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A Signal Through OX40 (CD134) Allows Anergic, Autoreactive T Cells to Acquire Effector Cell Functions

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To study mechanisms of peripheral self-tolerance, we injected small numbers of naive CD4+ TCR-transgenic T cells into mice expressing the MHC/peptide ligand under the control of an MHC class II promoter. The donor T cells expand rapidly to very large numbers, acquire memory markers, and go out into tissues, but the animals remain healthy, and the accumulated T cells are profoundly anergic to restimulation with Ag in vitro. Provision of a costimulatory signal by coinjection of an agonist Ab to OX40 (CD134), a TNFR family member expressed on activated CD4 T cells, results in death of the mice within 12 days. TCR-transgenic T cells recovered at 5 days from anti-OX40-treated mice have a unique phenotype: they remain unresponsive to Ag in vitro, but they are larger, more granular, and strongly IL-2R positive. Some spontaneously secrete IFN-γ directly ex vivo, and the majority make IFN-γ in response to PMA and ionomycin. Although they are anergic by conventional tests requiring Ag recognition, they respond vigorously to cytokines, proliferating in response to IL-2, and secreting IFN-γ when TCR signaling is bypassed with IL-12 and IL-18. We conclude that the costimulatory signal through OX40 allows otherwise harmless, proliferating, autoreactive T cells to acquire effector cell functions. The ability of these T cells to respond to cytokines by synthesizing additional inflammatory cytokines without a TCR signal may drive the fatal pathogenic process in vivo. *The Journal of Immunology, 2004, 172: 6735–6743.

In addition to clonal deletion of autoreactive T cells in the thymus, self-tolerance depends on inactivation of potentially responsive lymphocytes in peripheral lymphoid tissues, in which they are rendered harmless when they encounter Ags onAPC in the absence of infection or adjuvants. Mechanisms of peripheral tolerance are complex and involve various forms of deletion, inactivation, and suppression (1, 2). A number of investigators have studied the mechanisms of peripheral tolerance to self Ags by injecting naive TCR-transgenic T cells into otherwise syngeneic animals that express the Ag recognized by the T cells (3–11). In such experiments, the T cells undergo a period of rapid proliferation followed by the death of most of the cells. The surviving TCR-transgenic T cells are profoundly unresponsive in vivo or in vitro, a phenomenon called “in vivo anergy” or “adaptive tolerance” (12). The same cycle of T cell proliferation followed by T cell death occurs during a productive T cell immune response, but the proliferating cells differentiate into effector cells, and the rare surviving cells become functional memory cells (13). The innate immune response to infection or adjuvant tips the balance from tolerance to immunity by activating the APC, which then provides the additional cytokines and membrane costimulatory molecules that the T cells need to differentiate and survive as effector cells and memory cells (2, 14).

One of the costimulatory molecules that can determine the decision between immunity and tolerance is OX40 (CD134), a costimulatory TNFR family member expressed by activated CD4 T cells (15, 16). The ligand for OX40 (OX40L) is a membrane-bound member of the TNF family expressed on activated APC (17). A costimulatory signal through OX40 to activated CD4 T cells enhances T cell survival and memory cell formation (18–21), reverses CD4 T cell tolerance to peptide Ag (22), and promotes tumor rejection (23) and graft-vs-host disease (24). Although OX40 is strongly implicated in autoimmune disease (16, 25), the effect of a costimulatory signal through OX40 has not been investigated directly in peripheral self-tolerance. Therefore, we examined the effect of an agonist Ab to OX40 in a new model of peripheral CD4 T cell tolerance to ubiquitous self Ag.

The neo-self Ag in our model is a transgenic MHC class II molecule with an antigenic peptide covalently attached to the class II β-chain by a flexible linker. We follow the fate of naive TCR-transgenic T cells that recognize this peptide/MHC complex after i.v. transfer into the Ag-transgenic mice. For reasons that remain to be investigated, this model of peripheral tolerance differs from others because very large numbers of anergic donor T cells accumulate in the spleen and nonlymphoid tissues of unirradiated, nonlymphopenic, Ag-bearing recipients, facilitating the characterization of the tolerant T cells. In the other models, the recipients must be irradiated or deficient in T cells to recover large numbers of donor T cells, e.g., (6, 9). Several days after T cell injection, up to half of the CD4 T cells in spleen and liver are proliferating TCR-transgenic donor T cells, but the T cells are profoundly anergic in vitro, the animals appear healthy, and the T cells slowly disappear over the following weeks. However, when the animals are given a single injection of an agonist Ab to OX40 along with the transgenic T cells, the T cells differentiate into large, granular, IL-2R

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(CD25)-positive effector cells that secrete effector cytokines and cause the death of the animals within 12 days.

This simple model provides a tool to investigate how effector functions of CD4 T cells are turned off in peripheral tolerance and maintained during an immune response. Our results indicate that there are multiple levels of unresponsiveness in T cells rendered anergic in vivo. T cells recovered from anti-OX40-treated animals are unresponsive to Ag in vitro, but act like fully differentiated Th1 effector cells when stimulated instead with IL-12 plus IL-18.

Materials and Methods

Mice

Mice were housed under specific pathogen-free conditions at the Oregon Health and Science University animal facility. C57BL/6J mice expressing an MHC class II I-E\(^{\delta}\) molecule with an antigenic peptide covalently attached to the \(\alpha\)2m chain by a flexible linker were made by coinjection of plasmids encoding Env\(\alpha\) and Env\(\beta\) peptide driven by a class II promoter (26) as previously described (27) in the Transgenic Animal Core Facility of the University of Massachusetts Medical Center (Worcester, MA). The antigenic peptide is from pigeon cytochrome \(c\) (PCC)\(^3\) with a serine to threonine substitution at position 102 (PCC102S): ANERADLIRLYKQASAK. The founder was identified by Southern blot, and the progeny were maintained as heterozygotes and typed by PCR, using forward primer 5'-GGTTGTTGTTGCTGTCTCATC-3' and reverse primer 5'-AGGGCTCTCGGAGAGTAC-3'. CD4-deficient mice (28) on a C57BL/6 background were bred and backcrossed to the Ag-transgenic line to generate Ag-transgenic, CD40-deficient animals. C57BL/10 AND TCR-transgenic mice specific for PCC or moth cytochrome \(c\) (MCC) peptide on I-E\(^k\) (29) were obtained from S. Hedrick (University of California, San Diego, La Jolla, CA), and bred repeatedly to C57BL/6 recombinase-activating gene (RAG)-1 deficient mice obtained from The Jackson Laboratory (Bar Harbor, ME). AND TCR-transgenic T cells are efficiently selected in the thymus on I-A\(^b\) in C57BL/6 mice, and recognize PCC102S just as well as PCC on I-E\(^k\) (30). AD10 TCR-transgenic mice (30), also specific for PCC or MCC on I-E\(^k\), were maintained as heterozygotes on a B10.BR background.

Antibodies

PerCP anti-CD4 (RM4-5), FITC and biotin anti-Va11 (RR8-1), PE anti-V\(\delta\)3 (KJ25), biotin anti-CD25 (7D4), CD44 (IM7), and CD62L (MEL-14), FITC anti-I\(\delta\)2 (17-3-3), APC anti-I-L2 (JES6-5H4), FITC anti-TNF-\(\alpha\) (MP6-XT22), FITC anti-I-L10 (JESS-16E3), APC anti-I-L4 (11B11), PE anti-CTLA-4 (4F10-11) and some isotype controls were purchased from BD Pharmingen (San Diego, CA). APC anti-IFN-\(\gamma\) and some isotype controls were purchased from eBiosciences (San Diego, CA). Anti-OX40 Ab from clone OxX6 (European Cell Culture Collection, Porton Down, U.K.) was produced and purified for us by UniSyn (Hopkington, MA). Rat IgG was purchased from Cappel, ICN Pharmaceuticals (Costa Mesa, CA). Purified Abs to CD28 (PV-1, used at 100 \(\mu\)g/mouse), CD40 (FGK45, 200 \(\mu\)g/mouse), and 4-1BB (17B5, 100 \(\mu\)g/mouse) were kindly provided by Dr. S. Schoenberger (La Jolla Institute for Allergy and Immunology, San Diego, CA).

Transfer of TCR-transgenic T Cells

Spleen cells from AND RAG-1 knockout mice were suspended in HBES-buffered HBSS with 2% serum, and isolated on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) without hypotonic lysis. An aliquot was stained and analyzed by flow cytometry to determine the proportion of TCR-transgenic T cells (Va11, V\(\delta\)3, and CD4 positive) in the suspension. In some experiments, the cells were labeled for 10 min at 37 °C with 2 \(\mu\)M CFSE in 0.1% BSA in PBS. Cells were washed in HBSS with serum and resuspended in HBSS without serum for injection.

Flow cytometry

Single cell suspensions were prepared from spleens and lymph nodes of recipient animals, and red cells were lysed in hypotonic NH\(_4\)Cl medium. Liver cell suspensions were made from one liver lobe cut into pieces and pressed through a sieved, followed by incubation for 40 min at 37°C with 2 \(\mu\)M CFSE in 0.1% BSA in PBS. Cells were washed in HBSS with serum and resuspended in HBSS without serum for injection.

3 Abbreviations used in this paper: PCC, pigeon cytochrome \(c\); MCC, moth cytochrome \(c\); Ox40L, Ox40 ligand; RAG, recombination-activating gene.
lacked CD40 expression (Fig. 1B). Therefore, even in the absence of CD40 ligand/CD40 signaling, conventional costimulatory signals are not limiting for proliferation and survival of large numbers of autoreactive T cells in this system.

Anti-OX40 prevents functional tolerance of transferred TCR-transgenic T cells

Despite the large load of proliferating, autoreactive TCR-transgenic T cells, Ag-transgenic recipient mice remained active and apparently healthy (observed up to 6 wk). Because alternative costimulation through OX40 on activated CD4 T cells can prevent tolerance to superantigen or peptide Ag in vivo (18, 20, 22), we examined the effect of an agonist Ab to OX40 in this model of peripheral CD4 T cell tolerance to ubiquitous self Ag. Spleen cell suspensions containing 0.5–5 × 10^6 TCR-transgenic T cells were transferred into CD40-deficient or -sufficient, Ag-transgenic hosts (two mice per group). The percentages of total splenocytes in each mouse that were Vα11^+Vβ3^+CD4^+ on the indicated days after transfer are shown.

FIGURE 1. Ag-specific expansion of CD4 T cells in Ag-transgenic mice is CD40-independent. A. The thick lines show the expression levels of transgenic I-Ek on CD19^+ B cells and CD11c^+ dendritic cells of the Ag-transgenic mice. Also shown are background staining of nontransgenic mice (shaded histogram) and normal level staining of endogenous I-Ek on B cells and dendritic cells of B10.BR mice (thin lines). B, Proliferation of Ag-specific transgenic T cells transferred into Ag-transgenic hosts is not dependent upon CD40. A total of 5.9 × 10^6 transgenic T cells were transferred into CD40-deficient or -sufficient, Ag-transgenic hosts (two mice per group). The percentages of total splenocytes in each mouse that were Vα11^+Vβ3^+CD4^+ on the indicated days after transfer are shown.

FIGURE 2. Transferred T cells from anti-OX40-treated mice show an activated phenotype. TCR-transgenic T cells (5 × 10^5) were transferred into Ag-transgenic recipients with either anti-OX40 Ab or control rat IgG, then recovered from the spleen on day 5 and analyzed by flow cytometry for size, granularity, and expression of activation markers. A, Splenocytes were gated on CD4^+Vα11^+Vβ3^+ as in A. One representative mouse of three per group, from one experiment of eight, is shown.

very pale and speckled, whereas those from rat IgG controls remained pink. On days 5 and 8, livers of mice from anti-OX40-treated mice were extremely pale, spongy, and delicate, and showed microvesicular steatosis upon histological examination (see Fig. 5). Livers of control, rat-Ig-treated animals had white spots on the surface and were more pale than normal livers. Other organs appeared grossly normal in both groups. Nontransgenic recipients remained healthy following injection of TCR-transgenic T cells and anti-OX40, demonstrating a requirement for Ag in this system.

To examine the phenotype of the transferred T cells, spleen cell suspensions of recipient mice were analyzed by flow cytometry 5 days after transfer of 5 × 10^5 TCR-transgenic T cells. Representative mice from one of eight experiments are shown in Fig. 2. TCR-transgenic T cells were identified by expression of CD4, Vβ3, and Vα11, and surface expression of CD25, CD44, and CD62L was measured. Large numbers of donor TCR-transgenic T cells with memory/effector cell markers (CD44^high, CD62L^lo) accumulate in the spleens
of the recipient mice by day five, with or without anti-OX40 treatment, although the shift to memory/effector phenotype is more complete with anti-OX40 (Fig. 2). Spleens of anti-OX40-treated animals contain 1.5 to 5 times as many TCR-transgenic T cells, whereas total spleen cell numbers for both groups were 2 to 2.5 times greater than that of a nontransgenic control or untreated Ag-transgenic mouse. Averaging all experiments, the mean cell number of TCR-transgenic cells recovered from the spleen on day 5 was $49.7 \times 10^6$ (SE = $7.7 \times 10^3$, n = 18) with anti-OX40, and $15.3 \times 10^6$ (SE = $2.5 \times 10^3$, n = 18) with rat IgG. This fact implies that anti-OX40 may enhance the survival of the proliferating donor T cells or cause the retention of proliferating T cells in the spleen.

One striking characteristic of the donor T cells recovered from the anti-OX40-treated animals is their sustained, very high expression of the IL-2R α-chain, CD25, which declines to near background levels by day 5 in the rat IgG controls (Fig. 2B). Another feature of the T cells from OX40-treated animals is their large size (forward scatter) and granularity (side scatter) (Fig. 2A). Donor T cells recovered from the rat IgG-injected controls are smaller and less granular, resembling proliferating lymphoblasts recovered from cultures stimulated with peptide in vitro (data not shown).

The transgenic TCR is substantially down-modulated in both groups, but is slightly lower in the anti-OX40-treated animals (Fig. 2A). Although T cells from both groups show elevated CD44 and reduced CD62L compared with naive T cells, T cells from anti-OX40-treated animals are uniformly higher for CD44 and lower for CD62L, and the rat IgG control T cells include both CD62L high and CD62L low populations (Fig. 2B). Staining for intracellular CTLA-4 is weakly positive in both groups, although the higher background staining of the larger anti-OX40-treated T cells makes it difficult to directly compare levels of CTLA-4 expression between the groups (data not shown). When normal B6 recipients are used, few TCR-transgenic T cells are recovered with or without anti-OX40, and they retain all the properties of naive, small resting lymphocytes (data not shown).

Costimulation is adequate for maximal proliferation without anti-OX40

To investigate the effect of anti-OX40 on proliferation of the donor TCR-transgenic T cells, TCR transgenic spleen cells were labeled with CFSE before transfer, and mice were sacrificed on days 3, 5, and 8 (Fig. 3). The donor T cells in these Ag-transgenic animals divide very rapidly and uniformly, perhaps owing to the ubiquitous expression of the transgenic Ag. Significantly, there is no difference in the rate of cell division, as measured by the dilution of the CFSE label, between the anti-OX40-treated group and the rat IgG controls all the way out to day 8, when the CFSE has been diluted to background levels. Notice that proliferation continues unabated beyond day 5, when accumulation of TCR-transgenic T cells in the spleen is maximal, implying that donor T cells are leaving the spleen and/or dying in large numbers in both groups.

Donor T cells from anti-OX40-treated animals make IFN-γ

To attempt to explain the lethal effect of anti-OX40 in this model, we investigated the possibility that OX40 engagement promotes T cell differentiation to effector function by measuring production of effector cytokines. Fig. 4 shows that a portion of TCR-transgenic T cells recovered at day 5 from anti-OX40-treated animals spontaneously produces moderate amounts of IFN-γ during a five-hour incubation in vitro. When the T cells were stimulated with PMA and ionomycin, which bypasses the TCR and directly activates the TCR-transgenic, particularly in the anti-OX40 group. This finding is consistent with the absence of a CD62L high population of TCR-transgenic T cells in the spleens of anti-OX40-treated animals (Fig. 2B). These experiments show that the donor T cells do not need the OX40 signal to leave the spleen and go out into nonlymphoid tissues, but do require OX40 engagement to acquire effector cell functions and cause damage.

Donor T cells accumulate in nonlymphoid tissues with or without anti-OX40

Substantial mononuclear cell infiltrates were found in livers and lungs in Ag-transgenic animals whether or not they were treated with anti-OX40, but evidence of destruction of liver cells was found only in the anti-OX40-treated animals (Fig. 5). Most of the infiltrating CD4 T cells in the liver were TCR-transgenic in both groups, although only the anti-OX40-treated T cells showed the same high side scatter, high levels of CD25 expression, and intracellular IFN-γ as the T cells recovered from the spleen (Fig. 6 and data not shown). In comparison to the liver and spleen, a smaller proportion of the CD4 T cells recovered from the lymph nodes was TCR-transgenic, particularly in the anti-OX40 group. This finding is consistent with the absence of a CD62L high population of TCR-transgenic T cells in the spleens of anti-OX40-treated animals (Fig. 2B). These experiments show that the donor T cells do not need the OX40 signal to leave the spleen and go out into nonlymphoid tissues, but do require OX40 engagement to acquire effector cell functions and cause damage.
TCR-transgenic T cells from anti-OX40-treated animals are unresponsive to signals through the TCR, but proliferate vigorously in response to IL-2 and make copious IFN-γ in response to IL-12 plus IL-18. Although they continue to proliferate in vivo (Fig. 3), purified CD4 T cells recovered from Ag-transgenic animals were profoundly anergic to restimulation with peptide and fresh APC in vitro (Fig. 7). TCR-transgenic T cells from animals treated with anti-OX40 also showed little or no response to peptide Ag, although they had higher background proliferative responses that varied from mouse to mouse (Fig. 7). Proliferative responses of T cells from the rat IgG control animals could not be regained by addition of exogenous IL-2, while T cells from anti-OX40-treated animals proliferated vigorously to IL-2 with or without Ag (Fig. 7). When whole spleen cell suspensions instead of purified CD4+ T cells were tested in proliferation assays, cultures from anti-OX40-treated animals proliferated vigorously without addition of IL-2 or Ag (data not shown). When donor T cells recovered on day 5 from anti-OX40-treated or control mice were transferred into fresh Ag-transgenic recipients, they did not expand, even when transferred with additional anti-OX40 Ab (data not shown).

TCR-transgenic T cells recovered from Ag-transgenic animals also did not produce IFN-γ or IL-2 in response to APC with peptide Ag in vitro, regardless of anti-OX40 treatment, in 6 of 7 similar experiments (Fig. 8 and data not shown). Because IL-12 and IL-18 can induce IFN-γ secretion from effector T cells independently of TCR signals (33), we tested this stimulus in our system. IL-12 plus IL-18 induced very high levels of IFN-γ production from most of the TCR-transgenic cells recovered from anti-OX40-treated mice (Fig. 8). For the anti-OX40 T cells, IL-12 plus IL-18 was consistently more effective than PMA and ionomycin (Fig. 4). Very few rat IgG-treated control T cells responded to IL-12 plus IL-18. Therefore, the combination of ubiquitous Ag presentation and a persistent signal through OX40 results in accumulation of lethal CD4 effector cells with a unique phenotype: they are anergic to signals through their TCR, but remain very responsive to the cytokines IL-2, IL-12, and IL-18.

Among several Abs shown to block peripheral tolerance, only anti-4–1BB has effects similar to anti-OX40

Several other agonistic Abs have been reported to provide or induce costimulatory signals and block peripheral tolerance in T
cells, including anti-CD28 (34), anti-CD40 (8, 32, 35), and anti-4-1BB (CD137) (36). 4-1BB is a costimulatory TNFR family member closely related to OX40, which is expressed predominantly on CD8 cells, but has also been reported to be expressed and functional on CD4 T cells (36, 37). Although anti-4-1BB has complex effects on Ab formation and CD4-dependent autoimmunity in vivo (38), it blocks tolerance to peptide Ag in young mice and restores T cell priming in aged mice (36). We injected these other Abs in place of anti-OX40 together with TCR-transgenic T cells into Ag-transgenic mice, and analyzed the spleens by flow cytometry at day 5 as before. Only anti-4-1BB approached anti-OX40 in its ability to produce donor T cells with high CD25 expression and IFN-γ synthesis in response to IL-12 and IL-18 (Fig. 9). Donor T cell recovery was variable but comparable among mice treated with anti-OX40 and anti-4-1BB (data not shown). Anti-CD40 produced a few T cells with that phenotype, perhaps by inducing expression of endogenous OX40 ligand (17). Treatment with agonist anti-CD28 produced no change. Therefore, it seems likely that closely related OX40 and 4-1BB receptors deliver a similar costimulatory signal to activated T cells that is qualitatively different from that delivered by conventional costimulation through CD28.

**Discussion**

T cells that see their Ags on healthy tissues in the absence of adjuvant or infection are rendered tolerant. Induction of peripheral tolerance in naive T cells in vivo nearly always involves an abortive immune response with an initial period of T cell proliferation followed by T cell death (4, 6, 13, 39, 40). In tolerance, fewer Ag-specific T cells are recovered than in an immune response, and the recovered cells are functionally compromised compared with primed cells, even when assayed in isolation in the absence of possible regulatory cells (5, 6, 41, 42). The mechanisms of inactivation of tolerant T cells include proliferative anergy, due to the inability to synthesize IL-2, and a more general inability to recognize and respond to Ag because of compromised signaling through the TCR (12). This report points to an additional mechanism of tolerance at the level of effector cell cytokine production, by which tolerant T cells are rendered harmless because differentiation to effector function is blocked or cytokine and cytokine receptor expression is inhibited.

The tolerant T cells described in this report appear to be compromised in all the ways previously described. Nevertheless, they accumulate to large numbers in vivo by day 5, and appear to continue proliferating between day 5 and day 8 even as numbers decline (see Fig. 3). A signal through OX40 enables acquisition of effector cell functions by these otherwise harmless, tolerant, proliferating cells, and results in the death of the animals. However, the OX40 signal does not restore normal signaling through the Ag receptor, because cells recovered from anti-OX40-treated animals are anergic and fail to respond to Ag on APC (Figs. 7 and 8). Instead, like nontolerant armed effector Th1 cells, these cells respond vigorously to cytokines, proliferating in response to IL-2 without Ag, and making copious IFN-γ in response to IL-12 plus IL-18 without a signal through the TCR. T cells from anti-OX40-treated animals express high levels of CD25, the IL-2Rα chain (Fig. 2B). Messenger RNA levels for IL-2Rα, IL-12Rβ2, IL-7R, and IL-15Rα are increased 4- to 18-fold in donor T cells from
anti-OX40-treated animals vs rat IgG-treated controls on day 3.5 by preliminary Affymetrix gene chip analysis (average of two experiments, data not shown). Message for IFN-γ was increased 2.9-fold directly ex vivo, without in vitro activation. Messenger RNA for GM-CSF, lymphotoxin, and IL-3 was present in donor T cells from anti-OX40-treated animals but absent from rat IgG-treated controls.

Although the initiation of the T cell response is completely dependent on Ag recognition in our model, the ability to produce and respond to inflammatory cytokines in a positive feedback loop with accessory cells in target tissues may be adequate to sustain T cell effector functions and progressive disease in these animals after the signals through the TCR are compromised by continuous antigenic stimulation. In support of this idea, we found that T cells recovered on day 5 from anti-OX40-treated animals were unable to continue to expand or induce disease in secondary, Ag-transgenic hosts, even in the presence of anti-OX40 Ab (data not shown), implying that disease progression depends on activated accessory cells in the day 5 animals. Consistent with this view, it was recently reported that viral stromal keratitis can be caused by a T cell-dependent, non-TCR-mediated, cytokine-driven bystander mechanism (43). It is possible that similar, non-TCR-mediated, positive cytokine feedback loops could contribute to immunopathology in other kinds of infections and in graft-vs-host disease in which large numbers of activated T cells accumulate in tissues. We have not yet determined the cause of death in our anti-OX40-treated animals. The most obvious early pathological change is extensive liver damage, which could easily account for death, but other target organs could be involved. Similar acute liver damage can be induced by the combination of IL-12 and IL-18 in normal mice, and liver damage is IFN-γ-dependent in that model (44, 45). We have focused on IFN-γ in our experiments, but it is possible that other effector cytokines such as lymphotoxin, TNF-α, and GM-CSF could be involved in the disease process.

Our main finding is that T cell tolerance occurs and can fail at the level of differentiation to effector functions. The donor T cells recovered from anti-OX40-treated animals are “tolerant” because they are unresponsive to Ag in vitro, but otherwise resemble fully armed effector Th1 cells. They are larger and more granular than donor T cells from control animals, and the cytokine receptors and cytokine receptor signaling pathways leading to acute IFN-γ synthesis (33) are open and active. Other investigators have documented loss of effector functions in T cells rendered tolerant in vivo (3, 4, 7, 11, 46), but only a few experiments have distinguished such loss from compromised signaling through the TCR. Zajac et al. (46) found virus-specific CD8 T cells in persistently infected animals that were devoid of effector functions, and failed to make IFN-γ even when TCR signaling was bypassed with PMA and ionomycin. Similarly, a high frequency of PMA and ionomycin-unresponsive tumor-specific CD8 T cells was detected with tetramers in a melanoma patient (47). Adler and colleagues (10, 48) showed recently that fully differentiated, primed effector CD4 T cells are rapidly tolerated by exposure to soluble peptide or parenchymal self Ag in vivo, with early and selective loss of IFN-γ and TNF-α production in response to restimulation with Ag in vitro. Most previous studies have emphasized the ability of the OX40 signal to enhance T cell expansion and survival. OX40 was initially characterized as an activation marker on CD4 T cells (15, 49), and then shown to have potent costimulatory activity for activated CD4 T cells in vitro (50). Although the OX40/OX40L interaction has been implicated in T cell/B cell collaboration (51), mice deficient in OX40 or OX40L have normal or near normal Ab

FIGURE 8. Donor T cells from anti-OX40-treated mice secrete IFN-γ in response to IL-12 plus IL-18 treatment, but not in response to peptide Ag. Splenocytes recovered on day 5, or in vitro activated T cell blasts as a positive control, were stained for CD4, Vβ3, and intracellular IFN-γ. Plots shown are gated on CD4+ and the percentage of CD4+Vβ3+ cells that are positive for IFN-γ staining is shown. One representative mouse of three per group, from one experiment of three, is shown.

FIGURE 9. Treatment with anti-4-1BB Ab produces an effect similar to treatment with anti-OX40, whereas anti-CD28 has no effect, and anti-CD40 produces only a slight increase in IFN-γ production. A, Expression of CD25 on CD4+Vα11+Vβ3+ cells at 5 days after transfer of transgenic T cells into an Ag-transgenic host with the indicated antibody. B, Production of IFN-γ by CD4+Vα11+Vβ3+ cells during 5 h in culture with either IL-12 and IL-18 or no additions. One representative mouse of three per group, from one experiment of two, is shown.
responses, with defects in T cell proliferation and cellular immunity (17, 52–54). Agonist anti-OX40 Ab has striking adjuvant effects in enhancing memory cell formation and blocking acquired tolerance to superantigen and peptide Ags in CD4 cells (18–22). Rogers et al. (21) have shown that OX40 up-regulates expression of the antiapoptotic proteins, Bcl-xL and Bcl-2, under conditions of enhancement of survival of activated CD4 T cells. Moreover, they showed that viral transduction of Bcl-xL or Bcl-2 reverses the survival defect in OX40 knockout T cells. In contrast, Bansal-Pakala et al. (22) showed that agonist anti-OX40 can reverse established CD4 T cell tolerance in vivo and T cell clonal anergy in vitro, effects that may involve more than enhancing T cell expansion and survival.

In our system, it appears that the major effect of anti-OX40 is to induce T cell differentiation to effector function, rather than to promote T cell survival. Although 3-fold larger numbers of TCR-transgenic T cells accumulate in the spleens of anti-OX40-treated than of control animals, fewer TCR-transgenic T cells are found in lymph nodes, and similar numbers are found in liver and lungs. The striking difference was in the armed effector phenotype of the T cells in the anti-OX40-treated animals. When anti-OX40 is given 3 or 4 days after the injection of the TCR-transgenic T cells, the OX40 signal does not produce a phenotypic change overnight, but instead must be present for several days to allow accumulation of cells expressing high levels of CD25 and capable of secreting IFN-γ (data not shown). It appears that anti-OX40 may enhance T cell survival and numbers of memory cells when Ag is transient, as OX40 activates NFκB sequences of the same OX40 signaling pathway. For instance, to effector cells in other circumstances could be independent con-

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