiner, S.L., Birkeland, M.L., Locksley, R.M., Barclay, A.N., and Killeen, N. (1999). Robust B cell immunity but impaired T cell proliferation in the absence of CD134 (Ox40). J. Immunol. *163*, 6520–6529.

Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell *3*, 287–296.

Rathmell, J.C., and Thompson, C.B. (1999). The central effectors of cell death in the immune system. Annu. Rev. Immunol. *17*, 781–828.

Refaeli, Y., Van Parijs, L., London, C.A., Tschopp, J., and Abbas, A.K. (1998). Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. Immunity *8*, 615–623.

Rogers, P.R., and Croft, M. (2000). CD28, OX40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen. J. Immunol. *164*, 2955–2963.

Shinjyo, T., Kuribara, R., Inukai, T., Hosoi, H., Kinoshita, T., Miyajima, A., Houghton, P.J., Look, A.T., Ozawa, K., and Inaba, T. (2001). Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. Mol. Cell. Biol. *21*, 854–864.

Sperling, A.I., Auger, J.A., Ehst, B.D., Rulifson, I.C., Thompson, C.B., and Bluestone, J.A. (1996). CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. J. Immunol. *157*, 3909–3917.

Strasser, A., Harris, A.W., and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell *67*, 889–899.

Strasser, A., Harris, A.W., Huang, D.C., Krammer, P.H., and Cory, S. (1995). Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. *14*, 6136–6147.

Stuber, E., and Strober, W. (1996). The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. J. Exp. Med. *183*, 979–989.

Stuber, E., Neurath, M., Calderhead, D., Fell, H.P., and Strober, W. (1995). Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity *2*, 507–521.

Van Parijs, L., Peterson, D.A., and Abbas, A.K. (1998). The Fas/Fas

ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. Immunity *8*, 265–274.

Vasilakos, J.P., Ghayur, T., Carroll, R.T., Giegel, D.A., Saunders, J.M., Quintal, L., Keane, K.M., and Shivers, B.D. (1995). IL-1 β converting enzyme (ICE) is not required for apoptosis induced by lymphokine deprivation in an IL-2-dependent T cell line. J. Immunol. *155*, 3433–3442.

Walker, L.S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Forster, R., Lipp, M., and Lane, P. (1999). Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. J. Exp. Med. *190*, 1115–1122.

Watts, T.H., and DeBenedette, M.A. (1999). T cell co-stimulatory molecules other than CD28. Curr. Opin. Immunol. *11*, 286–293.

Weinberg, A.D., Wallin, J.J., Jones, R.E., Sullivan, T.J., Bourdette, D.N., Vandenbark, A.A., and Offner, H. (1994). Target organ-specific up-regulation of the MRC OX-40 marker and selective production of Th1 lymphokine mRNA by encephalitogenic T helper cells isolated from the spinal cord of rats with experimental autoimmune encephalomyelitis. J. Immunol. *152*, 4712–4721.

Weinberg, A.D., Rivera, M.M., Prell, R., Morris, A., Ramstad, T., Vetto, J.T., Urba, W.J., Alvord, G., Bunce, C., and Shields, J. (2000). Engagement of the OX-40 receptor in vivo enhances antitumor immunity. J. Immunol. *164*, 2160–2169.

Wong, B.R., Besser, D., Kim, N., Arron, J.R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999). TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol. Cell *4*, 1041–1049.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285–291.