OX40 Promotes Differentiation of $CD4^+$ T cells to Effector Cells

by

Cortny Ann Huddleston

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This is certify that the Ph.D. dissertation thesis of

Cortny A. Huddleston

has been approved

[redacted]

Mentor/Advisor

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Member

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Preface

I have prepared my dissertation in accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health & Science University. This manuscript consists of a general introduction, three chapters of original data, and a section with summary and general conclusions. The references cited for all chapters are listed together at the end of the text and follow the format of the Journal of Immunology.

Chapter two contains data, figures, and text as they appear in the original paper published in the Journal of Immunology (1). Stephanie Lathrop and I contributed most of the work to this manuscript and David Parker wrote the manuscript. Chapter three contains data, figures, and text as they appear in the original paper published in the European Journal of Immunology (2). Chapter four is a manuscript that has been submitted for publication to the Journal of Immunology.

Abstract

CD4 T cells play an important role in protection against viruses, bacteria, parasites, and cancers, but can also contribute to undesired immune responses such as autoimmunity, graft rejection, and allergic reactions. Understanding the mechanisms that control CD4 T cell effector function will lead to more effective vaccine design and the management of aberrant immune responses. The tumor necrosis factor receptor (TNFR) family member OX40 (CD134) is a costimulatory protein expressed exclusively on activated T cells that augments clonal expansion and survival of antigen-specific CD4 T cells, as well as enhancing the generation of effector and memory T cells. Mechanistically, it has been proposed that OX40 enhances CD4 T cell survival and memory cell generation by enhancing anti-apoptotic protein expression, as well as enhancing effector cytokine production. However, blocking OX40 signaling in vivo specifically reduces inflammation induced by cytokines, suggesting that OX40 may directly influence differentiation to effector function. I was interested in how OX40 regulates effector function in CD4 T cells, so I hypothesized that OX40 signaling could promote differentiation independent of T cell survival.

We have developed a model in which a peptide antigen covalently bound to MHC class II is expressed at low levels on all MHC class II positive cells in mice. Upon transfer of small numbers of antigen specific T cell receptor transgenic CD4 T cells, rapid expansion and infiltration of tissues is observed, but the T cells are tolerant and the animals remain healthy. Addition of an agonist antibody to OX40 at the time of T cell transfer induces accumulation of large, granular effector CD4 T cells that express the IL-

2 receptor alpha chain, CD25, and secrete interferon-γ directly ex vivo or in response to cytokine stimulation, and the animals die within one week. We have also developed a polyclonal model in which a small percentage of B6 CD4 T cells transferred into MHC class II disparate mice behave similarly to the monoclonal T cells described above. These adoptive transfer systems provide useful models in which to examine the immune consequences of OX40 signaling pathways.

I found that OX40 signaling induces effector cytokine production early in T cell priming, before changes in anti-apoptotic proteins could be detected. I also showed that genetically altered CD4 T cells with enhanced survival do not acquire effector function independent of OX40 costimulation, and OX40 deficient CD4 T cells can acquire effector function in the presence of OX40 sufficient cells. These experiments suggest that OX40 directly influences differentiation, but may also require cooperation with other factors.

I tested the requirement for additional costimulation in supporting OX40 signaling, and found that OX40 costimulation induces differentiation independent of CD28 and CD40 signaling. I also showed that OX40 signaling does not depend upon T-bet expression for differentiation, but enhances responsiveness to cytokine stimulation to promote effector function. However, I found that OX40 is dependent on IL-2 receptor signaling to promote effector cytokine production. While the mechanism of OX40 signaling is not completely understood, this evidence indicates that OX40 signaling can promote differentiation via induction of cytokine and cytokine receptor expression.

Chapter 1—Introduction

The broad goal of my research is to understand how CD4 T cell effector function is regulated during an immune response. Specifically, I am interested in how engagement of the tumor necrosis factor receptor (TNFR) family member CD134 (OX40) regulates survival and differentiation during CD4 T cell activation. To appreciate the influence of OX40 on CD4 T cells, it is important to first understand that CD4 T cells play a central role in coordinating the host's innate and adaptive immune response to infectious agents. CD4 T cells enhance both innate and adaptive immune cell effector function to destroy pathogens, and are conversely able to inhibit effector function when the pathogen has been cleared. CD4 T cells in turn receive activation, survival, and differentiation signals at each stage of an immune response that influence the decision to respond, and how to respond, to a foreign agent. Members of the TNFR family are emerging as key mediators of effector CD4 T cell development. In this thesis, I will address the role of OX40 in promoting accumulation of effector CD4 T cells, and will discuss how OX40 influences survival and differentiation during effector cell development.

1.1 Development of effector and memory CD4 T cells

The CD4 T helper cell compartment of the immune system plays an important role in the adaptive immune response to infectious agents, as well as contributing to autoimmune disease and anti-tumor immunity. Activated antigen-specific CD4 T cells release cytokines or directly interact with phagocytic cells such as macrophages to help

destroy intracellular pathogens. Similarly, CD4 T cells also help B cells and CD8 T cells in their responses to antigen (3, 4). Naïve T cells circulate in the periphery via lymph and blood and enter lymph nodes, Peyer's patches, and spleen where they are able to encounter DC presenting antigenic peptide bound to MHC class II complexes. Upon recognition of antigenic peptide through their unique T cell receptor (TCR), naïve T cells are able to initiate proliferation and develop into an expanded population of effector T cells (5). Phenotypically, naïve cells are small with little cytoplasm and express high levels of the lymph node homing receptor CD62L, interleukin-7 receptor alpha (IL-7Rα), important for homeostasis, and low levels of CD44. Activated effector cells are very large and granular and down-regulate CD62L and IL-7Rα, and express several activation markers such as CD69, an early product of mitogen activated protein kinase (MAPK) signaling, IL-2Rα (CD25), the high affinity IL-2 receptor that allows enhanced responsiveness to the growth factor IL-2, and higher levels of CD44 (6).

Effector T cells can be divided into functionally distinct populations based on their cytokine expression profile. CD4 T helper 1 (Th1) cells are generated in the presence of IL-12 and secrete interferon gamma (IFN-γ), lymphotoxin, IL-2, and tumor necrosis factor alpha (TNF-α) to help macrophages and CD8 T cells clear intracellular pathogens, while CD4 T helper 2 (Th2) cells develop under IL-4 stimulation and secrete IL-4, IL-5, IL- 9, and IL-13 to aid in clearance of extracellular pathogens and B cell activation and antibody production (5). Another subset of effector CD4 T cells has recently been described, known as CD4 T helper 17 (Th17) cells, which develop under cytokine stimulation from transforming growth factor beta (TGF-β1) and IL-6, and upon exposure to IL-23, secrete IL-17, and can recruit neutrophils to sites of inflammation (7).

The type of effector cell generated is dependent on a number of factors such as the nature and dose of antigen (8, 9), the duration of TCR engagement to cognate antigen (10-12), the availability, maturation state, and type of antigen presenting cell (APC) (12-14), costimulatory molecules (15), and the cytokine milieu initiated by innate immune cells (16).

After antigen withdrawal, effector T cells undergo a contraction phase in which most effector cells die by T cell apoptosis; however, the surviving effector cells differentiate into long-lived memory T cells (17, 18). Phenotypically, memory cells differ from effector cells in size and phenotype, and memory cells are also more resistant to apoptosis than effector cells. Memory cells are small resting cells that have regained IL-7Rα expression, maintain high CD44 expression, and do not express the activation markers CD69 and CD25. The quality of a memory T cell response is largely dependent on the size of the memory T cell population, generated after effector T cell contraction (6).

Although signals from the TCR dictate T cell specificity, optimal T cell activation and acquisition of effector function only occurs with additional receptor-ligand interactions between the T cell and APC (19, 20). When these signals occur at the same time as TCR engagement, they are known as costimulatory signals. Some costimulatory receptor-ligand pairs are expressed on naïve T cells, such as the Ig superfamily member CD28, which is a receptor for both B7-1 (CD80) and B7-2 (CD86), expressed on APC (19). CD28 signaling reduces the threshold for T cell activation by reducing the number of TCR:peptide:MHC interactions required to activate naïve T cells (21). T cell costimulation through CD28 amplifies signals initiated through the TCR and allows the T

cell to produce IL-2 (21, 22), proliferate (23), express effector cytokines (24), and enhance anti-apoptotic proteins that promote survival (25). Another Ig superfamily member, inhibitor of costimulation (ICOS) and its ligand (ICOSL) similarly promote expansion, survival, and differentiation, but ICOS is expressed after T cell activation. ICOS ligation also induces IL-10 production (26), which is an important suppressive cytokine discussed later. Despite broad T cell activation and differentiation via CD28 and ICOS, optimal immune responses occur when the APC has fully matured and more costimulatory ligands, such as ICOSL become available to activated T cells (27).

Immature DC are located throughout the periphery and continuously monitor their environment by endocytosing proteins and processing them into peptide antigens for display on the cell surface by MHC complexes (28). DCs express a variety of receptors that specifically recognize pathogens via pattern recognition motifs. Toll-like receptors (TLRs) are included in this category, and recognize bacterial cell wall components such as lipopolysaccharide (LPS), glycolipids, flagellin, and CpG DNA and double stranded RNA (29). Other receptors recognize carbohydrate structures such as the mannose receptor (30). DC maturation begins upon ligation of these pattern recognition receptors detecting "danger" signals that can induce IL-12 and other pro-inflammatory cytokines, increase expression of MHC complexes loaded with antigenic peptides, and increase costimulatory ligand expression (28). DCs also express the TNFR family member CD40, that when engaged, also serves as a "danger" signal to maturing DC. The CD40 ligand, CD154, is expressed on activated B and T cells, and is induced on other cell types during inflammatory responses. Although CD40 activation alone can induce DC maturation, co-activation through TLR signaling results in optimal DC activation (31). Maturing DC

migrate to T cell compartments of secondary lymphoid organs, and are able to secrete chemokines such as DC-CK1 (CCL18), which specifically attract naïve T cells (32). Mature DC are thus able to present cognate antigen in the presence of enhanced costimulatory ligand expression to naïve T cells to foster differentiation to effector T cell function (Figure 1-1).

CD28 costimulation activates the IL-2 promoter in T cells (33). IL-2 was originally characterized as a T cell growth factor, owing to its ability to promote antigen activated T cell proliferation in vitro (34). However, later studies showed that provision of IL-2 in vitro most efficiently promoted apoptosis or activation-induced cell death, and suggested that IL-2 functioned during the contraction phase of the immune response to restore T cell homeostasis (35). IL-2 and IL-2 receptor deficiency lead to early and aggressive autoimmune disease (36-38), suggesting that IL-2 functions in vivo as a regulator of immune suppression rather than T cell activation and proliferation. T regulatory cells, discussed in detail below, are absent or not functional with IL-2 or IL-2R deficiency. More recent experiments revealed that TCR and costimulatory receptor engagement was sufficient to promote T cell activation and several rounds of cell division without IL-2/IL-2R (39), but that IL-2 signaling in vivo is essential to promote effector cell development and enhance secondary immune responses (39-42).

In summary, optimal T cell activation and development to effector and memory cells requires recognition of peptide antigen presented on MHC class II complexes by mature DC or other APC. Furthermore, T cells require ligation of costimulatory receptors by ligands expressed on mature DC that induce proliferation and differentiation supported by subsequent cytokine receptor signaling.

1.2 Regulation of the immune response, tolerance induction

Self-reactive T cells that cause autoimmune disease are largely eliminated in the thymus before they fully mature into naïve T cells in a process called central tolerance. The mechanism of central tolerance is based on signal strength of the responding immature T cell. A T cell must be able to recognize self-MHC complexes, yet not become activated in response to self-peptides presented by MHC complexes. Thus, an immature T cell is eliminated via apoptosis if a signal through the TCR is too weak or too strong. A moderate TCR signal, signifying recognition of self-MHC complexes, but not full activation to self-peptides, warrants successful T cell maturation and release into the periphery (43). Central tolerance does not completely eliminate all self-reactive T cells, so mechanisms for regulating T cell responsiveness in the periphery are necessary to avoid autoimmunity.

A mature naïve T cell in the periphery that recognizes cognate antigen through the TCR, or signal one, without sufficient costimulation, or signal two, leads to a state of T cell hyporesponsiveness, or peripheral tolerance (44),(45). T cell activation induced cell death not only aids in the contraction phase of the T cell response, but also serves as a mechanism to preserve peripheral tolerance (46). Negative costimulatory signals delivered through cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed death-1 (PD-1) and PD-2, and BTLA (B and T lymphocyte attenuator), and T cell attenuation via suppressive cytokine production and regulatory T cells prevent self-reactive T cell functions as well (47), and will be discussed below.

The first example of peripheral tolerance, signal one without signal two, was originally termed clonal anergy and was characterized in CD4 T cell clones restimulated

with a TCR signal alone in vitro. The anergic CD4 T cells did not produce IL-2, although other protein synthesis could occur, and they could proliferate in response to exogenous IL-2, indicating that anergy induction was an active process, and not simply an inability of the T cell to respond to antigen (48). Proliferative non-responsiveness of effector CD4 T cells was also seen in vivo, as rapid loss of effector function was observed after transfer into naïve antigen-bearing recipients (49).

Naïve CD4 T cells can also be tolerized in vivo by injecting high dose antigen intravenously into a non-inflammatory environment that lacks costimulatory signals. The resulting T cells have undergone limited clonal expansion and are hyporesponsive, measured by proliferation and IL-2 production ex vivo, compared to cells primed in a pro-inflammatory environment complete with costimulatory signals (50-53). When antigen is transient, the anergic state can be reversed with time, but repeated stimulation with antigen in the absence of inflammation maintains peripheral tolerance (52). In other models, in which antigen presentation is persistent, naïve CD4 T cells become tolerant in the absence of inflammation (49, 54-58). This form of tolerance can be reversed following adoptive transfer of the tolerant T cells into a second recipient lacking antigen (59), similar to recovery of tolerance after transient antigen exposure in a single recipient (52). This confirms that persistence of antigen is required to maintain the tolerant state. In all of these studies, naïve T cells are able to respond to antigen, in that they expand and contract, but effector cells are absent or short-lived, and the few cells that survive the contraction phase are not true memory cells, because they are hyporesponsive to restimulation with antigen in vitro or in vivo. Thus, the balance between immunity and

tolerance is regulated by danger signals and inflammatory cytokines that enhance costimulatory signals delivered from APC to T cell.

Another consequence of TCR engagement is activation-induced cell death (AICD) mediated by the upregulation of CD95 (Fas) upon T cell activation, with enhanced expression by signaling through the interleukin-2 receptor (60). CD95 ligand (FasL) is expressed on activated antigen presenting cells, as well as activated T cells, and engagement of FasL with Fas initiates recruitment of procaspases via caspase adaptor proteins aggregated at the intracellular portion of Fas. This complex is known as the death inducing signaling complex. Once the initiator caspase, usually caspase-8, is activated, it mediates apoptosis directly through activation of caspase-3, or indirectly through activation of a caspase cascade that results in release of cytochrome c (61). Repeated TCR ligation results in enhanced Fas/FasL expression, but CD28 costimulation inhibits FasL expression and promotes Bcl-xL expression (25), which inhibits apoptosis by preventing cytochrome c release (62). Thus, another check and balance between immunity and tolerance induction, life and death in T cells, is dependent on engagement with FasL.

While AICD is dependent on death receptor signaling, activated T cell autonomous death (ACAD), or "death by neglect" is a programmed cell death driven by internal cellular factors, primarily by the Bcl-2 family (62). ACAD may be induced by, but not limited to, cytokine, growth factor, or antigen withdrawal. Anti-apoptotic proteins like Bcl-2 and Bcl-xL inhibit pro-apoptotic proteins like Bim induced by ACAD (63, 64). Therefore, a self-reactive T cell responding to antigen in the absence of

costimulation, growth factors, or pro-inflammatory cytokines will undergo ACAD, preserving peripheral tolerance.

CTLA-4 is an inhibitory protein that is induced on naïve T cells upon TCR engagement. The ligands for CTLA-4 are B7-1 and B7-2, the same ligands as for the costimulatory protein CD28, but CTLA-4 has a greater affinity and avidity for these ligands. Cross-linking CTLA-4 on activated T cells down regulates proliferation and IL-2 production, showing that CTLA-4 is a negative regulator of T cell activation, and promotes the preservation of peripheral tolerance (46). CTLA-4 antagonizes CD28 signaling, and has recently been shown to antagonize CD28-mediated extracellular signal-regulated kinase (ERK) signaling by activating Rap1, an inhibitor of the MAPK signaling pathway (65). CTLA-4 can directly inhibit T cell activation by negative signaling through its cytoplasmic tail, which prevents accumulation of AP-1, NFκB, and NFAT in the nucleus and induces cell cycle arrest (46). Other T cell coinhibitory proteins, PD-1, PD-2, and BTLA have also been characterized, but function as monomers instead of dimers, and have separate signaling pathways from CTLA-4 to dampen T cell effector function (66, 67).

CD4 T regulatory cells are a subdivision of the immune repertoire that regulate other T cell functions by direct cell-cell contact and/or by the release of negative regulatory cytokines like transforming growth factor beta (TGF-β1) and IL-10. T regulatory cells develop naturally in the thymus and express forkhead-winged-helix transcription factor 3 (Foxp3), CD25, CD103, and GITR (glucocorticoid-induced TNF receptor related gene) (47). Foxp3 appears to be the master regulator of T regulatory cell development. Expression of Foxp3 in thymocytes induces development of T regulatory

cells that enter the periphery and are able to suppress proliferation and effector functions in both CD4 and CD8 effector T cells. Deletion of Foxp3 results in an absence of natural T regulatory cells and severe autoimmune disease, while transgenic expression of Foxp3 enhances the number of T regulatory cells with suppressive functions (68). Furthermore, mutations in Foxp3 were found to cause immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) in humans. This suggests that lack of T regulatory cells or lack of T regulatory function allows hyperactivation of T cells responsive to self-antigens, commensal bacteria in the intestine, or innocuous environmental antigens and lead to autoimmune polyendocrinopathy, inflammatory bowel disease (IBD), or allergy (69). In vitro studies show that T regulatory cells suppress proliferation and cytokine production by effector cells via direct T-T cell contact (70-72), T_{reg}-APC cell contact (73), and by cytokine secretion (74-76). TCR engagement on T regulatory cells is required to induce suppressive functions (72, 77), but the antigen specificity is not always the same as the effector CD4 or CD8 T cell that is suppressed (72, 78, 79). Thus, with each exposure to a particular antigen, an effector T cell must combat suppressive effects of T regulatory cells, which ensures that only effector cells receiving strong positive costimulatory signals will mount a productive immune response.

1.3 OX40 and its ligand

CD28 costimulation is considered the primary signaling event in naïve T cells because it augments initial cell cycle entry and clonal expansion, and enhances expression of anti-apoptotic proteins like Bcl-xL to promote survival (23). CD28 signals

also induce early IL-2 production, and subsequent IL-2R signals further promote proliferation and differentiation of naïve T cells (39). However, provision of TCR signals and CD28 costimulation alone leads to apoptosis after initial T cell priming (80), suggesting that other signals must exist to drive long-term survival and differentiation to effector function. For CD4 T cells, expression of OX40, a TNFR superfamily member, provides a receptor for additional costimulatory signals that promote differentiation and survival after initial T cell priming (81).

OX40 (CD134) was originally characterized as a T cell activation marker, with preferential expression on CD4 T cells (82-84). Under strong antigenic stimulation, OX40 is expressed on CD8 T cells (85, 86), and gut $CD8^+$ intraepithelial cells express OX40 in conjunction with cytotoxic effector function (87). Unlike CD28, OX40 is not constitutively expressed, but induced after TCR engagement, and peak expression is observed 2-5 days after activation (81, 84, 88). OX40 can be induced on both naïve and effector T cells with TCR stimulation alone (89). Addition of an agonist OX40 antibody to in vitro mixed lymphocyte reactions promotes enhanced proliferation and effector cytokine production in T cells, although with a delayed response, reflecting the expression pattern (81).

OX40 ligand (OX40L) is expressed only on activated, not resting, APC (84, 90, 91). OX40L is expressed on several cell types, and was originally identified on human T cell leukemia virus type 1 (HTLV-1) transformed T cells (92, 93). Antigen and/or CD40 activated B cells express OX40L, and engagement of OX40L on B cells has been reported to drive differentiation to immunoglobulin secreting plasma cells (90, 94-96). CD40 ligand activated DC and macrophages express OX40L, and OX40L ligation also

provides a pro-inflammatory signal to the APC (97-99). In some cases, OX40L is expressed on NK cells and mast cells (100, 101). Recently, OX40L expression was found on a novel accessory cell in the T-B cell contact region of the spleen (102). OX40L is also expressed on vascular endothelial cells and thought to be involved in T cell migration to sites of inflammation (103, 104). The selective expression of both OX40 and OX40L suggest that they are highly regulated (80, 81, 91). OX40L and OX40 expression peak simultaneously, and persist for 5 to 7 days (81, 90). In vivo, OX40 and OX40L expression is sustained at sites of inflammation (105-108), suggesting that the expression pattern of OX40 and OX40L coincides with antigen stimulation and the persistence of inflammation during the effector phase of the immune response.

The costimulatory function of OX40 was initially shown in vitro by stimulating TCR transgenic T cells with peptide loaded MHC class II^+ fibroblast cell lines transfected with OX40L, B7-1, or both. Effector T cells stimulated with APC transfected with OX40L alone were able to proliferate and make effector cytokines, while naïve cells required co-expression of B7 and OX40L on APC to induce proliferation and acquisition of effector function (81). However, CD28 is not required for OX40 expression (89, 95), but in combination with TCR signals, CD28 can enhance OX40 expression (80). It is also important to note that OX40 does not replace the costimulatory effects of CD28 on initial cell division, but does augment CD4 T cell expansion later in an immune response (81). Furthermore, OX40 engagement results in decreased CTLA-4 expression (109), which may enhance the survival effects of CD28.

OX40 and OX40L deficient mice show no defects in viability of mice, organization of lymphoid tissue, or development of T or B cells (90, 98, 110, 111).

However, OX40 deficient mice have fewer T regulatory cells in the periphery (112). Initial T cell priming, proliferation, and effector cytokine production is also unabated with deficiency in OX40 signaling (81). However, OX40 deficient CD4 T cells are unable to maintain a primary T cell response after 3-5 days and show a defect in long-term survival and maintenance of effector function (80, 81, 113, 114). These observations are consistent with the expression pattern of OX40 and OX40L, discussed above. Furthermore, OX40 deficiency results in fewer memory T cells (115), suggesting that OX40 signaling promotes survival of effector cells entering the memory pool, or induces effector cells to differentiate into memory T cells (Figure 1-1).

1.4 Other TNFR family members that regulate immunity

Other members of the TNFR family also augment survival and differentiation subsequent to initial CD28 costimulation (15). CD40 ligation on APC may be the most important signal for initiating T cell costimulation because it enhances B7 ligands as well as upregulating several TNFR family ligands on DC, including OX40L (91). CD40 is triggered by CD40L, expressed on T cells, but it may also function as an important receptor in innate immunity by responding to ligation with heat shock proteins, contributing to DC maturation (31). Other members of the TNFR family directly modulate T cell responses, similarly to OX40, as described below.

4-1BB is expressed only after T cell activation (116). Expression occurs early in T cell priming, within 12-36 hours after TCR engagement (117), peaking at 48 hours and declining after 4-5 days (118). 4-1BB is expressed on both CD4 and CD8 T cells, but is induced faster and more robustly on CD8 T cells (119). 4-1BB is also expressed on

monocytes, DC, NK cells, eosinophils, and microglia. CD40 is a major regulator of 4-1BBL, inducing expression on B cells and DC, and can also be expressed on other cell types in the presence of inflammation (15). Similar to OX40, 4-1BB enhances survival and effector cytokine production from both CD4 and CD8 T cells (118, 120). An agonist antibody to 4-1BB delivered in vivo induces massive expansion of antigen responsive CD8 T cells, and also affects CD4 T cells (121, 122).

In contrast to OX40 and 4-1BB, CD27 is expressed on naïve CD4 and CD8 T cells, and is also found on NK cells and B cells (123). CD27 expression is enhanced transiently in correlation with antigen stimulation (124, 125). The ligand for CD27, CD70, is expressed on B cells, T cells, and DC, and is enhanced upon CD40 and TLR stimulation (126). Although CD27 promotes survival and differentiation in vitro (127, 128), it appears to primarily drive survival of CD4 and CD8 T cells in vivo (129). However, CD70 transgenic mice accumulate effector T cells by 4 weeks of age (130). This may suggest that CD27 simply promotes survival, and effector function is a byproduct of survival, or CD27 may augment effector function of surviving cells.

Herpes simplex virus-1 (HSV-1) gains entry into target cells via the receptor herpes virus entry mediator (HVEM), which also belongs to the TNFR family (131). HVEM is expressed on resting T cells, B cells, NK cells, and immature DC (15). HVEM associates with two TNF ligands, LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and lymphotoxin alpha (132). Unlike other TNF family members, HVEM is downregulated upon T cell activation. Interaction with LIGHT induces further downregulation of HVEM on T cells and DC (133, 134). However, costimulatory

signaling is still evident from in vitro studies showing a role for promoting proliferation, and in vivo in promoting allograft rejection (15).

Glucocorticoid induced TNFR family-related gene (GITR) is expressed at low levels on CD4 and CD8 T cells, and enhanced upon T cell activation (135, 136). GITR is also constitutively expressed at higher levels on $CD4^+$ $CD25^+$ T regulatory cells (137). Addition of anti-GITR antibody in vivo results in enhanced autoimmunity, suggesting that GITR signaling in T regulatory cells is important for their suppressive function (138). However, when T regulatory cells are cultured with responding effector cells sufficient or deficient in GITR, the function of GITR was not to reduce suppressive function of T regulatory cells, but to make effector cells more resistant to T regulatory cell suppression (139). This experiment, in conjunction with the fact that GITRL is transiently expressed on maturing DC and downregulated by 48 hours (139, 140), led to the hypothesis that GITR regulates immune responses by allowing T cell activation in the presence of danger and antigen, but as these signals disappear, effector T cells become susceptible to suppression by T regulatory cells.

Taken together, TNFR family members generally promote survival and differentiation in T cell responses, but the effects of each receptor is unique compared to CD28 costimulation, and to each other (15, 19). However, the redundancy in function paired with the temporal and spatial segregation of the TNFR family members point to an elaborate mechanism for inducing a specific immune response to each pathogenic insult.

1.5 OX40 Signal transduction

TNFR family members fall into two groups characterized by their intracellular signaling components. Death domain (DD) containing receptors like TNFR1, CD95, and death receptor 3 (DR3) allow formation of the death inducing signaling complex and recruit caspase activity that leads to apoptosis. The second group of receptors, to which OX40 belongs, do not have DD's, but have motifs that recruit TNF receptor associated factors (TRAFs) (141). TRAFs are adaptor proteins that serve as a platform for signal transduction that leads to inflammatory responses and promotes both cell survival and cell death (142). The intracellular tail of OX40 has 4-6 amino acid motifs that recruit TRAF2, TRAF3, and TRAF5 (15). TRAF2 recruitment leads to NFκB activation, and aggregation of TRAF2 also induces MAPK signaling and AP-1 activation (142, 143). TRAF2 deficiency results in early lethality, indicating the importance of TRAF signaling in other systems. OX40-mediated cytokine expression and survival is enhanced by TRAF2 signaling (109), and TNFR stimulated TRAF2 dominant negative T cells show a defect in MAPK signaling, cytokine production, and T cell longevity (109, 144). TRAF5 is a functional and structural homologue of TRAF2, but TRAF5 deficiency is not as severe as TRAF2 deficiency (142). In the absence of TRAF5, T cells stimulated with agonist anti-OX40 have exaggerated Th2 responses and poor proliferative responses and indicate that TRAF5 modulates TRAF2 induced cytokine production and proliferation (145). Finally, TRAF3 is a negative regulator of OX40 signaling, inhibiting NFκB activation (146), however, some TRAF3 splice variants do induce NFκB activation (147).

NFκB is composed of dimeric complexes of transcription factor members including Rel-a, c-Rel, Rel-B, NFκB1/p50, and NFκB2/p52. NFκB dimers are held in

the cytoplasm in unstimulated cells by cytoplasmic inhibitory proteins (IκBs), a family including IκBα, IκBβ, IκBε, and precursor forms of NFκB1 (p105) and NFκB2 (p100), which are proteolytically processed upon agonist NFκB activation signals that induce phosphorylation of IκB and ubiquitination, and allow NFκB dimers to translocate to the nucleus. NFκB activation leads to transcription of genes important for survival, cytokine and chemokine production, adhesion protein expression, and apoptosis (148). Two NFκB activation pathways have been defined in T cells; the classical pathway initiated by NFκB1 and Rel-A, and the alternative pathway, initiated by NFκB2 and Rel-B (149). NFκB1/Rel-A activation is important for IL-2 and IL-2R gene transcription (150, 151), while NFκB2/RelB activation is important for pro-inflammatory gene transcription (152). Two serine/threonine kinases have been implicated in TNFR signaling via TRAFs, NFκB-inducing kinase (NIK) and mitogen-activated protein kinase/extracellular signal regulatory kinase kinase (MEKK1) (153, 154). Studies have shown evidence that NIK is important for activation of the alternative NFκB pathway (155, 156), but regulation of each pathway is still not completely understood.

OX40 signaling also activates protein kinase B (PKB) and leads to upregulation of anti-apoptotic proteins, but only in previously activated CD4 T cells (113). This suggests that OX40 maintains the active form of PKB following CD28 costimulation, which activates phosphatidylinositol 3 kinase (PI3K) and PKB (157). Since OX40 inhibits CTLA-4 expression (109), OX40 could maintain active PKB by promoting additional CD28 signaling. OX40 also induces activation of p38 MAPK and PI3K that leads to enhanced stability of effector cytokine messenger RNA (158), indicating one

mechanism by which OX40 costimulation results in enhanced effector cytokine production.

1.6 OX40 in T cell expansion, survival, and memory

The observations that effector T cells are highly susceptible to activation induced cell death (AICD) and that effector cells responsive to OX40 proliferate and survive in vitro, led investigators to determine the role of OX40 signaling in T cell survival (159). Initial experiments employed a superantigen model, in which the expansion and contraction phase of antigen specific T cells is well characterized (160). Administration of agonist anti-OX40 after superantigen injection resulted in a 10-fold increase in cells surviving the contraction phase, indicating that OX40 signaling promotes survival during an immune response (161). Addition of danger signals via TLR-9 stimulation in addition to anti-OX40 boosted memory cell recovery by 60-fold over antigen alone. As previously discussed, danger signals can enhance T cell survival by inducing costimulatory ligands on APC, including OX40L, and induction of pro-inflammatory cytokines can also lead to enhanced T cell survival. CD4 T cells can also express TLR (162), and in this way danger signals can directly augment T cell differentiation and survival. In another study using adoptive transfer of TCR transgenic T cells followed by immunization of peptide in adjuvant, addition of anti-OX40 promoted accumulation of effector cells with enhanced cytokine production, and importantly, resulted in accumulation of functionally competent memory cells 35 days after immunization (115). In a parallel experiment, OX40 deficient T cells developed fewer effector cells and had 10-fold fewer memory cells than WT 35 days after T cell priming. These data confirm

the role of OX40 signaling in clonal expansion, promoting accumulation of effector cells that lead to augmentation in memory T cell populations.

Anti-apoptotic proteins can inhibit indirect AICD or ACAD that leads to release of cytochrome c, which is dependent on caspase activation but independent of death receptor signaling. OX40 deficient CD4 T cells primed in vitro show early expression of Bcl-2 and Bcl-xL early in T cell priming, but protein expression is reduced over time (80). Stimulation of wild type CD4 T cells with anti-OX40 enhances anti-apoptotic protein expression, and retroviral transduciton of Bcl-2 and Bcl-xL in OX40 deficient CD4 T cells restores the survival defect. Additional studies in the same manner show that OX40 maintains the active form of PKB, which promotes Bcl-2 expression (113), and OX40 signals also enhance the cell cycle regulator survivin (114). Furthermore, adoptive transfer of wild type or OX40 deficient CD4 T cells transduced with the active form of PKB impart enhanced effector cell accumulation, responsiveness to antigen, and enhanced lung pathology in an experimental model of asthma compared to vehicle transduced T cells (113). Taken together, these data show that OX40 signaling promotes survival after initial T cell priming by enhancing anti-apoptotic protein expression to enhance accumulation of effector CD4 T cells as well as memory T cells.

1.7 OX40 in T cell differentiation

OX40 is expressed on Th2 cells (102, 163), supporting the notion that OX40 drives Th2 differentiation (13, 86, 89, 99, 102, 108, 164, 165), but there are several examples in which OX40 enhances production of Th1 cytokines (81, 166-169). Naïve human CD4 T cells co-stimulated with anti-OX40 produce IL-4 and become Th2 cells

producing high levels of IL-4 (99). When CD4 T cells are stimulated by OX40L on activated B cells, IL-4 is produced and cells differentiate into Th2 cells (164). Inhibiting OX40 interactions in T-B contact zones in secondary lymphoid tissue inhibits germinal center formation (170), while OX40 ligation is important in driving Th2 responses and lung pathology in asthma models (106, 165). However, OX40 ligation also exacerbates CD4 T cell-mediated pathology in rheumatoid arthritis (168) and experimental autoimmune encephalomyelitis (EAE) (103, 105), two diseases normally mediated by activation of Th1 or Th17 cells. OX40 stimulation in the context of peptide and adjuvant immunization results in IL-2, IFN-γ, and IL-5 production, suggesting that OX40 can enhance both Th1 and Th2 cytokines in response to the same antigen (81). Taken together, these results suggest that OX40 does not directly influence T cell polarization to Th1 or Th2 cells, but enhances effector cell programs established early in T cell priming. The following sections on OX40 and disease will also highlight the role of OX40 in supporting differentiation programs.

1.8 OX40 in disease

OX40 expression on CD4 T cells is becoming a widely used marker for diagnosis of inflammatory and autoimmune diseases, and OX40:OX40L interactions are implicated in a growing number of disease models. Rheumatoid arthritis (RA) patients have OX40 expression on T cells from synovial fluid and synovial tissue, and OX40L is expressed on cells lining the synovial tissue (171), implicating a role for OX40 interactions in the development of RA. Spontaneous IBD in IL-2 deficient mice, or mice with hapten-induced IBD have $OX40^+$ cell infiltrates in the lamina propria, and treatment with OX40-

Ig fusion proteins ameliorates both diseases (107). CD4 T cell infiltrates at inflammatory sites in EAE in mice also express OX40 (172), and deletion of $OX40^+$ cells ameliorates disease (169), while OX40 engagement exacerbates disease (167, 173). In addition, blocking OX40:OX40L interactions reduces T cell function at inflammatory sites in EAE, also reducing disease incidence (105, 174). OX40 costimulation may be important for the development of autoimmune diseases. Agonist anti-OX40 promotes accumulation of effector CD4 T cells that had previously been rendered tolerant by administration of peptide in the absence of adjuvant in vivo (175). More experiments to confirm the feasibility of OX40 as a therapeutic target for disease are required, but evidence mentioned above show that blocking OX40:OX40L interactions in mouse models prevents the development and maintenance of autoimmune and inflammatory diseases.

OX40 signals enhance the immune response by promoting proliferation, survival, and effector cytokine production. In models of infectious disease, the influence of OX40 depends on the disease model, the type of pathogen, and the T helper polarization preference (176). The best example of the discrepancy in OX40 signaling is *Leishmania major* infection in BALB/c mice and in C57BL/6 mice. Th1 cells mediate *L major* parasite clearance, and BALB/c immune responses are generally skewed toward Th2, while C57BL/6 generally have Th1 immunity (108). A blockade or deficiency in OX40L ameliorates leishmaniasis in BALB/c mice, attributed to suppression of Th2 cells. Conversely, transgenic expression of OX40L in C57BL/6 mice resulted in an elevated Th2 response and decreased parasite clearance (177). In pulmonary infection, lung pathology is often caused by excessive inflammation rather than directly by the pathogen.

Blocking OX40L reduces lung pathology following influenza virus infection, consistent with a role for OX40L enhancing inflammation at the site of infection (178).

OX40:OX40L interactions are important for regulating Th2 cell and eosinophil accumulation and lung pathology in mouse models of asthma (165, 179). Recent evidence for OX40 signaling was found in asthma patients, which over express thymic stromal lymphoprotein (TLSP) in airway epithelium, which is critical for the development of asthma (180-182). TSLP induces human DC to express OX40L but not IL-12, and thus triggers naïve CD4 T cells to produce IL-4, IL-5, and IL-13 and differentiate into Th2 cells (183). OX40L selectively promotes TNF-α and inhibits IL-10 production in Th2 cells. The inhibition of IL-10 in Th2 cells results in inflammatory Th2 cells that may be the pathogenic cells inducing asthma, with TSLP induced OX40L being the key mediators of pathology. IL-10 is a suppressive cytokine that dampens APC function and induces differentiation of regulatory Th2 cells that do not cause overt pathology. This example of OX40 specifically inducing inflammatory Th2 cells may indicate that an important outcome of OX40 signaling is to promote pro-inflammatory cytokine production in a previously established development program.

In humans, an increase in $OX40^+$ $CD4^+$ T cells in peripheral blood precedes the onset of chronic graft versus host disease (GVHD) after transplantation. The magnitude of disease correlates with the number of $OX40^+$ cells, and measurement of $OX40^+$ T cells is useful for predicting the onset and therapeutic response to GVHD (184). There is also evidence that OX40 signaling is involved in allograft rejection. In mouse and rat models, $OX40^+$ cells also indicate chronic GVHD (185, 186), and administration of OX40 Ig fusion proteins in one model completely prevents the development of GVHD pathology,

reducing inflammation in target organs such as liver, gut, and skin, and prevents weight loss, diarrhea, and alopecia (187). OX40 and OX40L deficient donor cells transferred into MHC disparate recipients have delayed onset of GVHD, and a larger percentage of recipients do not develop GVHD (188). These data indicate that OX40 ligation promotes the pro-inflammatory environment that leads to GVHD. Interestingly, when recipient T cells are deficient in both CD28 and CD40L, two costimulatory molecules that act early in T cell priming, skin allograft rejection still occurs. $OX40^{+}$ $CD4^{hi}$ T cells were found in the skin graft, and administration of anti-OX40L induced long-term skin allograft survival. Furthermore, blocking 4-1BB, CD27, and inducible costimulatory (ICOS) costimulatory pathways did not promote skin allograft survival, indicating that OX40 is a critical mediator of organ rejection (189).

Tumor antigen specific T cells have the potential to eliminate tumors, but because tumors are derived from self, tumor specific T cells are often hyporesponsive due to peripheral tolerance mechanisms (190, 191). However, signaling through OX40 promotes pro-inflammatory cytokine production, and as mentioned before, OX40 ligation can overcome previously established peripheral tolerance (175). Transduction of OX40L into tumor cells or DC presenting tumor antigens enhances anti-tumor responses in several experimental models (192-194). Administration of an agonist OX40 antibody in vivo also enhances anti-tumor immunity to several types of tumors (195-199). Importantly, mice that cleared tumors in these cases were also resistant to challenge with the same tumor (195), indicating that OX40 ligation not only enhanced effector function but also increased memory T cell development of tumor specific T cells. Furthermore, adoptive transfer of tumor-specific memory T cells can prevent tumor growth in naïve

mice (195). Although OX40 is preferentially expressed on CD4 T cells, tumor infiltrating lymphocyte populations contain CD8 T cells that also express OX40 (199). This suggests that OX40 ligation can enhance both CD4 and CD8 tumor-specific T cells effector function, and in addition to CD4 T cell help to cytotoxic CD8 T cells, OX40 ligation may directly enhance CD8 effector function. Most human tumors are resistant to immune regulation, and although some $OX40^+$ T cells are found in tumors, targeting several costimulatory receptors in addition to OX40 may be the most effective anti-tumor treatment (200).

1.9 OX40 in persistent versus transient antigen stimulation

Naïve CD4 T cells undergo multiple rounds of proliferation before they differentiate, and several reports have addressed the relationship between cell division and acquisition of effector cell function. Initially, entry into cell cycle was proposed to be necessary to initiate differentiation in T cells (201, 202), but other experiments indicated that effector cytokine production could occur under cell cycle arrest in CD4 T cells (203, 204). Furthermore, a recent study indicates that CD40 is required for effector cytokine production in proliferating memory CD4 T cells, and implicates a role for costimulation during acquisition of effector function (205). In another study, initial exposure to antigen induced several rounds of cell division, but without continued exposure to antigen and cytokine stimulation, differentiation did not occur (11). In CD8 T cells, antigen and B7 costimulation allowed proliferation and clonal expansion, but additional signals were required for differentiation (206). CD4 T cells responding to antigen presented as self or as foreign had equal number of cell divisions, although self

presentation led to tolerance and foreign presentation led to effector function (56), again suggesting a disconnect between proliferation and differentiation. In a model of effector cell tolerance induction, effector CD4 T cells transferred into recipients bearing antigen in the absence of inflammation lost pro-inflammatory effector cytokine production in the first 24 hours, yet continued to proliferate through day 6 (207). These studies suggest that proliferation and survival can be segregated from differentiation, and regulated independently. OX40 regulates accumulation of effector CD4 T cells, and OX40 also promotes cytokine expression, so perhaps OX40 regulation of survival is separated from regulation of differentiation.

CD4 T cell responses to transient antigen in the presence of danger and costimulatory signals results in clearly defined expansion of naïve cells that acquire effector functions, followed by a contraction phase in which the surviving cells become memory T cells (161). In this case, OX40 signaling promotes survival and accumulation of effector cells that promote long-lived antigen specific memory cells, and additional signals induced by TLR-9 ligation enhance this effect. OX40 signaling appears to support enhanced survival of responding cells by upregulation of anti-apoptotic protein expression, and differentiation of surviving effector cells into memory cells (80, 113, 114).

CD4 T cell responses to persistent antigen, such as in GVHD models, result in alloresponses that induce pro-inflammatory cytokine expression and lead to extreme tissue damage and death of the recipient (57, 188). As previously discussed, blocking OX40:OX40L interactions reduces the severity of GVHD, but how OX40 signaling induces inflammation in these models is not known. The mechanism by which OX40

signaling potentiates GVHD may be through increasing the number and survival of alloresponsive cells entering cell cycle. However, OX40 signaling may have important direct effects on differentiation in response to persistent antigen. In a model of lethal acute GVHD, administration of anti-OX40L reduced lethality and manifestations of the disease, and specifically reduced inflammatory responses in target organs. After anti-OX40L treatment, T cells were recovered from the spleens of recipient mice, and were hyporesponsive upon in vitro restimulation (187).

The molecular mechanism of OX40 signaling has been studied both in vitro and in vivo, and indicates that OX40 promotes survival and effector function by enhancing anti-apoptotic protein expression (80, 113, 114). However, the specific reduction in pro-inflammatory cytokines by OX40 blockade under persistent antigen presentation suggests that OX40 may also have a direct effect on differentiation (187). What is the phenotype of CD4 T cells directly stimulated with anti-OX40 under persistent antigen presentation? Does OX40 promote survival of differentiating CD4 T cells, or does OX40 induce differentiation in CD4 T cells responding to antigen? Does OX40 directly induce accumulation of effector cells, or do other costimulatory or cytokine signals support or enhance signals through OX40? Based on these questions, I hypothesized that OX40 ligation could directly promote the differentiation of proliferating donor cells in the context of persistent antigen presentation.

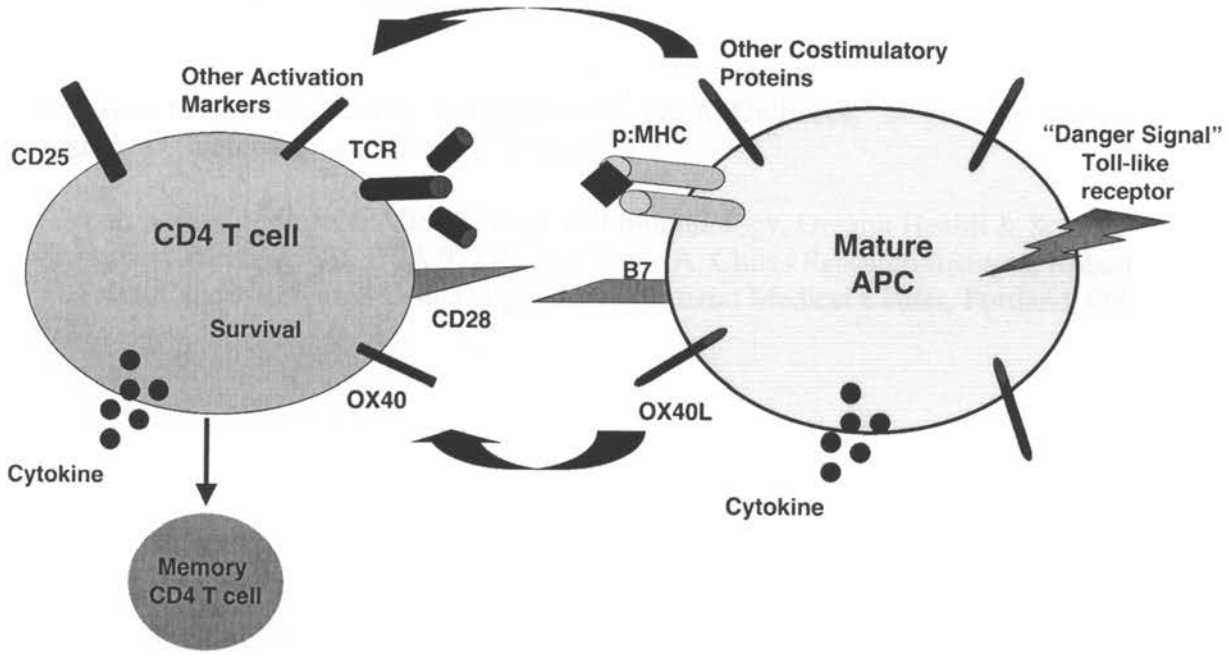

FIGURE 1-1. CD4 T cell activation, effector cell, and memory cell development. APC present antigenic peptide on MHC class II proteins that are recognized by the TCR on naïve CD4 T cells. At the same time, constitutively expressed B7:CD28 interactions provide a costimulatory signal to the T cell that amplifies signals from the TCR and promotes expression of activation markers and initiates gene transcription for cytokine and cytokine receptors. Mature APC that have received a signal from toll-like receptors, CD40 signaling, and similar signals, activate NFκB, which enhances peptide:MHC complex expression and gene expression of cytokines and other costimulatory ligands. The activated CD4 T cell then receives signals from receptor-ligand interactions such as OX40:OX40L to promote CD4 T cell survival, effector cytokine production, and to enhance memory CD4 T cells populations.

Chapter 2—Manuscript #1

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

Stephanie K. Lathrop,[*] Cortny A. Huddleston,[*] Per A. Dullforce,[*] Megan J. Montfort,[*] Andrew D. Weinberg,[†] and David C. Parker [2*]

*Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR, USA 97239, and [†]Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center, Portland, OR 97213

Abstract

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 antigen *in vitro*. Provision of a costimulatory signal by co-injection of an agonist antibody to OX40 (CD134), a TNF receptor 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 antigen *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 antigen 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*.

Introduction

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, where they are rendered harmless when they encounter antigens on resting APC in the absence of infection or adjuvants. Mechanisms of peripheral tolerance are complex and involve various forms of deletion, inactivation, and suppression (208, 209). A number of investigators have studied the mechanisms of peripheral tolerance to self antigens by injecting naïve TCR-transgenic T cells into otherwise syngeneic animals that express the antigen recognized by the T cells (49, 210-217). 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"(48). 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 (218). The innate immune response to infection or adjuvant tips the balance from tolerance to immunity by activating the APC, which then provide the additional cytokines and membrane costimulatory molecules that the T cells need to differentiate and survive as effector cells and memory cells (209, 219).

One of the costimulatory molecules that can determine the decision between immunity and tolerance is OX40 (CD134), a costimulatory TNF receptor family member expressed by activated CD4 T cells (220, 221). The ligand for OX40 (OX40L) is a membrane-bound member of the TNF family expressed on activated APC (222). A costimulatory signal through OX40 to activated CD4 T cells enhances T cell survival and

memory cell formation (223-226), reverses CD4 T cell tolerance to peptide antigen (227), and promotes tumor rejection (228) and graft versus host disease (229). Although OX40 is strongly implicated in autoimmune disease (221, 230), 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 antibody to OX40 in a new model of peripheral CD4 T cell tolerance to ubiquitous self antigen.

The neo-self antigen 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 naïve TCR-transgenic T cells that recognize this peptide/MHC complex after intravenous transfer into the Ag-transgenic mice. For reasons which 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 non-lymphoid tissues of unirradiated, non-lymphopenic, 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 in order to recover large numbers of donor T cells, e.g. (213, 216). 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 antibody to OX40 along with the transgenic T cells, the T cells differentiate into large, granular, IL-2R (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 antigen 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 & Science University animal facility. C57BL/6J mice expressing an MHC class II I-E^k molecule with an antigenic peptide covalently attached to the Eβ chain by a flexible linker were made by coinjection of plasmids encoding Eα^k and Eβ^k/peptide driven by a class II promoter (231) as previously described (232) in the Transgenic Animal Core Facility of the University of Massachusetts Medical Center (Worcester, MA). The antigenic peptide is from pigeon cytochrome C (PCC) with a serine to threonine substitution at position 102 (PCC102S): ANERADLIAYLKQASAK. The founder was identified by Southern blot, and the progeny were maintained as heterozygotes and typed by PCR, using forward primer 5'-GGTTGTTGTGCTGTCTCATC-3' and reverse primer 5'-AGGGCTTCTGGAGAGTAC-3'. CD40-deficient mice (233) 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 peptide (MCC) on I-E^k (234) were obtained from Steve Hedrick (U of California at San Diego, La Jolla, CA), and bred repeatedly to C57BL/6 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 (235). AD10 TCR-transgenic mice (235), 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-Vα11 (RR8-1), PE anti-Vβ3 (KJ25), biotin anti-CD25 (7D4), CD44 (IM7), and CD62L (MEL-14), FITC anti-I-E^k (17-3-3), APC anti-IL-2 (JES6-5H4), FITC anti-TNFα (MP6-XT22), FITC anti-IL-10 (JES5-16E3), APC anti-IL-4 (11B11), PE anti-CTLA-4 (4F10-11) and some isotype controls were purchased from BD PharMingen (San Diego, CA). APC anti-IFN-γ and some isotype controls were purchased from eBiosciences (San Diego, CA). Anti-OX40 antibody from clone OX86 (European Cell Culture Collection, Porton Down, UK) was produced and purified for us by UniSyn (Hopkington, MA). Rat IgG was purchased from Cappel, ICN Pharmaceuticals (Costa Mesa, CA). Purified antibodies to CD28 (PV-1, used at 100 μg/mouse), CD40 (FGK45, 200 μg/mouse), and 4-1BB (17B5, 100 μg/mouse) were kindly provided by Dr. Stephen 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 HEPES-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 (Vα11, Vβ3, and CD4 positive) in the suspension. In some experiments, the cells were labeled for 10 minutes at 37° with 2 μM 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_4Cl medium. Liver cell suspensions were made from one liver lobe cut into pieces and pressed through a sieve, followed by incubation for 40 minutes at 37° with mixing in serum-free RPMI culture medium with 0.002% DNAse I (Sigma, St. Louis, MO) and 0.02% collagenase D (Boehringer-Mannheim, from Roche, Indianapolis, IN). Liver lymphocytes were enriched by sedimentation of hepatocytes at 30 X g for 3 minutes, followed by isolation on Lympholyte-M. A total of 1-2 x 10^6 cells were incubated on ice in 120 ml PBS with 1% serum and 0.01% sodium azide with the indicated antibodies or strepavidin at saturating concentrations, before analysis on a FACSCalibur flow cytometer using CellQuest acquisition/analysis software (BD Immunocytometry, San Jose, CA). Cells were stained for surface antigens and then stained for intracellular CTLA-4 or cytokines using the Cytofix/Cytoperm kit from BD PharMingen (San Jose, CA).

Cell culture

For intracellular cytokine staining, spleen cell suspensions were stimulated in RPMI 1640 culture medium containing 10% FBS and 10mM monensin for 5 hours with 20ng/ml PMA (Sigma) plus 500ng/ml ionomycin (Sigma) or equal numbers of irradiated B10.BR spleen cells as APCs with or without 1μM MCC peptide. Alternatively, the cells were cultured in the same medium with 10ng/ml IL-12 (Cell Sciences, Inc., Norwood, MA) and100ng/ml IL-18 (R & D Systems, Minneapolis, MN) for five hours, with monensin added for the last hour of culture. For the proliferation assays, spleen cell

suspensions were enriched for $CD4^+$ cells by negative selection with the SpinSep murine $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, Canada). 2.5×10^4 purified CD4 T cells were cultured with 10^5 irradiated B10.BR spleen cells and varying amounts of MCC peptide in RPMI 1640 with 10% FBS, with or without 10U/ml IL-2 (as a culture supernatant of a murine IL-2 cDNA transfected plasmacytoma line (236)) for approximately 72 hours. 1 μCi [^{3}H]-thymidine (6.7 Ci/mMol, Perkin Elmer Life Sciences, Boston, MA) was added per well for the last 12-18 hours of culture. Cells were harvested onto glass fiber filters and counted on a Topcount scintillation counter (Packard Instrument Company, Meriden, CT). T cells blasts were made from AD10 TCR-transgenic mouse splenocytes prepared by cell dissociation and red blood cell lysis, and cultured at an initial cell density of approximately 4×10^6 cells/ml with 2.5 μM MCC peptide for 5-7 days. Live cells were isolated with Lympholyte-M before use.

Results

Ag-transgenic and TCR-transgenic mice

For these experiments, we made Ag-transgenic mice that express an MHC class II I-E^k/peptide complex from a class II promoter (see Materials and Methods). The antigenic peptide is covalently attached to the transgenic $E\beta^k$ chain by a flexible linker. I-E^k could be detected at very low levels on B cells and CD11c positive dendritic cells from the Ag-transgenic mice by flow cytometry (Fig. 1A), and is likely to be expressed on other class II positive cells as well using this promoter (231, 232). Spleen cells from the Ag-transgenic mice stimulate vigorous proliferation of naïve AND TCR-transgenic T cells *in vitro* (data not shown). Although the AND TCR-transgenic T cells are specific for PCC peptide on I-E^k (234), they can be efficiently selected in the thymus on I-A^b in C57BL/6 mice (235). They were bred to C57BL/6 RAG-1 deficient mice, so only transgenic TCRs are expressed on the T cells used in these experiments.

Adequate costimulation in vivo for T cell accumulation even in the absence of CD40

When spleen cells containing 5-6 x 10^6 TCR-transgenic T cells are transferred intravenously into unirradiated, Ag-transgenic recipients, they expand rapidly to maximal numbers, constituting 15 to 25% of total spleen cells by day 3, and then slowly disappear (Fig. 1B and data not shown). Similar peak numbers are reached by day 5 when only 5 x 10^5 TCR-transgenic T cells are injected (data not shown). Very few donor T cells were recovered from non-transgenic recipients (<0.3% of total spleen). The rapid proliferation and accumulation of donor T cells in Ag-transgenic animals, in the absence of activation of APC by adjuvant or infection, might be expected to depend on the ability of the T cells

to activate APC directly through the interaction of CD40L, expressed on activated CD4 T cells, with CD40 on dendritic cells (237). However, we found that CD40 expression in recipient animals was not necessary for T cell expansion, because similar numbers of donor T cells were recovered at days 3 and 7 from Ag-transgenic recipients that lacked CD40 expression (Fig. 1B). Therefore, even in the absence of CD40L/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

In spite of the large load of proliferating, autoreactive TCR-transgenic T cells, Ag-transgenic recipient mice remained active and apparently healthy (observed up to 6 weeks). Because alternative costimulation through OX40 on activated CD4 T cells can prevent tolerance to superantigen or peptide Ag *in vivo* (223, 225, 227), we examined the effect of an agonist antibody to OX40 in this model of peripheral CD4 T cell tolerance to ubiquitous self antigen. Spleen cell suspensions containing 0.5-5 x 10^6 TCR-transgenic T cells were transferred intravenously into unirradiated, Ag-transgenic recipients together with 50 μg of agonist anti-OX40 antibody or control rat IgG. Mice receiving rat IgG remained healthy, while all Ag-transgenic mice receiving anti-OX40 became less active and hunched or moribund in 6 to 8 days, and were euthanized or dead within twelve days (8 of 8 mice). Spleens from both groups were enlarged (2 to 4 times normal size) but those from anti-OX40 treated mice were delicate and pale, while those from rat IgG controls retained normal structure and color. 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. Non-transgenic recipients remained healthy following injection of TCR-transgenic T cells and anti-OX40, demonstrating a requirement for antigen in this system.

To examine the phenotype of the transferred T cells, spleen cell suspensions of recipient mice were analyzed by flow cytometry five 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, CD62L was measured. Large numbers of donor TCR-transgenic T cells with memory/effector cell markers ($CD44^{hi}$, $CD62L^{lo}$) 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, while total spleen cell numbers for both groups were 2 to 2.5 times greater than that of a non-transgenic 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 49.7×10^6 (S.E. = 7.7×10^6, n = 18) with anti-OX40, and 15.3×10^6 (S.E. = 2.5×10^6, n = 18) with rat IgG. This observation 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-2Rα chain, CD25,

which declines to near background levels by day five 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 downmodulated 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 to naïve 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 $CD62L^{hi}$ and $CD62L^{lo}$ populations (Figure 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 naïve, 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 antigen. 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 signaling pathways leading to cytokine transcription, the majority of the T cells made large amounts of IFN-γ, showing that the IFN-γ gene is accessible and the transcriptional machinery for IFN-γ production is intact. In contrast, T cells from the rat IgG control animals did not make IFN-γ spontaneously, and many fewer cells produced IFN-γ in response to PMA and ionomycin. In both groups, PMA and ionomycin stimulation induced only small increases in TNFα production and induced IL-2 synthesis in a small proportion of cells, which was consistently slightly lower in T cells from the anti-OX40-treated animals. We were unable to detect IL-10 or IL-4 by intracellular cytokine staining in donor T cells under any conditions (data not shown).

Donor T cells accumulate in non-lymphoid 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 $CD62L^{hi}$ 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 non-lymphoid 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 antigen, 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 antigen (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 antigen (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 antigen *in vitro*, regardless of anti-OX40 treatment, in 6 of 7 similar experiments(Fig. 8 and data not shown). Since IL-12 and IL-18 can induce IFN-γ secretion from effector T cells independent of TCR signals (238), 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 antigen 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 antibodies shown to block peripheral tolerance, only anti-4-1BB has effects similar to anti-OX40

Several other agonistic antibodies have been reported to provide or induce costimulatory signals and block peripheral tolerance in T cells, including anti-CD28 (239), anti-CD40 (215, 237, 240), and anti-4-1BB (CD137) (241). 4-1BB is a costimulatory TNF receptor 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 (241, 242). Although anti-4-1BB has complex effects on antibody formation and CD4-dependent autoimmunity *in vivo* (243), it blocks tolerance to peptide antigen in young mice and restores T cell priming in aged mice (241). We injected these other antibodies 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 OX40L (222). 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 antigens on healthy tissues in the absence of adjuvant or infection are rendered tolerant. Induction of peripheral tolerance in naïve T cells *in vivo* nearly always involves an abortive immune response with an initial period of T cell proliferation followed by T cell death (211, 213, 218, 244, 245). In tolerance, fewer Ag-specific T cells are recovered than in an immune response, and the recovered cells are functionally compromised compared to primed cells, even when assayed in isolation in the absence of possible regulatory cells (212, 213, 246, 247). 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 antigen because of compromised signaling through the TCR (48). 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 described above. 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 antigen receptor, because cells recovered from anti-OX40-treated animals are anergic and fail to respond to antigen on APC (Figs. 7 and 8). Instead, like non-tolerant armed effector Th1 cells, these cells respond vigorously to cytokines, proliferating in response

to IL-2 without antigen, 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 four to eighteen-fold in donor T cells from anti-OX40-treated animals versus 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 antigen 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, antigen-transgenic hosts, even in the presence of anti-OX40 antibody (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 (248). It is possible that similar, non-TCR-mediated, positive cytokine feedback loops could contribute to immunopathology in other kinds of infections and in graft-versus-host disease where 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 (249, 250). 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" since they are unresponsive to antigen 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 INF-γ synthesis (251) are open and active. Other investigators have documented loss of effector functions in T cells rendered tolerant in vivo (210, 211, 214, 217, 252), but only a few experiments have distinguished such loss from compromised signaling through the TCR. Zajac et al. (252) 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 (253). Adler and colleagues showed recently that fully differentiated, primed effector CD4 T cells are rapidly tolerized by exposure to soluble peptide or parenchymal self antigen in vivo, with early

and selective loss of INF-γ and TNF-α production in response to restimulation with antigen in vitro (49, 254).

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 (220, 255), and then shown to have potent costimulatory activity for activated CD4 T cells *in vitro* (256). Although the OX40/OX40L interaction has been implicated in T cell/B cell collaboration (257), mice deficient in OX40 or OX40L have normal or near normal antibody responses, with defects in T cell proliferation and cellular immunity (222, 258-260). Agonist anti-OX40 antibody has striking adjuvant effects in enhancing memory cell formation and blocking acquired tolerance to superantigens and peptide antigens in CD4 cells (223-227). Rogers et al. (226) have shown that OX40 upregulates expression of the anti-apoptotic 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. On the other hand, Bansal-Pakala showed that agonist anti-OX40 can reverse established CD4 T cell tolerance in vivo and T cell clonal anergy in vitro (227), 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 three-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 antigen is transient, as in the peptide and superantigen systems described above (223-227), and may enhance T cell differentiation to effector function when antigen is continuous, as in the models of graft versus host disease (229), autoimmunity (230), cancer (228), and this report.

T cell survival in some circumstances and T cell differentiation to effector cells in other circumstances could be independent consequences of the same OX40 signaling pathway. For instance, OX40 activates NFκB (261, 262), which is known to promote both cell survival and cytokine synthesis (263-265). Similar signals promoting survival and differentiation of activated T cells have been reported to be delivered by other TNFR family members, which also activate NFκB, including CD27 (127, 266) and 4-1BB (CD137) (241, 242, 261, and this report). Alternatively, both survival and differentiation could be secondary to upregulation of cytokine receptors via the OX40 signal. Engagement of OX40 upregulates expression of the IL-2 receptor (225 and this report) and of mRNA for the receptors for IL-15, IL-12, and IL-7 (data not shown), all of which have been reported to enable survival and/or drive differentiation of T cells (238, 267-271). In our model in which the OX40-driven effector CD4 T cells kill the animals, these cytokine receptors are likely to be essential to a positive feedback loop with APC in the pathogenic process, as described above. We are currently testing this hypothesis and addressing the mechanism by which the OX40 signal allows accumulation of armed

effector T cells using donor T cells with transgenes or mutations affecting T cell survival, cytokine receptors, and signaling pathways.

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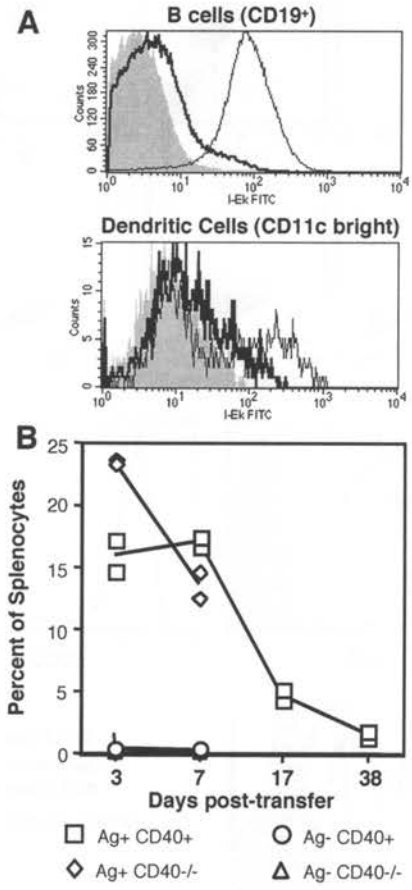

FIGURE 2-1. Antigen-specific expansion of CD4 T cells in Ag-transgenic mice is CD40-independent. (A) The thick lines show the expression levels of transgenic I-E^k on $CD19^+$ B cells and $CD11c^+$ dendritic cells of the Ag-transgenic mice. Also shown are background staining of non-transgenic mice (shaded histograms) and normal level staining of endogenous I-E^k 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. 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 $V\alpha11^+/V\beta3^+/CD4^+$ on the indicated days after transfer are shown.

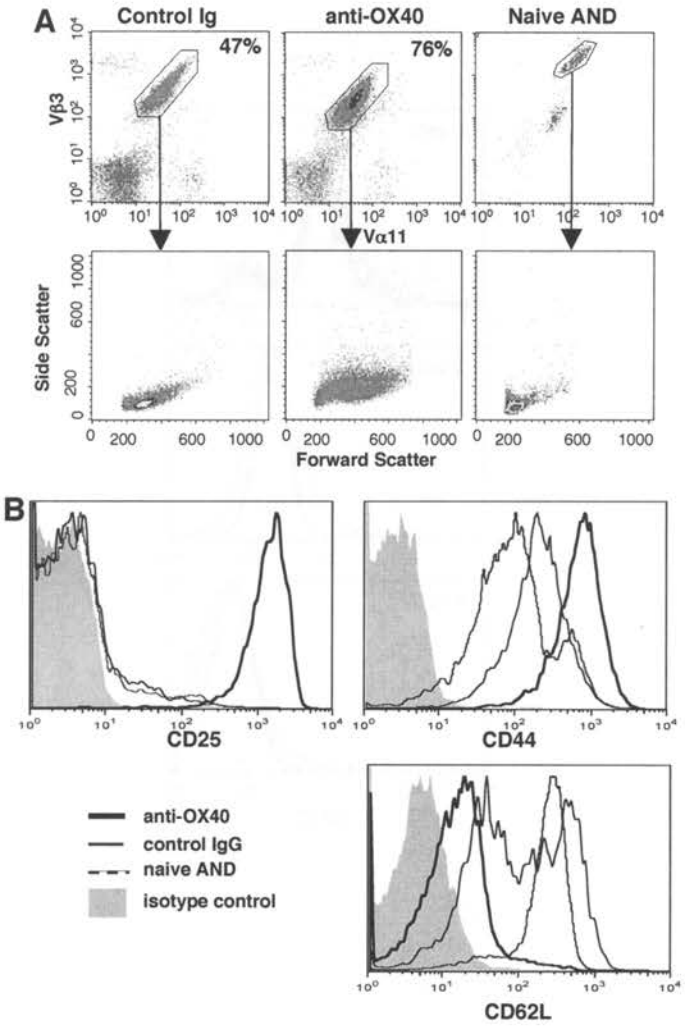

FIGURE 2-2. Transferred T cells from anti-OX40-treated mice show an activated phenotype. TCR-transgenic T cells (5 x 10^5) were transferred into Ag-transgenic recipients with either anti-OX40 Ab or control rat IgG, then recovered from the spleen on day 5 and analyzed by flow cytometry for size, granularity, and expression of activation markers. (A) Splenocytes were gated on $CD4^+$ and donor T cells were detected by staining for Vα11 and Vβ3, the transgenic TCR chains. The percentage of $CD4^+$ cells that were $V\alpha11^+/V\beta3^+$ is indicated, and the forward and side scatter profile of each $V\alpha11^+/V\beta3^+$ population is shown. For comparison, $CD4^+$ splenocytes from naive donor TCR-transgenic $RAG^{-/-}$ mice are also shown. (B) The histograms show staining for CD25, CD44, or CD62L of splenocytes gated on $CD4^+/V\alpha11^+/V\beta3^+$ as in (A). The figure shows one representative mouse of three per group, from one experiment of eight.

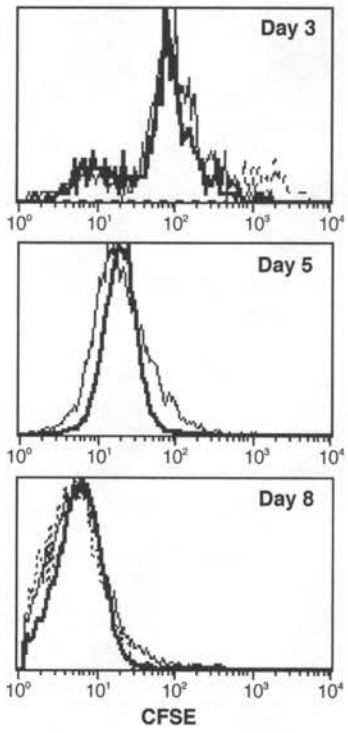

FIGURE 2-3. Anti-OX40 does not affect the proliferation rate of TCR-transgenic T cells transferred into Ag-transgenic hosts. T cells were CFSE labeled before transfer with anti-OX40 Ab (bold lines) or control rat IgG (thin lines), and the dilution of CFSE examined in $CD4^+$, $V\alpha11^+$ and $V\beta3^+$ splenocytes after 3, 5 or 8 days. The dashed line on day 3 shows CFSE-labeled donor $CD4^+$ cells that have not divided; on day 8 it shows the background level of fluorescence of unlabeled host T cells.

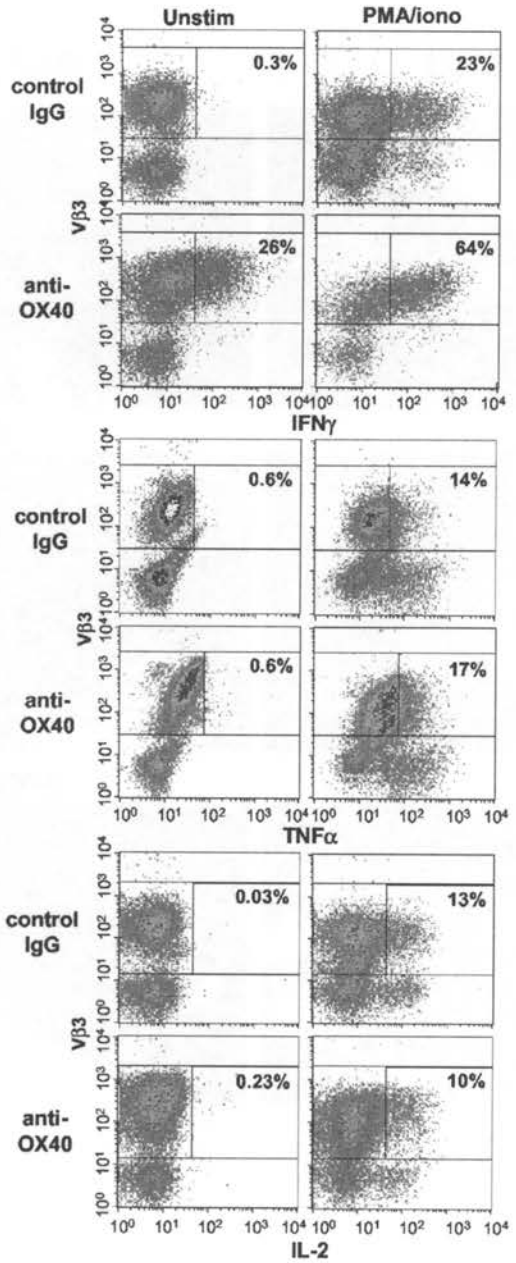

FIGURE 2-4. Donor T cells from Ag-transgenic hosts treated with anti-OX40 secrete IFN-γ directly *ex vivo* and in response to activation with PMA and ionomycin. Splenocytes recovered from host animals on day 5 were cultured for 5 hours in the presence of monensin, with or without PMA and ionomycin, and then stained for CD4, Vβ3, and intracellular IFN-γ, TNFα, or IL-2. The percentage of $CD4^+/Vb3^+$ cells expressing the cytokine is indicated on the plots, which are gated on $CD4^+$ cells. the figure shows one representative mouse of three per group, from one experiment of five.

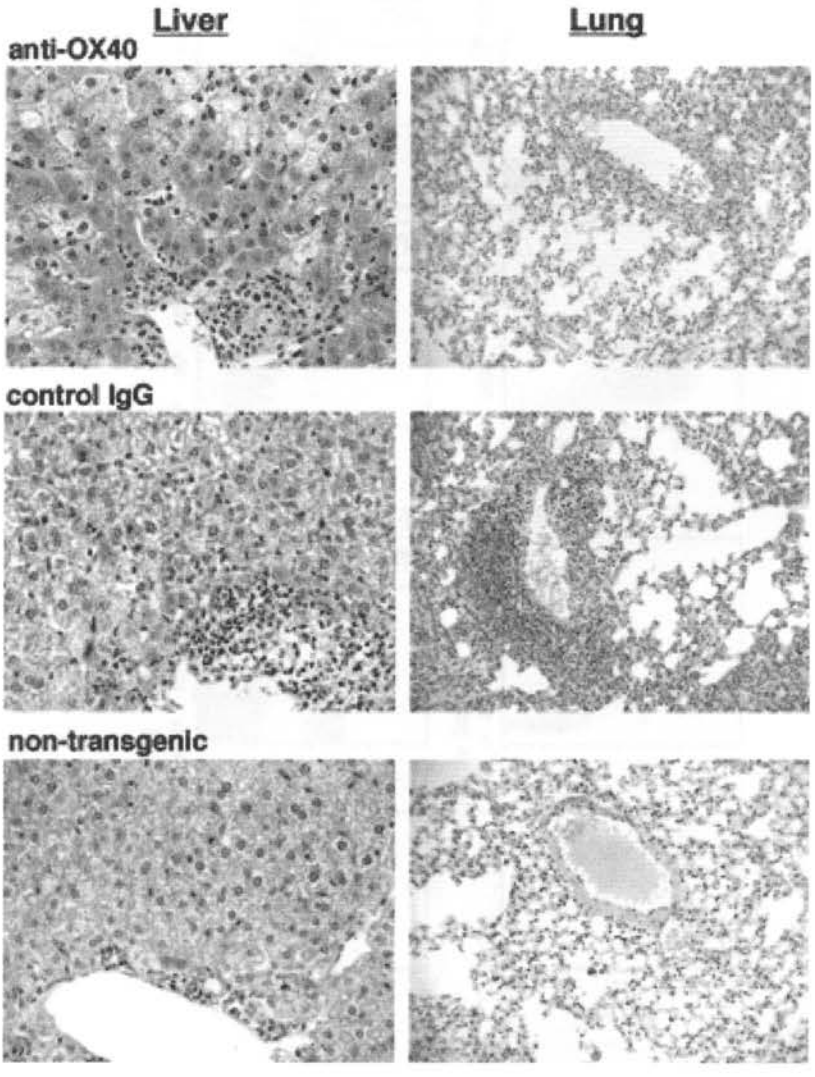

FIGURE 2-5. Treatment with anti-OX40 results in mononuclear cell infiltrate and hepatocyte damage, while control livers show infiltrate without damage. Infiltration of similar numbers of lymphocytes into the lungs is apparent in both anti-OX40 and rat IgG treated mice. The figure shows H&E staining of recipient livers (400X) and lungs (200X) on day 8 after transfer of 5 x 10^5 TCR transgenic T cells into Ag-transgenic recipients, with and without anti-OX40, or non-transgenic recipient with anti-OX40.

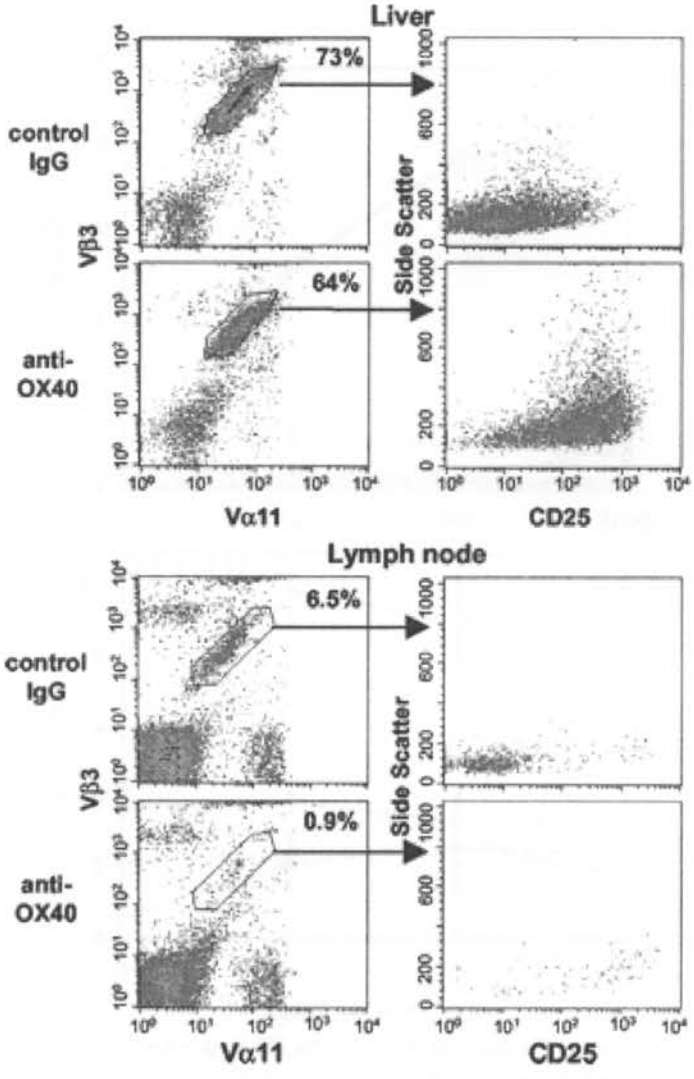

FIGURE 2-6. Transgenic T cells are found at a lower frequency in the lymph node in anti-OX40-treated hosts than in control animals, and retain their large, $CD25^+$ phenotype in both livers and lymph nodes. The percentage of $CD4^+$ liver or lymph node lymphocytes that are $V\alpha11^+/V\beta3^+$ is shown. The CD25 expression and side scatter of this donor T cell population is shown in the plots to the right. The figure shows one representative mouse of three per group, from one experiment of two (livers) or four (lymph nodes).

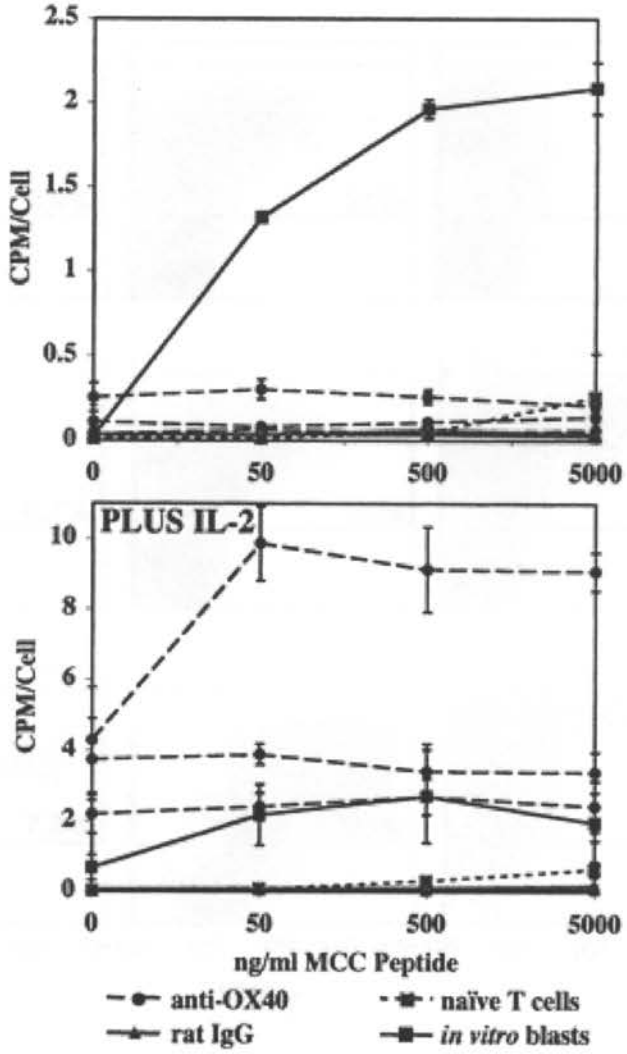

FIGURE 2-7. Purified $CD4^+$ donor T cells recovered from Ag-transgenic hosts on day 5 do not proliferate in response to peptide antigen, even with addition of exogenous IL-2 (lower panel). Those from anti-OX40-treated mice proliferate upon addition of IL-2, with or without addition of peptide antigen. Thymidine incorporation assay results from one experiment of four, with three mice per group, is shown. At the end of the culture period, counts per minute in each well were divided by the number of TCR-transgenic T cells in that well, as calculated by flow cytometry analysis.

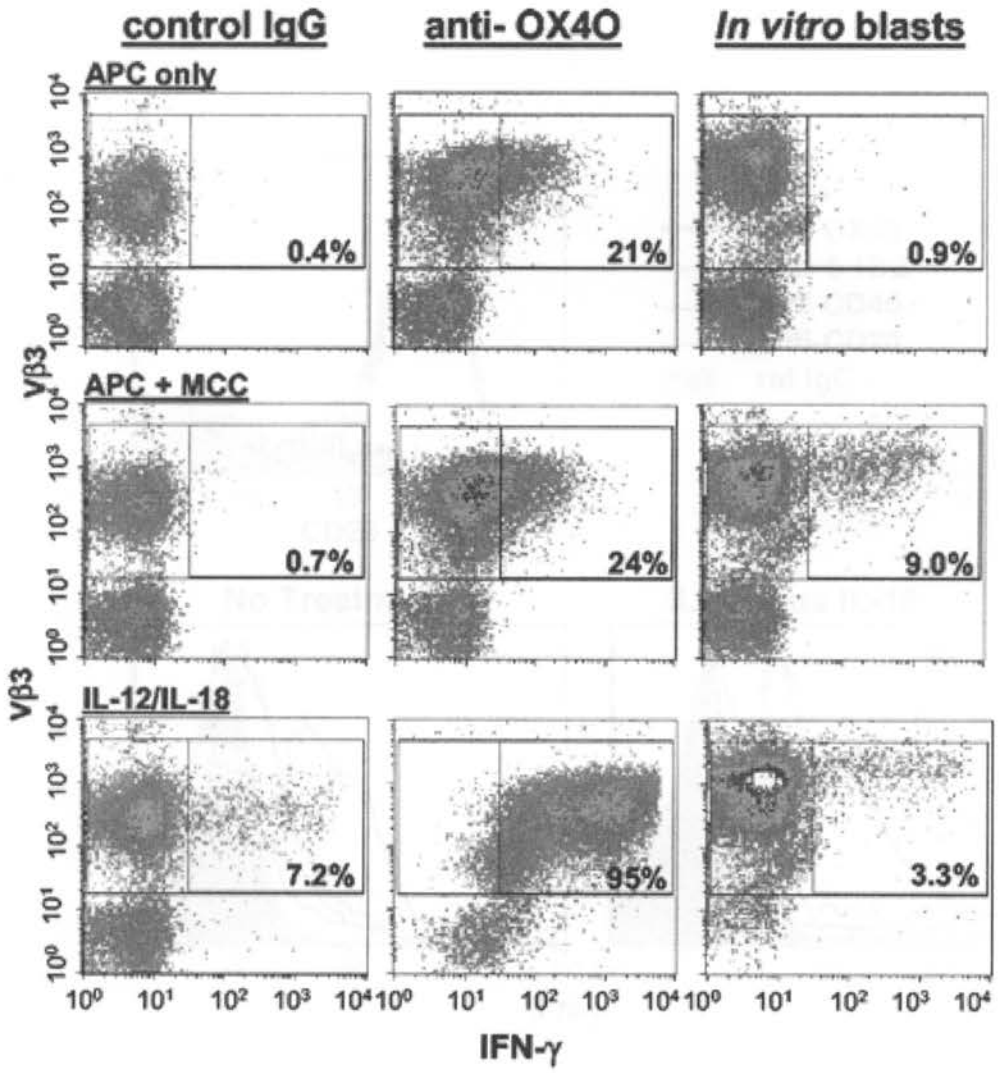

FIGURE 2-8. Donor T cells from anti-OX40-treated mice secrete IFN-γ in response to IL-12 plus IL-18 treatment, but not in response to 1 μM peptide antigen. Splenocytes recovered on day 5, or *in vitro* activated T cell blasts as a positive control, were stained for CD4, Vβ3, and intracellular IFN-γ. Plots shown are gated on $CD4^+$ and the percentage of $CD4^+/V\beta3^+$ cells that are positive for IFN-γ staining is shown. The figure shows one representative mouse of three per group, from one experiment of three.

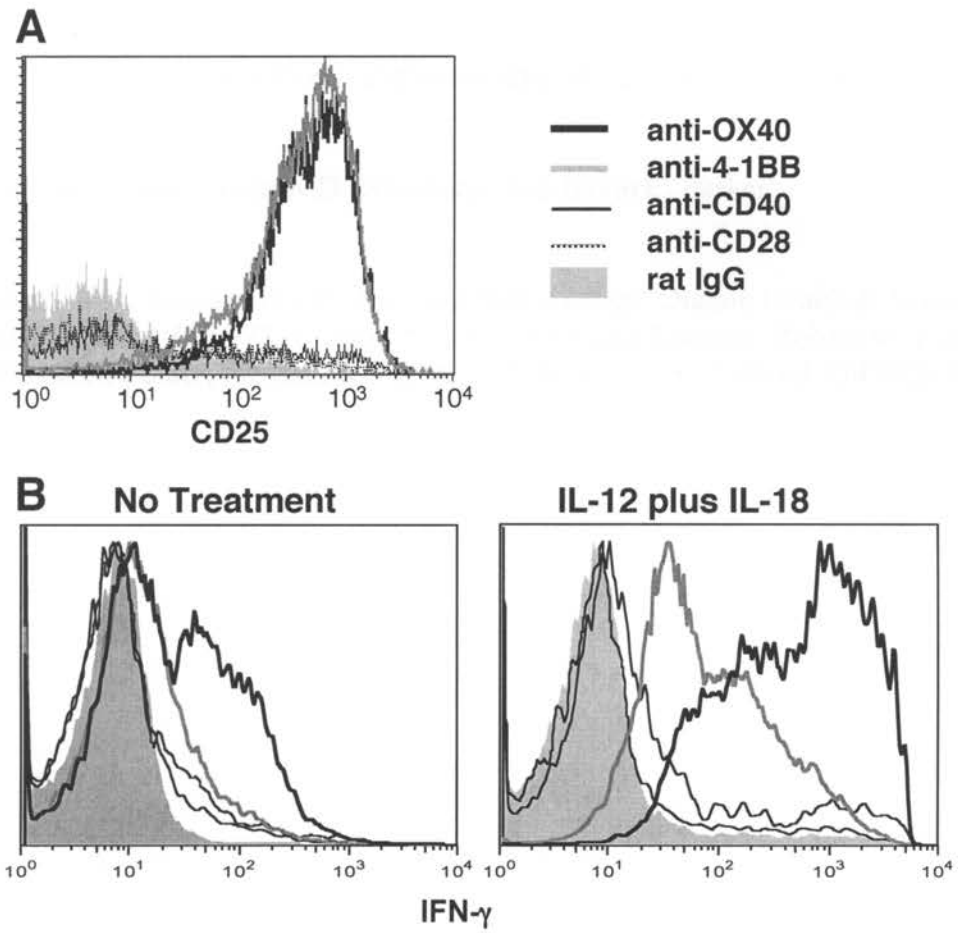

FIGURE 2-9. Treatment with anti-4-1BB Ab produces an effect similar to treatment with anti-OX40, while anti-CD28 has no effect, and anti-CD40 produces only a slight increase in IFN-γ production. (A) Expression of CD25 on $CD4^+/V\alpha11^+/V\beta3^+$ cells at 5 days after transfer of transgenic T cells into an Ag-transgenic host with the indicated antibody. (B) Production of IFN-γ by $CD4^+/V\alpha11^+/V\beta3^+$ cells during 5 hours in culture with either IL-12 and IL-18 or media alone. The figure shows one representative mouse of three per group, from one experiment of two.

Chapter 3—Manuscript #2

OX40 (CD134) Engagement Drives Differentiation of $CD4^+$ T Cells to Effector Cells

Cortny A. Huddleston[1], Andrew D. Weinberg[2], and David C. Parker[1]

[1]Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR 97239 [2]Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center, Portland, OR 97213

Summary

Naïve, $CD4^+$ T cells proliferate extensively but fail to differentiate when they are transferred into unirradiated recipients that express alloantigen or transgenic antigen on all MHC class II^+ cells. Addition of an agonist antibody to OX40 (CD134), a costimulatory TNF receptor family member expressed on activated $CD4^+$ T cells, enables the proliferating T cells to accumulate as differentiated effector cells and kill the host animals. The donor T cells from anti-OX40 treated animals express high levels of IL-2Rα (CD25) and acquire the ability secrete IFN-γ when stimulated with IL-12 and IL-18. OX40 promotes differentiation by 48 hours in T cell priming, before changes in Bcl-2 expression or cell recovery become apparent. We found that a Bcl-2 transgene or deficiency in Fas or TNFR1 failed to influence accumulation of differentiated donor cells, and found larger changes in expression of cytokine and cytokine receptor genes than in survival genes. Accumulation of differentiated $CD4^+$ effector T cells is initiated directly through OX40, but some OX40-deficient donor cells can gain effector function as bystanders to $OX40^{+/+}$ cells. Taken together, these data suggest that $CD4^+$ T cell differentiation to effector function is an important effect of OX40 engagement under conditions of ubiquitous antigen presentation.

Introduction

Optimal $CD4^+$ T cell activation requires recognition of cognate antigen together with costimulatory signals. However, costimulatory signals are not limiting for initial T cell proliferation *in vivo*, even with resting APC under conditions of tolerance induction (51, 56). *In vivo*, additional costimulatory signals from activated APC are necessary to promote $CD4^+$ T cell survival and acquisition of effector function. Without these additional costimulatory signals, proliferating $CD4^+$ T cells fail to accumulate, differentiate into effector T cells, or generate long-lived memory T cells, and the surviving tolerant T cells are hyporesponsive upon subsequent engagement of cognate antigen (48, 209). Several costimulatory receptors that belong to the tumor-necrosis factor receptor (TNFR) superfamily, including CD27, 4-1BB, and OX40, can enhance $CD4^+$ T cell responses upon antigen recognition *in vivo*, allowing responding T cells to survive as well as to acquire differentiated effector functions (15).

OX40 is a costimulatory receptor expressed predominately on $CD4^+$ T cells that appears on the cell surface 24-48 hours after antigen recognition (81), and engages OX40 ligand (OX40L) expressed on the surface of activated APC (94). Mice deficient in OX40 or OX40L have diminished antigen specific clonal expansion and memory T cell populations (90, 98, 110, 111, 115, 165). Blocking OX40/OX40L interactions *in vivo* decreases the severity of inflammatory diseases such as graft-versus-host disease (188), experimental autoimmune encephalomyelitis (105), and asthma (163, 165). In contrast, agonist OX40 antibody increases clonal expansion (115), promotes robust memory T cell populations (161, 272), and enhances anti-tumor immunity (195, 273, 274). In addition,

a signal through OX40 has been reported to reverse previously established peripheral tolerance (175).

Although OX40 costimulation can promote acquisition of effector function, the mechanism by which this occurs is not clear. Previous reports demonstrate that OX40 promotes T cell longevity, which can lead to a robust memory T cell population (80, 113-115, 161, 272). More specifically, OX40 signaling results in enhanced Akt activity and Bcl-2, Bcl-x_L, and survivin expression, which lead to survival of activated T cells and enlarges the memory T cell populations (80, 113, 114). OX40 engagement has also been shown to enhance secretion of IL-4 and IL-5 (165) and germinal center formation (89) in Th2 responses, and IL-2 and IFN-γ production in Th1 responses (1, 167). Here, we examine whether OX40 engagement enhances effector function by promoting survival of differentiating cells, or by driving T cell differentiation independent of survival.

To determine the role of OX40 signaling in acquisition of effector function, we used two similar models of ubiquitous antigen presentation. We had previously established that agonist anti-OX40 promoted accumulation of differentiated effector $CD4^+$ T cells when TCR transgenic T cells are transferred into unirradiated, antigen transgenic hosts (1). In this study, we examined the polyclonal responses of alloreactive C57BL/6 (B6) $CD4^+$ T cells, and showed that agonist anti-OX40 antibody can promote the accumulation of differentiated effector $CD4^+$ T cells in unirradiated recipients. To determine the role of OX40 costimulation in a polyclonal population of $CD4^+$ T cells, we used Bcl-2 transgenic or death receptor and OX40 deficient mice available on the common B6 background in the alloantigen model. We used the TCR transgenic model to investigate the effects of OX40 signaling in the initial phases of T cell priming. The data

herein suggest that OX40 directs differentiation to effector $CD4^+$ T cells under conditions of ubiquitous antigen presentation.

Materials and Methods

Mice and Adoptive Transfers

Mice were housed under specific pathogen-free conditions at the Oregon Health & Science University animal facility. (B6 x bm12) F1 mice were made by crossing female B6.Ly5.1 (C57Bl/6 congenic for CD45.1), obtained from the National Cancer Institute (Frederick, Maryland), to B6.C-H2^{bm12}/KhEg mice, obtained from the Jackson Laboratory (Bar Harbor, ME). C57Bl/6J, TNFR1$^{-/-}$ (C57BL/6-tnfrsf1a), B6^{lpr}, Thy1.1 congenic (C57BL/6J-Igh^{a}Thy1^{a}Gpi^{a}), and Bcl-2 transgenic (C57BL/6-TgN(Bcl2)36wehi) were obtained from the Jackson Laboratory. OX40$^{-/-}$ mice were kindly provided by Nigel Kileen (111). Pigeon cytochrome C (PCC) specific AND TCR transgenic, Rag-1 deficient mice, and antigen transgenic mice expressing I-E^{k} plus covalently associated PCC with the amino acid T102S substitution on the C57BL/6 background have been described previously (1). Donor splenocytes for each experiment were prepared for intravenous injection as previously described (1). In some experiments, donor cells were labeled with 2 μM CFSE in 0.1% BSA in PBS for 10 minutes at 37° and washed in Hanks Buffered Saline Solution (HBSS) with 2% serum. Cells were injected i.v. with 50 μg anti-OX40 or control IgG in HBSS without serum into unirradiated (B6 x bm12)F1 recipients or into antigen transgenic recipients.

Antibodies

PerCP anti-CD4 (RM4-5), biotin anti-CD25 (7D4), allophycocyanin (APC) anti-IL-2 (JES6-5H4), PE anti-Bcl-2 (3F11), and labeled isotype controls were purchased

from BD PharMingen (San Diego, CA). APC anti-IFN-γ (XMG1.2), FITC anti-CD90.1 (HIS51), PE anti-CD45.1 (A20), and appropriate isotype controls were purchased from eBiosciences (San Diego, CA). PE anti-Bcl-x_L (7B2.5) and labeled isotype control were purchased from Southern Biotechnology (Birmingham, AL). Anti-OX40 antibody from clone OX86 (European Cell Culture Collection, Porton Down, UK) was produced and purified for i.v. injection. Rat IgG was purchased from Cappel, ICN Pharmaceuticals (Costa Mesa, CA). Recombinant mouse IL-12 was purchased from Cell Sciences (Norwood, MA), and recombinant mouse IL-18 was purchased from R & D Systems (Minneapolis, MN).

Cell Culture and Flow Cytometry

Spleen cell suspensions were prepared for intracellular cytokine staining as previously described (1). Labeled cells were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry, San Jose, CA) and analyzed using FlowJo (Tree Star, Inc., San Jose, CA).

Microarrays

$5x10^6$ (first experiment) or $3.5x10^6$ (second experiment) AND $Rag1^{-/-}$ TCR transgenic T cells were transferred into antigen transgenic recipients with 50 μg of anti-OX40 or control IgG, as previously described (1). The TCR transgenic T cells were enriched from antigen transgenic spleen cell suspensions on day 3.5 by labeling cells with anti-Vb3 biotinylated antibodies (BD PharMingen) followed by anti-biotin magnetic beads (Miltenyi Biotec, Auburn, CA) and separation with an autoMACS magnetic

column (Miltenyi Biotec). We found the anti-OX40-treated T cells difficult to isolate, perhaps because they are very large and granular and tend to aggregate. Thus, we obtained 90% $V\beta3^+$ cells in the enriched population from control IgG-treated animals, but only 75% $V\beta3^+$ cells from anti-OX40-treated animals in two independent experiments. RNA was prepared from the enriched cells using the RNAeasy kit from Qiagen (Valencia, CA). Isolated total RNA was checked for quality and used at 1 μg/μL to prepare labeled cDNA, which was hybridized to the murine genome MG-U74Av2 array (12,488 genes) according to manufacture's guidelines (Affymetrix, Santa Clara, CA) at the OHSU Gene Microarray Shared Resource. Data generated from the duplicate gene chip experiments were analyzed using Affymetrix Microarray Suite 5.0 (MAS 5.0) software, and the gene documentation and probe match software are available on the Affymetrix website, http://www.affymetrix.com/. Briefly, we excluded all genes with absent detection calls on all four chips and genes that showed no change in both comparisons. Of the remaining 3,653 genes, we selected genes that increased or decreased at least 2.9 fold (mean fold change derived from signal $\log_2$ ratio) and had p-values for the present detection call less than 0.1 in anti-OX40 increased or control control IgG decreased in either experiment. We chose 2.9 fold as the limit of detection because that is the change in IFN-γ expression on the gene chip, of which we regularly measure its protein expression level. The genes that increased or decreased according to these criteria are listed in Supplemental Table 1 (96 genes) and Supplemental Table 2 (88 genes). Some genes reported to affect T cell survival or differentiation that did not meet these criteria for robust change, but did not have an absent detection call on all four chips, are listed in Supplemental Table 3.

Results

OX40 engagement drives CD4$^+$T cells to accumulate and differentiate to effector cells in unirradiated allogeneic recipients.

To examine the role of OX40 costimulation, we employed a murine model of GVH, in which we transferred B6 splenocytes into unirradiated, MHC class II disparate (B6 x bm12)F1 recipients. A population of donor B6 CD4$^+$ T cells mount an alloreactive immune response to the MHC class II I-A^{bm12} molecule, which differs from I-A^b due to mutations in the peptide-binding domain (275, 276). In the majority of GVH models, the recipient must be irradiated prior to transfer of donor T cells in order to promote effector cell development of donor B6 CD4$^+$ T cells (57). In this report, we examined the effects of OX40 costimulation in unirradiated recipients by injecting an agonist antibody against OX40 or control IgG at the time of donor CD4$^+$ cell transfer. In Figure 1, 4.5x10^6 B6 CD4$^+$ splenocytes were transferred with control IgG into unirradiated F1 recipients in the absence of exogenous costimulation or adjuvant. Five days later, the donor CD4$^+$ T cells had proliferated extensively, as measured by CFSE dilution (Fig. 1A, Control IgG), and accumulated (Table I, Control IgG, B6 group), while CD4$^+$ T cells did not proliferate in control syngeneic recipients (data not shown). Consistent with the rapid expansion, on day five control IgG treated donor CD4$^+$ T cells expressed uniformly increased CD44 levels and heterogenous levels of CD62L, but did not express the activation marker IL-2Ra (CD25), while undivided CD4$^+$ donor cells remained CD25$^-$, CD44^{low}, and CD62L^{high} (Fig. 1A, Control IgG). The alloreactive CD4$^+$ cells developed poor effector T cell function measured as IFN-g production when stimulated with PMA and ionomycin or recombinant IL-12 plus IL-18, a cytokine combination that has been shown to

stimulate IFN-g production from effector T cells independent of the TCR (277) (Fig. 1B, Control IgG).

With agonist anti-OX40 treatment, the donor $CD4^+$ cells proliferated and accumulated to larger numbers compared to the control IgG treated donor T cells by day 5 (Table I, anti-OX40, B6 group). These alloreactive $CD4^+$ T cells were larger and more granular than control cells (data not shown), and approximately 90% of divided cells had very high levels of CD25 expression (Fig. 1A, OX40). Upon stimulation in vitro with PMA and ionomycin, approximately 40% of the divided donor $CD4^+$ cells produced IFN-g, and stimulation with recombinant IL-12 and IL-18 drove more than 70% of the donor cells to produce IFN-g (Fig. 1B, OX40). These data show that costimulation in the form of anti-OX40 allows accumulation of donor $CD4^+$ T cells which have gained effector function in an unirradiated recipient.

Five days after donor cell transfer, the mice appeared outwardly healthy with anti-OX40 treatment even though the alloreactive $CD4^+$ T cells had acquired robust effector T cell function (Fig. 1A and B, OX40). By day eleven, fewer donor $CD4^+$ T cells were recovered (Table I, anti-OX40, B6 group), and the percent of cells expressing CD25 had decreased from 89% to 40%, and IFN-g producing cells following stimulation with IL-12 and IL-18 also decreased from 76% to 42% (Fig. 1C, OX40). These experiments showed that although anti-OX40 treatment resulted in accumulation of effector T cells in an otherwise tolerizing environment, the effect was transient and did not lead to overt disease.

Next, we wished to test the hypothesis that transfer of more donor cells would result in greater accumulation of effector cells and cause disease, as we reported earlier in

a similar, TCR transgenic model (1). When we transferred 10^7 $CD4^+$ T cells, donor cell recovery on day five doubled (Table 1, anti-OX40, B6 group), acquisition of effector function was similar to cells recovered at 5 days (data not shown), and the mice appeared outwardly healthy. However, at day seven, six of six mice became moribund and were euthanized. Fewer donor $CD4^+$ T cells were recovered from day 7 spleens (Table I, anti-OX40 day 7), but these cells still expressed very high levels of CD25 and produced IFN-g upon stimulation with PMA and ionomycin or with recombinant IL-12 and IL-18 (data not shown). As in our previously published experiments using TCR transgenic T cells and antigen transgenic mice, providing a single injection of anti-OX40 allowed accumulation of donor cells with a Th1 differentiated phenotype that led to lethal disease in the recipient (1).

Anti-OX40 directly activates donor $CD4^+$ T cells bearing the OX40 receptor

To determine whether anti-OX40 is acting directly on donor $CD4^+$ T cells, and not on cells in the recipient, we transferred OX40-deficient ($OX40^{-/-}$) B6 $CD4^+$ T cells with agonist anti-OX40 antibody into unirradiated F1 recipients. The $OX40^{-/-}$ donor $CD4^+$ cells accumulated (Table 1, $OX40^{-/-}$) but did not differentiate to effector T cells, as measured by CD25 expression and IFN-g production upon stimulation with IL-12 and IL-18 (Fig. 2A). $OX40^{-/-}$ donor $CD4^+$ T cells treated with or without anti-OX40 are phenotypically the same as control IgG $OX40^+$ donor cells as shown in Figure 1 A and B, control IgG panels (Fig. 2A). These data demonstrate that anti-OX40 is exerting its costimulatory effect on the donor T cells to allow accumulation of differentiated effector T cells, rather than acting on cells in the recipient.

To determine whether OX40 engagement on activated donor cells is capable of eliciting costimulatory functions in the recipient that then act independent of OX40, we mixed $4.5x10^6$ $CD4^+$ B6 cells congenic for the Thy1.1 marker with $4.5x10^6$ $CD4^+$ $OX40^{-/-}$ cells and transferred them into unirradiated F1 recipients with anti-OX40 or control IgG. After five days, both donor $CD4^+$ populations accumulated in the spleen (Table 1, $OX40^{-/-}$ and Figure 2B). In control IgG treated animals, the $OX40^{-/-}$ donor cells accumulated more than $OX40^+$ donor cells (Table 1), but both populations expressed low levels of CD25 and produced little IFN-g (Fig. 2B, bottom panels). The enhanced accumulation of $OX40^{-/-}$ T cells compared to their normal counterparts has been reported before in other systems (278, 279). With anti-OX40, $CD4^+$ cells bearing the OX40 receptor differentiated to effector T cells as before (Fig. 2B, top panels). In the presence of OX40 positive donor cells, a portion of $OX40^{-/-}$ $CD4^+$ T cells reproducibly expressed CD25 and produced IFN-g upon IL-12 and IL-18 stimulation in six of six mice (Fig. 2B, top panels). It must be noted that fewer $OX40^{-/-}$ donor T cells were recovered, and of those, a smaller proportion acquired the effector cell phenotype (Fig. 2B). These data show that anti-OX40 mainly acts on $OX40^+$ donor cells to initiate acquisition of effector function. However, bystander differentiation does occur in OX40-deficient donor T cells, implying that the $OX40^+$ donor cells can induce OX40-independent effector function in bystander T cells, perhaps by eliciting other costimulatory signals in the recipients.

CD4 T cells that have a transgene or mutation that enhances survival do not acquire effector T cell function without OX40 engagement

We have shown that anti-OX40 acts directly through OX40 on the alloreactive donor $CD4^+$ T cells (Fig. 2), however, the mechanism of OX40-mediated accumulation of effector $CD4^+$ T cells is unknown. Triggering OX40 could promote the differentiation of proliferating donor cells or, alternatively, OX40 could promote survival and accumulation of differentiated donor cells. It is known that over expression of proteins that promote survival directly rescue $OX40^{-/-}$ defects in accumulation of activated T cells and memory cell generation in other systems (80, 113, 114). To test the hypothesis that OX40 engagement enhances T cell survival in our system of ubiquitous antigen presentation, we transferred $4.5x10^6$ splenic $CD4^+$ cells from B6 mice transgenic for the anti-apoptotic protein Bcl-2 into unirradiated F1 recipients. Although Bcl-2 over expression was shown to promote survival in a previous report (80), the transgenic T cells failed to accumulate to larger numbers than wild type donor $CD4^+$ cells in our model (Table 1, Bcl-2 and B6). Donor cell recovery, CD25 expression, and IFN-g production upon stimulation with IL-12 and IL-18 from Bcl-2 transgenic $CD4^+$ cells were similar to wild type in the presence or absence of anti-OX40 (Figure 3A and Table 1). Enhanced expression of Bcl-2 did not increase the numbers of IFN-g secreting effector cells in the absence of anti-OX40, showing that Bcl-2 over expression does not mimic anti-OX40 treatment in this system.

It is possible that Bcl-2 failed to protect differentiating donor cells from activation-induced T cell death owing to Fas (CD95) or TNFR1 in our model. To test this hypothesis, we transferred donor B6 splenocytes with a natural mutation in CD95

($B6^{lpr}$), or splenocytes deficient in TNFR1, into unirradiated F1 hosts with or without anti-OX40. After five days, alloreactive $CD4^+$ T cell accumulation, CD25 expression, and IFN-g production in response to recombinant IL-12 and IL-18 was similar to wild type responses with or without anti-OX40 (Table 1 and Fig. 3B and C). The most straightforward explanation of these results is that OX40 engagement promotes differentiation to effector function in this model of ubiquitous antigen presentation, rather than to prevent death of differentiating $CD4^+$ T cells.

OX40 signaling leads to increased cytokine and cytokine receptor expression

To further explore the consequences of OX40 signaling, we compared changes in gene expression profiles between anti-OX40 and control IgG treated antigen-specific donor cells using Affymetrix oligonuceotide arrays. To obtain a homogenous population of antigen-specific donor $CD4^+$ T cells, we employed our previously published model in which TCR transgenic T cells are transferred into antigen-transgenic mice (1). This model yields results similar to the parent into F1 model described in this report. Data from two independent experiments using the MG-U74Av2 chip were analyzed for comparison of RNA levels in purified donor T cells from animals treated with anti-OX40 or control IgG for 3.5 days. We looked for genes encoding cytokines and cytokine receptors as measures of T cell differentiation, as well as for genes known to be involved in apoptosis and survival.

Consistent with the flow cytometry data, OX40 ligation induced expression of mRNA for CD25 (Table 2). Two other cytokine receptors acting through the common gamma chain, IL-15Rα and IL-7R, were also induced (Table 2). Like IL-2R, the IL-15

and IL-7 receptors have been implicated in survival and differentiation of T cells (39, 267, 280).

Notably, anti-OX40 also induced mRNA for IL-12Rβ2 (Table 2), consistent with the strong response to stimulation with IL-12 and IL-18 in the anti-OX40-treated T cells. In addition to IFN-γ production measured by intracellular cytokine staining (Fig. 1B, OX40), the OX40 signal increased expression of mRNA for several other pro-inflammatory T cell cytokines: GM-CSF, lymphotoxin-α (also known as TNFβ), and IL-3 (Table 2 and Supplementary Table 1). Cytokines and receptors that decreased with OX40 costimulation included IL-4, IL-16, and gp130 (the signal transducer subunit of IL-6R family members). CD153 (CD30 ligand) and CD27 were also decreased with anti-OX40 administration, while OX40 and 4-1BB expression increased (Table 2 and Supplementary Table 2).

Because the OX40 signal has been shown to induce expression of Bcl-2 and Bcl-x_L in a model of transient antigen exposure (80, 113), we were surprised to find that Bcl-2 and some other Bcl-2 family members (Mcl-1 and Bim) scored as "no change" with anti-OX40 treatment (Table 2). However, we did see a 1.8-fold increase in Bcl-x_L expression, which was below our analysis threshold of 2-fold change in expression (Table 2). Two genes involved in glucose transport and metabolism, Glut-1 and hexokinase-2, which are reported to decrease in T cells upon growth factor withdrawal (281), were increased greater than 2.9 fold with OX40 engagement (Table 2). Messages for the death receptors Fas, TNFRI, and DR6 (Tnfrsf21) also scored as "no change" with anti-OX40, as did message for the negative regulatory molecules, CTLA-4 and PD-1

(Supplementary Table 3). Overall, these data support the idea that OX40 drives the differentiation of proliferating T cells to Th1 effector cells in this model.

OX40 drives differentiation early in T cell priming

We wished to test the hypothesis that OX40 signaling promotes acquisition of effector function early in T cell priming, during exponential expansion and before extensive death of proliferating T cells. Using the AND TCR transgenic mice, we transferred $3.5x10^6$ CFSE-labeled splenic AND $Rag1^{-/-}$ T cells with a single injection of anti-OX40 or control IgG into antigen transgenic recipients (1) and harvested splenocytes 30, 48, 72, and 120 hours after injection. Anti-OX40 treated donor $CD4^+$ T cells expressed CD25 30 hours after injection and maintained uniformly high CD25 expression through 120 hours, while control cells initially expressed CD25, but became CD25 intermediate at 48 hours, and CD25 low to negative by 72 hours (Fig. 4A). IFN-γ production directly *ex vivo* or after stimulation with PMA and ionomycin or IL-12 and IL-18 was equal with or without anti-OX40 at 30 hours after injection. However, at the 48, 72, and 120 hour time points, anti-OX40 treated donor cells produced markedly increased IFN-γ upon stimulation compared to control IgG treated cells (Fig. 4B). Donor $CD4^+$ T cells recovered from both anti-OX40 and control IgG populations uniformly proliferate with the same kinetics at each time point as shown by CFSE dilution (Fig 4B, bottom). While donor cell recovery is similar, if not greater, in control IgG-treated animals in the first 72 hours, greater accumulation of anti-OX40 treated donor cells over control cells occurred between 72 and 120 hours (Table 1, TCR Tg group), consistent with previously published results (1). These data show that OX40 engagement induces

differentiation early in T cell priming before effects of enhanced survival can be measured.

To determine whether there is a correlation between differentiation and survival protein expression at early time points, we examined Bcl-2 by flow cytometry on donor cells 30, 48, 72, and 120 hours post injection in anti-OX40 or control IgG treated recipients. We also examined Bcl-x_L protein expression at 72 hours, a time at which Bcl-x_L mRNA levels are increased in OX40 over control IgG treated animals (Table 2). Consistent with increased mRNA, Bcl-x_L protein expression at 72 hours was significantly enhanced in OX40 treated recipients (Fig. 4C and Table 2). We found no difference in the mean fluorescence intensity (ΔMFI) of Bcl-2 protein expression between anti-OX40 and control IgG treated cells at 30 and 48 hours (Fig. 4C). We were surprised to find that Bcl-2 expression decreased significantly at 72 hours in the control IgG group compared to OX40 treated cells, since the gene chip analysis at 3.5 days showed no difference in Bcl-2 RNA expression (Fig. 4C and Table 2). However, at 120 hours, Bcl-2 expression was very low in both anti-OX40 and control IgG treated cells (Fig. 4C). Acquisition of IFN-γ effector cytokine production is apparent at 48 hours, before a difference in CFSE dilution, cell recovery, or Bcl-2 expression. These data suggest that the primary effect of OX40 signaling may be to promote cytokine receptor expression and differentiation to effector function, since these are the earliest responses we measured during T cell priming *in vivo* in our system.

Discussion

Recent literature on the role of OX40 costimulation indicates that a signal through OX40 enhances survival proteins to promote $CD4^+$ T cell accumulation and memory (80, 113, 114, 161). Data in this report clearly show that OX40 costimulation promotes $CD4^+$ T cell differentiation, measured as the ability to produce IFN-γ, independent of survival. OX40 promotes differentiation at early time points in T cell priming, before differences in Bcl-2 expression or cell recovery can be measured. We have also shown that donor $CD4^+$ T cells with genetic alterations that prolong survival are not able to differentiate without anti-OX40. In addition, we found that anti-OX40 acts directly through OX40 on responding $CD4^+$ T cells to initiate acquisition of effector function, although OX40-deficient $CD4^+$ T cells are able to acquire effector function as bystanders to $OX40^+$ donor cells.

We show for the first time that OX40 engagement promotes enhanced effector function beginning at 48 hours of T cell priming in vivo. Perhaps differentiation early in T cell priming leads first to cytokine and cytokine receptor expression and then to acquisition of effector function and enhanced survival of effector and memory cells as a secondary outcome. In fact, we saw much larger increases at 3.5 days in expression of cytokine and cytokine receptor genes than genes for anti-apoptotic proteins. While we see acquisition of effector function with anti-OX40 as early as 48 hours, only a small percentage of donor cells are able to make IFN-g at that time point. In another report, differentiation to IFN-g synthesis in a large population of antigen specific T cells occurs early and is maintained in response to immunization (58). While effector cell

differentiation directly mediated by OX40 may take longer to develop in a large population compared to viral priming, it is possible that OX40 engagement can promote only a small fraction of CD4 cells to become effectors, and that the ability to promote survival is also focused selectively on this small population. However, it is possible that OX40 does not have an effect on survival of this small population, as there was no detectable advantage in the ability of Bcl-2 transgene, Fas or TNFR1 deficient genetic mutants to accumulate or differentiate in our system. We prefer a simpler model in which effector cell differentiation driven by OX40 occurs in most of the proliferating donor cells, but takes several days for many of the cells to acquire effector function.

The outcome of OX40 costimulation could favor acquisition of effector function or enhanced survival depending on the context of T cell activation, such as ubiquitous versus localized antigen, or persistent versus transient antigen presentation. While it is clear that a signal through OX40 promotes T cell survival and memory upon transient or localized antigen stimulation in vitro as well as in vivo (80, 81, 113-115, 161, 272, 282, 283), data in this report indicate that differentiation may be the primary effect of OX40 engagement upon persistent and ubiquitous antigen presentation. Croft and co-workers reported that OX40 costimulation promotes Bcl-2 and Bcl-x_L expression upon stimulation in vitro (80) and that Bcl-2 or Bcl-x_L overexpression in $CD4^+$ donor cells enhances survival and memory T cell generation in OX40-deficient $CD4^+$ T cells (80, 113). When we tested this possibility in our system, we discovered that Bcl-2 overexpression did not promote differentiation or significantly enhance cell recovery compared to control $CD4^+$ T cells in unirradiated F1 recipients without OX40 costimulation. However, expression of the anti-apoptotic Bcl-2 protein rescues growth

factor withdrawal mediated apoptosis but has little effect on apoptosis through the death receptor pathway (60). We considered that triggering OX40 could prevent apoptosis mediated by death receptors rather than growth factor withdrawal in vivo, and so allow accumulation of differentiated antigen specific donor cells. However, when we transferred $CD4^+$ T cells that were deficient in the death receptors Fas or TNFR1, they did not acquire effector function in unirradiated F1 recipients without anti-OX40, and we observed no differences with anti-OX40.

In addition to data in this paper, there are other examples in which differentiation may be the primary result of OX40 signaling (1, 108, 158, 175, 177). Administration of agonist OX40 antibody after the onset of tolerance in vivo can reverse the tolerant phenotype (175). Recently, OX40 signaling has been shown to prolong IL-2, IL-3, and IFN-g expression by promoting stabilization of the cytokine mRNA in human $CD4^+$ memory cells (158).

We and others have shown that OX40 induces sustained expression of IL-2R (272), IL-7R, and IL-15R (1)(and Fig. 4). Perhaps both differentiation to IFN-g synthesis and the increase in T cell survival and memory cell generation are results of cytokine receptor signaling rather than a direct effect of OX40, as the common gamma chain receptors are known to enhance both survival and effector function (39, 177, 280). OX40 signaling may drive expression of common gamma chain receptors such as IL-2 receptor, whose signals would then promote differentiation or survival, depending on the context of T cell activation. A recent report has shown that OX40 costimulation activates Akt to promote T cell longevity by enhancing anti-apoptotic protein expression (113). Akt activity has also been reported to be downstream of common gamma chain receptors such

as IL-2, IL-7, and IL-15 (284, 285). Thus, increased T cell longevity following OX40 ligation may depend on signaling through this family of cytokine receptors.

Notably, our data indicate that OX40 costimulation is not the only signal capable of permitting accumulation of differentiated effector donor T cells in unirradiated recipients. OX40-deficient $CD4^+$ T cells have a modest ability to acquire effector function when mixed with $OX40^{+/+}$ donor cells in the presence of agonist anti-OX40. Differentiation seen in $OX40^{-/-}$ $CD4^+$ T cells mixed with $OX40^{+/+}$ depends on OX40-mediated T cell differentiation. Differentiating $OX40^+$ cells in this mixing experiment may initiate a positive feedback loop by licensing APC to promote expression of other costimulatory molecules and cytokines that could indirectly induce effector function in $OX40^{-/-}$ $CD4^+$ T cells. In concordance with this idea, one report has shown cytokine-driven bystander activation of $CD4^+$ T cells mediated in this way (286).

In summary, we provide evidence that T cell differentiation is an important effect of OX40 ligation. We show that anti-OX40 promotes accumulation of $CD4^+$ T cells with effector function in a model that otherwise promotes tolerance, and can result in fatal GVHD in unirradiated recipients. We also found that other signaling pathways can drive differentiation of proliferating T cells, because a portion of OX40 deficient donor $CD4^+$ T cells can differentiate into effector cells when mixed with normal donor T cells in our model. Thus, both T cell survival and acquisition of effector functions must be considered when costimulatory signals are manipulated in approaches to treating or preventing chronic infections, autoimmune diseases, GVH, allogeneic bone marrow graft rejection, and cancer.

Table 1: *Donor $CD4^+$ Cell Recoveries from Spleen*[a]

		Donor $CD4^+$ Input ($x10^6$)	Donor $CD4^+$ Recovered ± SD ($x10^6$)	
			Anti-OX40	Rat IgG Control
B6 (n=12)	Day 5	4.5	19 ± 5.6	4.9 ± 1.6
B6 (n=3)	Day11	4.5	1.5 ± 1	2.2 ± 0.9
B6 (n=3)	Day 5	10	42 ± 7.5	4.6 ± 1.4
B6 (n=6)	Day 7[b]	10	8.6 ± 0.74	3.2 ± 0.24
$OX40^{-/-}$ (n=6)[c]	Day 5	4.5	2.9 ± 1.4	3.4 ± 0.5
$OX40^{-/-}$ (n=6)[d]	Day 5	4.5	6.9 ± 0.9	4.1 ± 0.3
$OX40^{+/+}$ (n=6)[d]	Day 5	4.5	18.2 ± 3.1	1.8 ± 0.5
Bcl-2 Tg (n=6)	Day 5	4.5	18.6 ± 3.2	2.9 ± 0.31
$B6^{lpr}$ (n=6)	Day 5	4.5	9.2 ± 0.95	2.8 ± 0.22
$TNFR1^{-/-}$ (n=6)	Day 5	4.5	10.5 ± 0.1	1.8 ± 0.67
TCR Tg (n=6)	Day 1	3.5	0.33± 0.34	0.15 ± 0.02
TCR Tg (n=6)	Day 2	3.5	1.1 ± 0.67	1.56 ± 0.67
TCR Tg (n=9)	Day 3	3.5	8.5 ± 4.2	10.8 ± 6.5
TCR Tg (n=9)	Day 5	3.5	96.7 ± 7.6	43.8 ± 4.2

[a] $CD4^+$ T cells in a B6 or TCR Tg spleen cell suspension were transferred with anti-OX40 or control IgG i.v. into unirradiated (B6.Ly5.1 x bm12)F1 or antigen transgenic recipients, and spleens were harvested at the indicated day. The number of donor $CD4^+$ cells recovered was determined by multiplying the percent $CD4^+$ donor cells determined by flow cytometry by the total cells recovered from the spleen. n equals the number of animals in each treatment group, combining data from one to 4 experiments.

[b] All six OX40 treated mice were moribund and euthanized.

[c] $OX40^{-/-}$ donor cells transferred alone.

[d] $OX40^{-/-}$ and $OX40^{+/+}$ were mixed together and injected into a single recipient.

Table 2: *Effects of the OX40 signal on cytokine, cytokine receptor, and survival gene expression in donor T cells*[a]

Gene or protein name	Accession Number	Fold change
	Genes that increase	
IL-2Rα	NM_008367	(18.2)
IL-7R	NM_008372	5.5
IL-15Rα	NM_133836	(4.4)
IL-12Rβ2	NM_008354	(15.4)
IFN-γ	NM_008337	2.9
Lymphotoxin-α	NM_010735	(4.0)
GM-CSF	NM_009969	(19.5)
IL-3	NM_010556	(3.9)
OX40	NM_011659	4.0
4-1BB	NM_011612	6.1
Bcl-x	NM_009743	1.8
Hexokinase-2	NM_013820	5.0
Glut-1	NM_011400	(3.5)
	Genes that decrease	
IL-4	NM_021283	(-20.0)
IL-16	NM_010551	-3.2
gp-130	NM_010560	-8.9
CD27	XM_284241	-3.6
CD30L	NM_009403	(-13.5)
	Genes that do not change	
Bcl-2	NM_177410	1.1
Mcl-1	NM_008562	-1.5
Bim	NM_009754	0.0
Fas	NM_007987	1.3
TNFR1	NM_011609	0.1
DR6	NM_178589	(-2.0)

[a]AND TCR transgenic T cells were injected into antigen transgenic mice with or without anti-OX40. 3.5 days later, transgenic T cells were enriched and total RNA was purified for hybridization on an Affymetrix MG-U74Av2 gene chip as described in materials and methods. Fold change numbers in parenthesis represent genes with absent detection calls for control treatment in genes that increase, and for anti-OX40 treatment in genes that decrease. Additional data from this analysis are presented in Tables 1-3 in the online supplement.

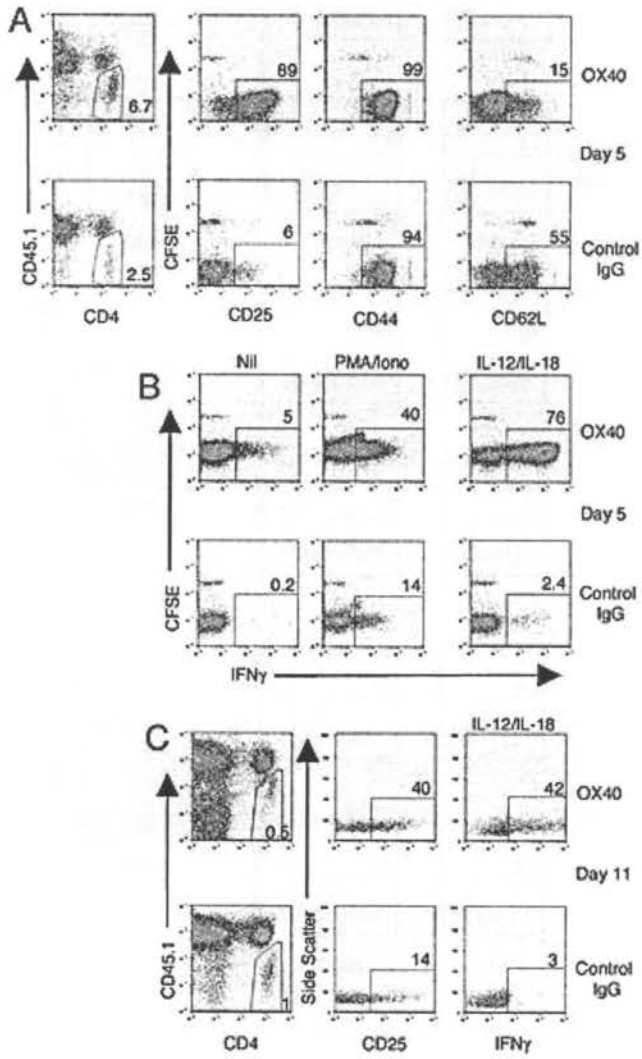

FIGURE 3-1. Anti-OX40 promotes acquisition of effector function in donor $CD4^+$ alloreactive T cells. $4.5x10^6$ $CD4^+$ T cells in a B6 spleen cell suspension were transferred with 50 μg anti-OX40 or rat IgG i.v. into unirradiated (B6.CD45.1 x bm12)F1 recipients, and spleens were harvested 5 days (A and B) or 11 days (C) later. A) Percent of CD45.1 negative donor $CD4^+$ T cells in a representative sample is shown in the left panel. Among the $CD4^+$ donor cells, T cell activation surface markers CD25, CD44, and CD62L, on $CFSE^{lo}$, divided donor cells are compared to $CFSE^{hi}$, undivided, donor cells directly *ex vivo*, as shown in the right panels. B) Percent IFN-γ production by donor $CD4^+$ T cells restimulated *in vitro* for 5 hours with media, 20 ng PMA and 500 ng ionomycin (PMA/iono), or 10 ng IL-12 and 100 ng IL-18 (IL-12/18). C) Recipient spleens were harvested 11 days after T cell transfer. Percent of CD45.1 negative donor $CD4^+$ T cells is shown in the left panel. Percent of fresh CD25 positive donor $CD4^+$ T cells and IFN-γ positive donor cells after stimulation with IL-12 and IL-18 is shown in the right panels.

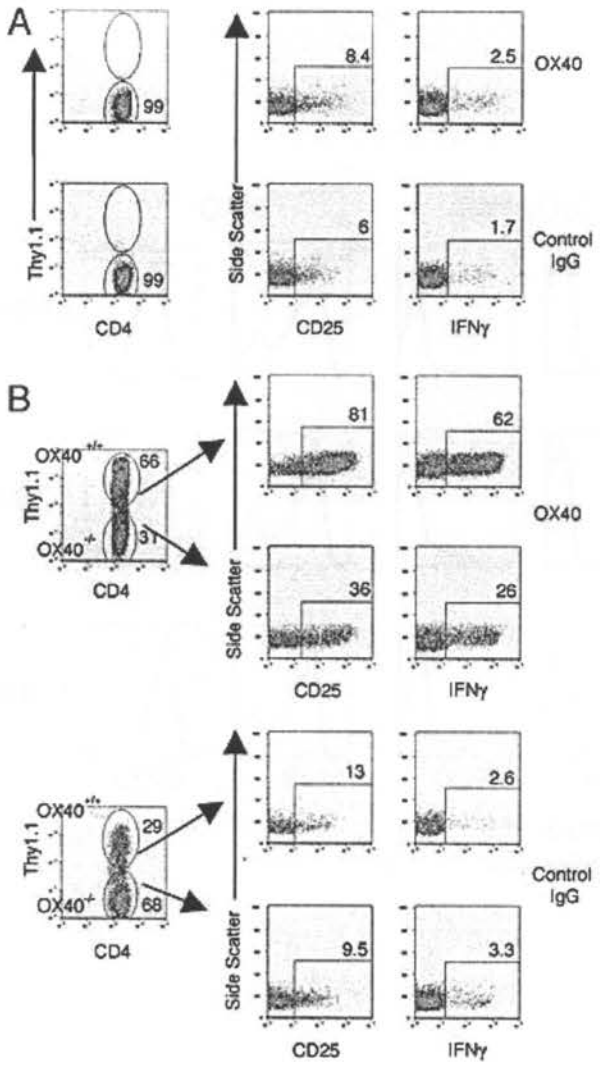

FIGURE 3-2. OX40 acts directly and indirectly on donor T cells to promote effector function. All graphs are gated on $CD4^+$, $CD45.1^-$ donor lymphocytes. $4x10^6$ B6.Thy1.1 $CD4^+$ T cells and $4x10^6$ B6 $OX40^{-/-}$ $CD4^+$ T cells in a spleen cell mixture or $4x10^6$ $OX40^{-/-}$ alone were transferred with 50 μg anti-OX40 or rat IgG into a non-irradiated (B6.ly5.1 x bm12)F1 recipient for 5 days. A) Top row represents mice treated with anti-OX40, bottom row represents mice treated with rat IgG. CD25 expression of donor $CD4^+$ cells versus side scatter is shown in the middle panel. Percent of IFN-γ positive cells after stimulation with IL-12 and IL-18 for 5 hours is shown on the right. B) Top two rows represent mice treated with anti-OX40, bottom two rows represent mice treated with rat IgG. Percent of donor B6 ($Thy1.1^+$) and donor $OX40^{-/-}$ ($Thy1.1^-$) is shown in the left panels. CD25 expression of each donor population is shown center. Percent IFN-γ positive cells after stimulation with IL-12 and IL-18 for 5 hours is shown on the right.

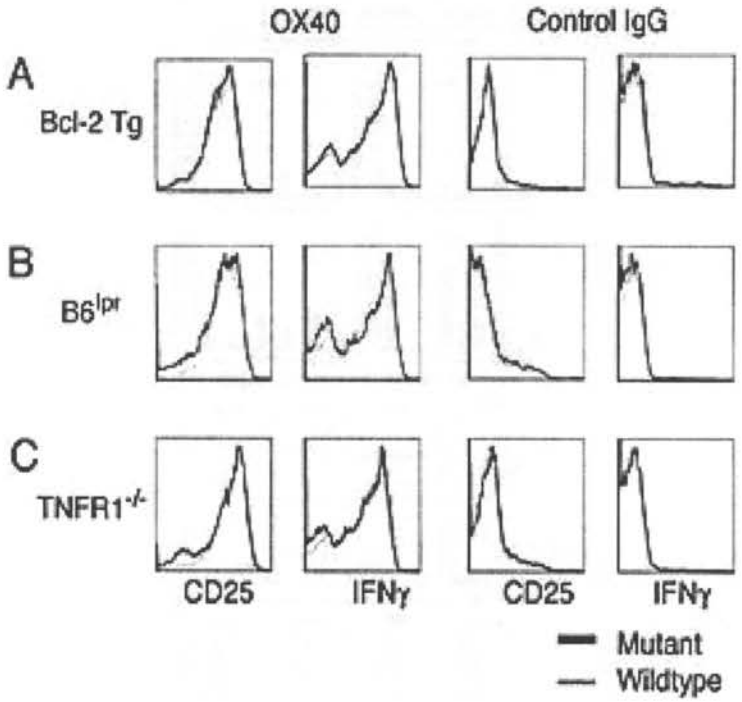

FIGURE 3-3. Gain of effector function is not influenced by the Bcl-2 transgene or mutations in death receptors. All histograms represent spleen cells gated on $CD4^+$, CD45.1 negative donor T cells. $4x10^6$ $CD4^+$ T cells were transferred in a spleen cell suspension into (B6 x bm12)F1 hosts for 5 days with 50 μg anti-OX40 or rat IgG. CD25 expression on freshly isolated cells and IFN-γ production of donor cells restimulated with 10 ng IL-12 and 100 ng IL-18 for 5 hours are shown for each mutant donor (thick line) and compared to wild type donor (thin line) $CD4^+$ T cells.

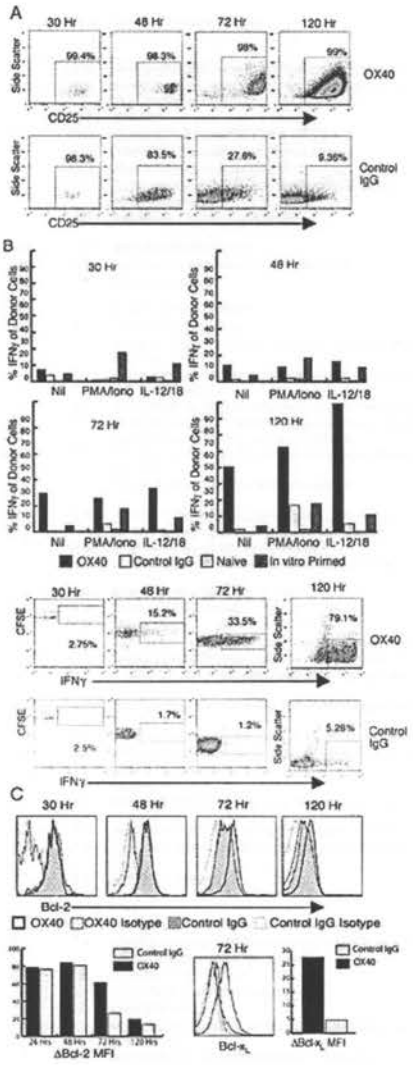

FIGURE 3-4. Anti-OX40 promotes acquisition of effector function early in T cell priming. $3.5x10^6$ AND TCR transgenic T cells in a spleen cell suspension were transferred with 50 μg anti-OX40 or control IgG i.v. into antigen transgenic recipients, and spleens were harvested 30, 48, 72, or 120 hours later. A) Forward scatter and percent of CD25 positive donor cells gated on CD4, Vα11, and Vβ3 for each time point is shown. B) Percent IFN-γ production of donor $CD4^+$ T cells stimulated for 5 hours *in vitro* with media, 20 ng PMA and 500 ng ionomycin (PMA/Iono), or 10 ng IL-12 and 100 ng IL-18 (IL-12/IL-18). For each condition, the bars show mean percent IFN-γ^+ cells for donor cells from animals treated with anti-OX40, control IgG, naïve, and day 5 or 6 *in vitro* primed TCR transgenic cells. In the bottom 8 panels, a representative plot shows cell division (CFSE) and IFN-γ production upon IL-12 and IL-18 stimulation for anti-OX40 and control IgG treated donor cells. C) Mean fluorescence intensity (MFI) and representative histograms of Bcl-2 or Bcl-x_L protein expression and isotype-matched staining controls in donor cells gated on CD4, Vα11, and Vβ3. In the bar graphs, ΔMFI represent the change in intensity between protein and isotype control MFI for each sample.

Supplement Table 1. *Genes with increased expression in donor T cell preparations from OX40-treated animals*[a]

Genes in bold are mentioned in the article text.

		Experiment #1				Experiment #2					
Affymetrix probe set	RefSeq or transcript ID	rat Ig	P or A	anti OX40	P or A	fold change	rat Ig	P or A	anti OX40	P or A	fold change	mean fold change	Gene or Protein Product	Reference
Possibly growth- or differentiation-related and expressed in lymphocytes:														
100030_at	NM_009477	8.2	A	381.1	P	36.8	10	A	439	P	21.1	28.9	uridine phosphorylase 1, upregulated by TNFalpha, IL-1alpha, and IFNg	(1)
102658_at	NM_010555	73.1	P	1107.6	P	14.9	73	P	1797	P	26.0	20.5	interleukin 1 receptor, type II, decoy receptor, expressed by activated T cells and shed by neutrophils	(2) (3)
92948_at	**NM_009969**	2.9	A	**58**	**P**	**32.0**	**10**	**A**	**55.1**	**P**	**7.0**	**19.5**	**GM-CSF**	
101917_at	**NM_008367**	7.8	A	**168.8**	**P**	**24.3**	**18**	**A**	**150**	**P**	**12.1**	**18.2**	**interleukin 2 receptor alpha, CD25**	
161689_f_at	NM_010555	51.6	A	924.7	P	13.9	63	P	1477	P	18.4	16.2	interleukin 1 receptor, type II (see above)	
99323_at	**NM_008354**	22.5	A	**209.7**	**P**	6.5	5.4	A	**226**	**P**	**24.3**	**15.4**	**Interleukin 12 receptor beta2**	
98045_s_at	NM_023118	17.7	A	79.7	P	7.0	17	A	210	P	11.3	9.1	Dab2, disabled-2, a tumor suppressor and an adaptor protein for TGFbeta receptor signaling	(4, 5)
93871_at	NM_031167	143	P	1233.9	P	8.0	120	P	1204	P	9.2	8.6	interleukin 1 receptor antagonist	(2) (3) (6)
94688_at	NM_010751	7.1	A	83.7	P	6.1	7.9	A	140	P	10.6	8.3	Max dimerization protein	
97487_at	NM_009255	18.6	A	62.4	P	3.0	11	A	101	P	9.6	5.8	serine proteinase inhibitor, spi4, serpine2	(7)
AFFXMur/ X57349_M_at	NM_011638	3.7	A	22.5	P	7.5	11	A	42.3	P	3.7	5.6	transferrin receptor	
103509_at	**NM_011612**	20.9	P	**136.2**	**P**	4.9	29	P	**254**	**P**	6.1	**5.5**	**4-1BB, CD137, Tnfrsf9**	
99030_at	**NM_008372**	56.9	P	**300.4**	**P**	5.7	44	P	**261**	**P**	5.3	**5.5**	**interleukin 7 receptor**	
93895_s_at	NM_008789/ NM_010585	58	P	167.4	P	3.5	43	P	287	P	7.0	5.2	inositol 1,4,5-trisphosphate receptor I, mediates the release of intracellular calcium	
94881_at	NM_007669	32.9	A	228.7	P	6.5	55	M	216	P	3.7	5.1	cyclin-dependent kinase inhibitor 1A (P21), intermediate in p53-mediated cell cycle arrest	(8, 9)
94375_at	**NM_013820**	87.5	P	**425.3**	**P**	4.3	102	P	**587**	**P**	5.7	**5.0**	**hexokinase 2, disappears upon growth factor withdrawal**	**(10)**
104701_at	NM_011498	239	P	979.3	P	3.7	205	P	1315	P	6.1	4.9	Stra13/Clast5/DEC1, transcriptional repressor, Stra13 deficiency results in systemic autoimmunity, target of TGFbeta	(11) (12)
99475_at	NM_007706	45.2	P	403.5	P	5.7	79	P	455	P	4.0	4.8	SOCS-2, suppressor of cytokine signalling-2, negative regulator of cytokine signaling	(13)
96596_at	NM_008681/ NM_010884	155	P	519.9	P	4.0	144	P	915	P	5.3	4.6	TDD5, closely related to Ndr1 (see below)	(14)
160464_s_at	NM_008681	151	P	555	P	3.2	136	P	795	P	5.7	4.5	Ndr1, homologous to human NDRG1, differentiation-associated gene, putative tumor suppressor	(15)(16) (17)
161023_at	**NM_133836**	86	A	**383.1**	**P**	4.3	107	A	**402**	**P**	4.6	**4.4**	**interleukin 15 receptor, alpha chain**	
94977_at	NM_008789/ NM_010585	27.5	P	196.7	P	3.5	57	P	498	P	5.3	4.4	inositol 1,4,5-triphosphate receptor 1 (see above)	
102630_s_at	**NM_010735**	25.6	A	**171.5**	**P**	3.7	28	A	**151**	**P**	4.3	**4.0**	**lymphotoxin alpha, TNF-beta**	
102681_at	**NM_011659**	258	P	**1040.5**	**P**	3.5	209	P	**1031**	**P**	4.6	**4.0**	**OX40, CD134, Tnfrsf4**	
94086_at	**NM_010556**	7.7	A	**36.5**	**P**	4.0	13	A	**64.1**	**P**	3.7	**3.9**	**interleukin 3**	
93465_at	NM_030060	185	P	686.3	P	2.6	140	P	1004	P	4.9	3.8	bZIP family transcription factor, homologous to human Jun dimerization protein p21SNFT which represses IL-2 promoter distal NF-AT/AP-1 site	(18)
160829_at	NM_009344	30.5	P	151.2	P	3.7	49	P	144	P	3.7	3.7	TDAG51, T cell death associated gene, upregulates Fas and T cell death	(19) (20)
93738_at	**NM_011400**	29.8	A	**128.3**	**P**	3.5	33	A	**135**	**P**	3.5	**3.5**	**glut-1, solute carrier family 2 (facilitated glucose transporter), member 1**	
98087_at	NM_007669	83.1	A	242.6	P	3.7	83	A	258	P	3.0	3.4	cyclin-dependent kinase inhibitor 1A (P21), see above	
98405_at	NM_009256	292	P	843.2	P	2.8	262	P	1066	P	4.0	3.4	serine protease inhibitor 6, SPI6, lymphocyte granzyme B inhibitor that may protect CTL from lysis	(21) (22)
93931_at	NM_011073	70.5	P	255.6	P	3.5	93	P	291	P	3.0	3.3	perforin	
98299_s_at	NM_011409/ NM_011410	37.5	P	119.5	P	2.6	29	P	86.2	P	3.5	3.1	schlafen3 (Slfn3)/schlafen 4, growth regulatory genes in T lymphocytes	(23)
92315_at	NM_011410	193	P	964	P	2.6	135	P	663	P	3.2	3.0	schlafen4 (Slfn4), growth regulatory gene in T lymphocytes	(23)
103715_at	NM_009132	59.8	A	143.9	P	3.2	46	A	133	P	2.6	2.9	adseverin, scinderin, member of the gelsolvin family, induced in Th cells by IL-9	(24)
99334_at	**NM_008337**	36.9	P	**112.4**	**P**	3.5	49	P	**120**	**P**	2.3	**2.9**	**interferon gamma**	
Other genes of interest:														
160564_at	NM_008491	114	P	1481.8	P	10.6	74	P	1946	P	22.6	16.6	lipocalin 2, induced by SV40, dexamethasone, and retinoic acid	(25)
92368_at	NM_019511	37	A	174	P	3.5	35	A	351	P	11.3	7.4	RAMP3, receptor activity modifying protein 3, regulates ligand specificty of the calcitonin-receptor-like receptor	
160084_at	NM_013614	134	P	395.5	P	3.2	155	P	622	P	4.3	3.8	ornithine decarboxylase	
104371_at	NM_010046	39.1	P	98.4	P	2.6	53	P	278	P	4.0	3.3	diacylglycerol acyltransferase (Dgat)	(26)
97820_at	NM_016905	48.3	P	131.3	P	3.0	51	P	234	P	3.5	3.3	galactokinase	
94419_at	NM_031196	97.5	A	219.2	P	2.6	63	A	268	P	3.7	3.2	solute carrier family 19 (sodium/hydrogen exchanger), member 1, reduced folate carrier	(27)
100026_at	BC053706	79.3	A	327.6	P	3.5	117	P	348	P	2.6	3.1	branched chain aminotransferase 1, cytosolic, target of c-myc	(28)
94855_at	NM_008831	33.4	A	89.1	P	3.5	43	A	108	P	2.3	2.9	prohibitin, intracellular antiproliferative protein, c-myc target, chaperone in assembly of mitochodrial protein complexes	(29) (30) (31)
95148_at	NM_016895	148	P	522.5	P	2.6	231	P	952	P	3.2	2.9	adenylate kinase isozyme 2	
Likely made by contaminating inflammatory cells in the T cell preparation from antiOX40-treated animals:														
93097_at	NM_007482	13.9	A	280.8	P	19.7	4.1	A	725	P	194.0	106.9	liver arginase 1, shapes granulomatous pathology	(32)
102733_at	NM_010371	2.9	A	41.9	P	12.1	2	A	84.9	P	34.3	23.2	granzyme C, cytotoxic T lymphocyte-specific serine protease	
94769_at	NM_008611	92	A	543.9	P	6.5	12	A	323	P	29.9	18.2	matrix metalloproteinase 8, neutrophil collagenase	
103024_at	NM_007403	56.9	A	342.8	P	4.8	16	A	951	P	18.4	11.5	CD156, ADAM 8, a disintegrin and metalloprotease involved in leukocyte extravasion	(33)
96153_at	NM_008694	401	P	1199.6	P	3.2	255	P	3732	P	13.0	8.1	neutrophilic granule protein	(34)
101160_at	NM_009140	27.3	P	194.8	P	5.3	20	P	199	P	9.8	7.6	MIP-2, macrophage inflammatory protein 2, CXCL2 chemokine	
92694_at	NM_009892	473	P	3087.4	P	6.1	525	P	4930	P	9.2	7.6	eosinophil chemotactic cytokine, ECF-L, Ym1, potent mediator of osteoclast formation produced by inflammatory macrophages	(35) (36)
102712_at	NM_011315	48.7	A	421.1	P	11.3	102	P	440	P	3.5	7.4	serum amyloid A 3, SAA3, acute response protein, induces transcription of matrix metalloproteinases, target of NFkB	(37) (38) (39)
98773_s_at	AI323667	51.7	P	376.3	P	7.0	33	P	179	P	5.3	6.1	immunoresponsive gene 1 (Irg1) induced by LPS	(40)
98088_at	NM_009841	314	P	1229.7	P	3.5	213	P	1669	P	7.0	5.2	CD14, LPS receptor and myeloid cell marker	**(41)**
104388_at	NM_011338	190	P	1066.8	P	3.2	296	P	2269	P	6.5	4.9	MIP-1gamma, CCL9 chemokine	
94755_at	NM_010554	59.4	P	184.5	P	3.2	21	A	259	P	6.5	4.9	interleukin 1 alpha	
98589_at	NM_007408	177	P	1386.8	P	4.6	211	P	2006	P	6.3	4.9	adipose differentiation related protein, adipohilin, lipid droplet metabolism	(42)
95338_s_at	NM_008605	25.9	A	62.8	P	2.6	13	A	142	P	7.0	4.8	matrix metalloproteinase 12, macrophage metalloelastase	
95951_at	NM_010819	48.4	P	228.4	P	4.6	84	P	169	P	4.3	4.4	macrophage C-type lectin (Mcl), C-type lectin, superfamily member 8	(43)
103977_at	NM_007972	24.5	P	93.3	P	4.0	32	P	181	P	4.3	4.1	coagulation factor X	
93497_at	NM_009778	342	P	1065.5	P	3.0	210	P	1133	P	4.9	4.0	complement component C3	
93500_at	NM_020559	269	P	1051.8	P	4.0	293	P	1315	P	4.0	4.0	aminolevulinic acid synthase 1	
98774_at	L38281	3	A	26.7	P	5.3	7.9	A	19	P	2.5	3.9	immunoresponsive gene 1 (Irg1) induced by LPS	(40)
99413_at	NM_009912	68.1	P	319	P	4.0	96	P	444	P	3.7	3.9	CCR1, monocyte and eosinophil chemokine receptor for MIP-1alpha and RANTES	
103759_at	NM_009763	15.6	P	63.6	P	3.7	14	P	50.3	P	3.5	3.6	bone marrow stromal cell antigen 1, BP-3, CD157, ADP-ribosyl cyclase ectoenzyme, marker for lymphoid progenitors and myeloid cells	
92217_s_at	NM_008147/N M_013532	138	P	408.1	P	2.8	104	P	553	P	4.3	3.6	gp49A/B1, KIR family proteins on mast cells, gp49B1 on activated T cells with 2 ITIM domains regulates IFNg response of T and NK cells	(44)
98828_at	NM_008401	97.2	P	234.4	P	2.6	88	M	393	P	4.6	3.6	CD11b, Mac-1 alpha-chain, integrin alpha M, complement receptor type 3	
102877_at	NM_013542	550	P	2278.8	P	4.0	960	P	3611	P	2.8	3.4	granzyme B	
101820_at	X70920	42.8	P	91.5	P	2.3	29	P	142	P	4.6	3.4	Ly-6G, myeloid marker Gr-1, marker for a subsets of DC and neutrophils that makes large amounts of IL-12	**(45) (46)**
104025_at	NM_022653	43.9	P	125.8	P	2.5	42	A	161	P	3.5	3.0	thimet oligopeptidase 1 (Thop1) cytosolic endopeptidase that degrades MHC I peptides, may limit antigen presentation	(47)
93608_at	NM_015766	136	P	319.7	P	2.5	110	A	376	P	3.5	3.0	IL-27, EBI3, IL-12 p40 homolog, secreted cytokine, made by lymphocytes,and dendritic cells, induces proliferation without IL-2 in naïve T cells.	**(48, 49)**
92849_at	NM_009139	81.5	P	97.2	P	2.3	89	P	289	P	3.5	2.9	C10, MRP-1, CCL6 chemokine	**(50)**
95339_r_at	NM_008605	25	A	156.3	P	3.2	51	P	341	P	2.6	2.9	macrophage metalloelastase	
Other increased genes:														

		Experiment #1					Experiment #2						
Affymetrix probe set	RefSeq or transcript ID	rat Ig	P or A	anti OX40	P or A	fold change	rat Ig	P or A	anti OX40	P or A	fold change	mean fold change	Gene or Protein Product	Reference
97413_at	NM_029639	6.8	A	70.1	P	13.9	7.4	A	82	P	13.9	13.9	RIKEN cDNA 1600029D21 gene	
95019_at	NM_008185	8.1	A	212.9	P	19.7	35	A	136	P	3.5	11.6	glutathione S-transferase, theta 1	
95603_at	NM_138595	15.4	A	152.8	P	11.3	46	A	214	P	6.1	8.7	glycine decarboxylase	
94154_at	NM_009375	13.3	A	138.9	P	8.6	29	A	186	P	7.5	8.0	thyroglobulin	
92715_at	NM_023137	80.3	P	1870.1	P	9.8	71	P	692	P	4.9	7.4	ubiquitin D, diubiquitin, induced by TNF alpha and IFN gamma	(51)
101912_at	NM_183249	76.4	P	536.5	P	7.0	59	P	529	P	6.5	6.7	RIKEN cDNA 1100001G20 gene	
101800_at	NM_008039	95.3	P	585.9	P	4.6	67	P	623	P	8.0	6.3	formyl peptide receptor-like 2	(52)
93234_at	NM_010827	8.5	A	66.2	P	6.1	9.4	A	82.6	P	6.5	6.3	musculin	
160469_at	NM_011580	23.4	P	144.7	P	4.9	16	P	196	P	7.0	5.9	thrombospondin 1	
96634_at	NM_027464	39.1	P	146.3	P	4.3	16	P	162	P	7.5	5.9	RIKEN cDNA 5730469M10 gene	
102375_at	NM_144918	49.4	A	111	P	2.5	19	A	142	P	6.6	5.5	SET and MYND domain containing 5, retinoic acid responsive gene 1	(53)
99198_at	NM_172086	8.4	A	37	P	3.7	4.9	A	33.5	P	6.5	5.1	ribosomal protein L32, translationally regulated by mitogens in T lymphocytes	(54)
98018_at	NM_011171	48.8	P	301.9	P	4.9	36	P	194	P	4.6	4.8	protein C receptor, endothelial, plays a role in the protein c pathway controlling blood coagulation	
92918_at	NM_010172	31.3	P	254.8	P	4.0	40	P	330	P	5.3	4.6	coagulation factor VII	
93353_at	NM_008524	23.5	A	126.8	P	4.0	21	A	118	P	4.5	4.3	lumican, keratin sulfate proteoglycan	(55)
95911_at	AI585872	6.5	A	34.3	P	5.3	12	A	42.8	P	3.0	4.2	RIKEN cDNA 3010025C11 gene	
100949_at	NM_030565	16.8	A	133.9	P	4.9	49	A	164	P	2.6	3.8	cDNA sequence BC004044	
101115_at	NM_008522	102	P	262.6	P	2.3	89	P	528	P	5.3	3.8	lactotransferrin	
97252_at	NM_023536	157	A	214.7	P	2.3	88	A	262	P	4.9	3.6	RIKEN cDNA 2610012O22 gene	
95927_f_at	AA222883	29.1	P	53.8	P	2.1	13	A	76.3	P	4.9	3.5	RIKEN cDNA 2610201A13 gene	
103563_at	AW125713	186	P	497.5	P	2.3	180	P	751	P	4.3	3.3	RIKEN cDNA 4930555L03 gene	
162482_at	AV109962	103	A	433.4	P	3.5	157	A	652	P	3.0	3.3	85% homology to a 205 bp region of murine Traf4	
98459_at	NM_009171	80.3	P	257.3	P	2.8	81	P	314	P	3.5	3.2	serine hydroxymethyl transferase 1 (soluble)	
104342_i_at	NM_023196/NM_183423	5.8	A	37.5	P	3.7	29	A	51.7	P	2.5	3.1	phospholipase A2, group XIIA	
95137_at	NM_133706	136	P	510.4	P	3.0	167	P	637	P	3.2	3.1	RIKEN cDNA 1810014L12 gene	
92540_f_at	NM_009272	140	P	522.6	P	3.2	221	P	746	P	2.8	3.0	spermidine synthase	
95109_at	NM_024193	262	P	842.9	P	2.8	178	P	737	P	3.2	3.0	nucleolar protein 5A, myc regulated	(56)

[Supplemental Table 1 footnote]
*TCR transgenic T cells were injected into antigen transgenic mice with or without anti-OX40. 3.5 days later, transgenic T cells were enriched and total RNA was purified for hybridization on an Affymetrix MG-74Av2 gene chip. This table shows genes whose expression increases ≥ 2.9-fold with anti-OX40 treatment. The hybridization signal and detection call (P for present and A for absent) for anti-OX40 and control rat IgG for genes that increased in two independent experiments are shown. The fold-change for each experiment, the mean fold change for the two experiments, gene or protein name, and references for some genes are also reported. Please refer to the materials and methods for a description of the data analysis.

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Affymetrix probe set	RefSeq or transcript ID	Experiment #1 rat Ig	P or A	anti OX40	P or A	fold change	Experiment #2 rat Ig	P or A	anti OX40	P or A	fold change	mean fold change	Gene or Protein Product	Reference

Supplement Table 2. *Genes with decreased expression in donor T cell preparations from OX40-treated animals*[a]

Genes in bold are mentioned in the article text.

Affymetrix probe set	RefSeq or transcript ID	Experiment #1 rat Ig	P or A	anti OX40	P or A	fold change	Experiment #2 rat Ig	P or A	anti OX40	P or A	fold change	mean fold change	Gene or Protein Product	Reference
Possibly growth- or differentiation-related and expressed in lymphocytes:														
92283_s_at	**NM_021283**	15.3	P	0.8	A	-12.1	19.9	P	0.3	A	-27.9	**-20.0**	**interleukin 4**	
96109_at	NM_008452	298.2	P	49.5	A	-5.7	288.2	P	7.7	A	-22.6	-14.1	LKLF, Kruppel-like factor 2 (lung), T cell quiescence via c-myc, survival of memory T cells	(1-4)
101136_at	**NM_009403**	71.4	P	5.4	A	-13.0	69.4	P	6.1	A	-13.9	**-13.5**	**CD153, CD30 ligand, can promote T cell function and survival or induce cell death via TNF-alpha**	**(5, 6)**
94345_at	**NM_010560**	316.4	P	37.2	P	-8.5	192.4	P	25.7	P	-11.3	**-8.9**	**gp130, interleukin 6 signal transducer**	
94401_s_at	NM_053149	69.2	P	3.5	A	-16.0	158.7	P	19.6	P	-8.6	-12.3	hemogen, homologous to EDAG	(7)
97994_at	NM_009331	2887.5	P	512	P	-5.7	2868	P	460	P	-6.5	-6.1	TCF-1, Tsf7, transcription factor for thymocyte survival, regulates proliferation and apoptosis	(8, 9)
161788_f_at	NM_007901	56.7	P	17.9	P	-3.7	85.4	P	5.3	A	-8.0	-5.9	endothelial differentiation sphingolipid G-protein-coupled receptor 1 or sphigosine-1-phosphate ($S1P_1$ or Edg1), required for egress from lymph node	(10)
979995_at	NM_009331	792.6	P	139.6	P	-4.9	896.2	P	130.4	P	-7.0	-5.9	TCF-1 (see above)	(see above)
93728_at	NM_009366 NM_207652	101.1	P	20.5	P	-3.2	116	P	14.7	A	-8.6	-5.9	TSC-22, growth inhibitory transcriptional repressor, early response gene, homologous to GILZ, decreases with B cell activation	(4, 11)
93445_at	NM_009690	409.1	P	83.3	P	-5.1	247.4	P	51.8	P	-4.9	-5.5	CD5 antigen-like, apoptosis inhibitory 6, CT-2, also called Sp-a, Api6, and AIM, inhibits T cell apoptosis	(12-15)
160629_at	NM_026418	765.3	P	179.3	P	-4.3	615.4	P	78.2	P	-8.5	-5.4	RGS10, regulator of G-protein signalling 10	(16, 17)
93202_at	NM_011851	223.7	P	39.4	P	-4.3	215	P	30.1	P	-8.5	-5.4	CD73, ecto-5' nucleotidase, regulates avidity of LFA-1	(18)
102655_at	NM_010576	130.3	P	48.4	P	-2.5	163.9	P	19.3	P	-8.5	-4.5	integrin alpha 4, VLA4 alpha subunit, CD49d, regulates T lymphocyte traffic and activation	
95292_at	NM_010576	148.5	P	64.7	P	-2.1	202.5	P	39.5	P	-5.1	-4.1	integrin alpha 4, VLA4 alpha subunit, CD49d	
103015_at	NM_009744	208.9	P	80.6	P	-2.6	267.7	P	42.4	P	-4.9	-3.8	Bcl6, B-cell leukemia/lymphoma 6, oncogene and potent transcriptional repressor	(19, 20)
97844_at	NM_009061	223.3	P	44.6	P	-4.0	183.1	P	35.8	P	-3.5	-3.7	RGS2, regulator of G-protein signaling 2, regulated by IL-2, knockout is deficient in T cell activation	(21, 22)
102209_at	NM_016791	1172.1	P	366	P	-3.2	1258.2	P	332.7	P	-4.0	-3.6	NFATc1, nuclear factor of activated T-cells. This probeset detects 3' untranslated region of isoform NFATc1/A.	(23)
102282_g_at	**XM_284241**	692.9	P	257.6	P	-2.8	756	P	160.6	P	-4.3	**-3.6**	**CD27, TNF receptor family member 7, induces effector T cell differentiation; central memory marker, cooexpressed with CD62L**	**(24, 25)**
102397_at	NM_009824	93.9	P	23.6	P	-3.0	82.2	P	16.6	P	-4.0	-3.5	CBFA2T3, MTGR2, MT16, A-kinase anchoring protein in T lymphocytes	(26)
161765_f_at	NM_026418	267.1	P	75	P	-3.2	311.4	P	58	P	-3.7	-3.5	regulator of G-protein signalling 10	(27)
93319_at	NM_009025 NM_174859	858.1	P	232.2	P	-2.8	729.3	P	182.6	P	-3.7	-3.3	RAS p21 protein activator 3. Ras-GAP 3, Ras GTPase-activating protein 3, may bind IP4	(28)
102029_at	**NM_010551**	279.7	P	139.5	P	-2.1	324.3	P	84.1	P	-4.3	**-3.2**	**interleukin 16**	**(29)**
103454_at	NM_011461	373.5	P	130.7	P	-3.0	249.4	P	74.1	P	-3.2	-3.1	Spi-C transcription factor (Spi-1/PU.1 related)	
93915_at	NM_011136	351.6	P	192.4	P	-2.0	678.3	P	123.5	P	-4.3	-3.1	OCA-B, BOB.1/OBF.1, Pou2af1, required for B cell differentiation	(30, 31)
99024_at	NM_010753	423.9	P	113	P	-2.3	329.8	P	65.5	P	-4.0	-3.1	Max dimerization protein 4, Max-interacting transcriptional repressor	(32, 33)
98766_at	NM_011894	45.8	P	13.4	A	-3.2	35.8	P	13.7	A	-2.8	-3.0	SH3-domain binding protein 5 (BTK-associated)	(34)
100924_at	NM_008091	213.6	P	83	P	-2.6	161.9	P	41.5	P	-3.2	-2.9	GATA binding protein 3, required for Th2 differentiation	(35)
93397_at	NM_009915	606.1	P	166.7	P	-3.2	420.9	P	108	P	-2.6	-2.9	chemokine receptor CCR2, binds CCL2 (MCP)	(36)
Other genes:														
101587_at	NM_010145	407.7	P	12	A	-29.9	433.9	P	10.6	A	-32.0	-30.9	epoxide hydrolase 1, microsomal	
93351_at	NM_008278	204	P	15.2	A	-13.9	71.1	P	2.9	A	-36.8	-25.3	hydroxyprostaglandin dehydrogenase 15 (NAD)	
100068_at	NM_013467	61.5	P	8.7	A	-5.3	109.5	P	2.6	A	-39.4	-22.3	aldehyde dehydrogenase family 1, subfamily A1	
104375_at	NM_052994	151.9	P	4.2	A	-16.0	124	P	4.8	A	-22.6	-19.3	Spock2	
98098_at	NM_009799	178.7	P	9.5	A	-12.1	288	P	8.7	A	-21.1	-16.6	carbonic anhydrase 1	
103534_at	NM_016956	738.3	P	91.7	P	-8.0	556.9	P	13.3	A	-21.1	-14.6	hemoglobin, beta adult minor chain	
101869_s_at	NM_008220 NM_016956	3015.8	P	391.8	P	-8.0	1807.9	P	115.4	M	-16.0	-12.0	hemoglobin, beta adult major chain	
96122_at	NM_181588	185.2	P	14.9	A	-11.3	53	P	4	A	-10.6	-10.9	RIKEN cDNA 2310016A09 gene	
104696_at	NM_007799	247.4	P	18.8	A	-11.3	326.4	P	37	P	-9.8	-10.6	cathepsin E	
103340_at	NM_011270/ NM_021321	123.3	P	22.8	A	-4.0	166	P	8.3	A	-16.0	-10.0	Rhesus blood group CE and D	
103257_at	NM_178936	17.2	P	2.9	A	-3.7	30.3	P	1.5	A	-16.0	-9.9	RIKEN cDNA 4930577M16 gene	
160413_at	NM_008741	106.5	P	6.9	A	-9.2	71.2	P	6.1	A	-10.6	-9.9	neuron specific gene family member 2	
94781_at	NM_008218	1643.8	P	251.4	P	-5.7	853.6	P	66.3	A	-12.1	-8.9	hemoglobin alpha, adult chain 1	
99446_at	NM_007641	657.2	P	243.1	P	-2.6	1534.4	P	123.9	P	-13.0	-7.8	CD20, B cell marker	
97779_at	NM_008572	95.7	P	6.6	A	-13.0	132.5	P	58.2	M	-2.3	-7.6	mast cell protease 8	
98475_at	NM_016762	45.7	P	9.4	A	-4.0	40.4	P	3.6	A	-9.8	-6.9	matrilin 2	
103507_at	NM_010130	232.1	P	26.1	A	-9.2	86.1	P	25.5	A	-2.8	-6.0	EMR1,F480, macrophage marker	
93101_s_at	U96635	35.8	P	9.7	A	-3.5	37.9	P	4.4	A	-8.0	-5.7	E3 ubiquitin-protein ligase Nedd-4	
94247_at	AA600542	223.5	P	29.9	P	-8.5	175.4	P	19.8	P	-4.6	-5.5	RIKEN cDNA 5730453H04 gene	
100629_at	NM_010360	24.9	P	11.6	A	-2.0	23.8	P	2.6	A	-8.6	-5.3	glutathione S-transferase, mu 5	
96735_at	NM_019990	283.5	P	64.6	A	-4.3	191.2	P	24.1	A	-8.1	-5.2	START domain containing 10	
102065_at	NM_007995	258.1	P	41.4	P	-7.0	72.8	P	28.8	M	-3.0	-5.0	ficolin A	
94136_at	XM_488664	101.6	P	14.1	P	-4.9	107.2	P	13.3	A	-4.9	-4.9	RIKEN cDNA 5830431A10 gene	
103556_at	AI840158	1616.8	P	305.8	P	-4.9	1029.2	P	263.6	P	-4.6	-4.8		
92642_at	NM_009801	892.8	P	153.3	P	-5.3	1330.9	P	259	P	-4.3	-4.8	carbonic anhydrase 2	
103200_at	AK122269	528.7	P	93.5	P	-3.2	449.9	P	79.9	P	-5.7	-4.5	RIKEN cDNA 6330500D04 gene	
97519_at	NM_009263	848.7	P	124	P	-6.1	695	P	238	P	-3.0	-4.5	osteropontin, secreted phosphoprotein 1	
92614_at	NM_008321	461.5	P	93.8	P	-5.3	342.5	P	83.9	P	-3.5	-4.4	inhibitor of DNA binding 3	
102762_r_at	NM_011269	174.2	P	36.2	P	-3.2	229	P	47.2	P	-5.3	-4.3	Rhesus blood group-associated A glycoprotein	
104173_at	NM_007641	608.6	P	196.2	P	-2.5	1182.5	P	108.7	P	-6.1	-4.3	CD20, B cell marker	
94991_at	AW046661	142.9	P	36.9	P	-4.0	149.9	P	29.3	P	-4.3	-4.1	synaptopodin	
102254_f_at	AA28958	79.1	P	39.7	M	-2.3	69	P	12.5	A	-5.7	-4.0		
104000_at	NM_197999	100.9	P	23.3	A	-4.0	118.4	P	30.2	A	-4.0	-4.0	RIKEN cDNA 2210023G05 gene	
160255_at	AA657044	1303.2	P	409	P	-3.0	1238	P	261.7	P	-4.9	-4.0	RIKEN cDNA 1110004P15 gene	
92198_s_at	NM_007827	38.3	P	17.7	A	-2.0	46.6	P	7.2	A	-6.1	-4.0	DAF 2, decay accelerating factor 2	
98976_at	NM_021475	194.6	P	34	P	-4.9	74.5	P	26.2	M	-3.0	-4.0	Decysin, disintegrin metalloprotease	
103299_at	NM_178911	360.4	P	101.9	P	-3.2	297.6	P	55.9	P	-4.6	-3.9	expressed sequence AI132321	
160486_at	NM_025455	173.9	P	48.1	P	-3.7	186.3	P	35.9	P	-4.0	-3.9	RIKEN cDNA 1810010N17 gene	
162172_f_at	XM_486230	23.1	P	12.5	P	-2.5	21.2	P	1.7	A	-5.3	-3.9	E3 ubiquitin-protein ligase Nedd-4	
96481_at	NM_178877	58	P	15.4	A	-4.0	46.7	P	11.8	A	-3.7	-3.9	expressed sequence C80638	
93507_at	NM_011594	119.3	P	26.6	A	-4.6	81.6	P	26.3	A	-3.0	-3.8	TIMP-2, tissue inhibitor of metalloproteinase 2	
92661_at	NM_007955	28.7	P	9	P	-2.8	24.1	P	4.5	A	-4.6	-3.7	embryonic stem cell phosphatase, mOST-PTP	
98855_r_at	AA014745	38.3	P	16.1	P	-3.0	73.2	P	16.1	P	-4.3	-3.7		
104239_at	NM_020286	714	P	210.1	P	-2.8	770.3	P	170.4	P	-4.3	-3.6	phemx, pan hematopoietic expression	
93963_at	NM_172514	246.8	P	64.2	P	-3.5	232.9	P	78.8	P	-3.7	-3.6	expressed sequence AI661017	
94332_at	NM_011808	310.9	P	111	P	-2.8	389.5	P	86.3	P	-4.6	-3.6	Ets-1, transcription factor	

		Experiment #1				Experiment #2						
Affymetrix probe set	RefSeq or transcript ID	rat Ig	P or A	anti OX40	P or A	fold change	rat Ig	P or A	anti OX40	P or A	fold change	mean fold change	Gene or Protein Product	Reference
100528_at	NM_009459	186.2	P	58.1	P	-3.0	257.8	P	52.4	P	-4.0	**-3.5**	ubiquitin-conjugating enzyme E2H	
104014_at	NM_010424	215.5	P	34.5	P	-4.0	130.9	P	42.9	M	-3.0	**-3.5**	hemochromatosis	
160261_i_at	AI849416	702.1	P	221.9	P	-3.2	705.7	P	198.3	P	-3.7	**-3.5**		
93321_at	NM_008328	412.1	P	201.7	P	-2.0	372	P	31.3	P	-4.9	**-3.5**	interferon activated gene 203	
95356_at	NM_009696	641.6	P	142.3	P	-4.0	452.6	P	200.5	P	-3.0	**-3.5**	apolipoprotein E	
96710_at	XM_126043	1194.6	P	370.4	P	-3.2	991.6	P	276.7	P	-3.7	**-3.5**	H2A histone family, member V	
98033_at	NM_025806	1078	P	375.3	P	-3.2	613.6	P	227.2	P	-3.7	**-3.5**	RIKEN cDNA 1100001H23 gene	
92507_at	NM_011682	66.6	P	15.6	P	-3.5	69.2	P	12	M	-3.2	**-3.4**	utrophin	
102094_f_at	AI841270	513.1	P	216.7	P	-4.3	434.3	P	141.2	P	-2.0	**-3.1**		
103617_at	NM_010016	33.5	P	10.9	P	-2.3	30.9	P	7.7	A	-4.0	**-3.1**	DAF 1, decay accelerating factor 1	
95984_at	AI58616	67	P	23.7	P	-2.1	53.1	P	12.4	A	-4.0	**-3.1**		
161010_r_at	AI843786	18.9	P	2.7	A	-3.2	18.1	P	8.6	A	-2.8	**-3.0**		
95940_f_at	NM_198301	307.9	P	107.4	P	-2.5	261.5	P	61	P	-3.5	**-3.0**	cDNA sequence BC052328	
104612_g_at	AI854008	496.1	P	156.5	P	-3.0	551.1	P	178.8	P	-2.8	**-2.9**		
94432_at	NM_009175 NM_145933	494.2	P	179.4	P	-2.6	608.2	P	204.8	P	-3.2	**-2.9**	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	
95386_at	XM_128781.5	326.2	P	95.1	P	-3.2	182	P	34.1	A	-2.5	**-2.9**	predicted lysocardiolipin acyltransferase	
96615_at	NM_025347 NM_026875	751.5	P	222.2	P	-3.2	859.3	P	230.7	P	-2.8	**-2.9**	yippee-like 3, Ypel3	
99465_at	NM_010788	75.7	P	36.3	P	-2.0	72.3	P	15.5	A	-3.7	**-2.9**	methyl CpG binding protein 2	

[Supplemental Table 2 footnote]
*TCR transgenic T cells were injected into antigen transgenic mice with or without anti-OX40. 3.5 days later, transgenic T cells were enriched and total RNA was purified for hybridization on an Affymetrix MG-74Av2 gene chip. This table shows genes whose expression decreases ≥ 2.9-fold with anti-OX40 treatment. The hybridization signal and detection call (P for present and A for absent) for anti-OX40 and control rat IgG for genes that decreased in two independent experiments are shown. The fold-change for each experiment, the mean fold change for the two experiments, gene or protein name, and references for some genes are also reported. Please refer to the materials and methods for a description of the data analysis.

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Supplement Table 3. *Selected genes of interest that change less than 2.9-fold in T cell preparations from OX40-treated animals.*
Genes in bold are mentioned in the article text.

		Experiment # 1					Experiment # 2							
Affymetrix probe set	RefSeq or transcript ID	rat Ig	P or A	anti-OX40	P or A	fold change	rat Ig	P or A	anti-OX40	P or A	fold change	comparison call and mean fold change		Gene or Protein Product	Reference
98868_at	**NM_177410**	45.4	P	**46.4**	P	1.1	36	P	**35.2**	P	1.1	**NC**	**1.1**	**Bcl-2**	(1, 2)
99027_at	**NM_009743**	171.1	P	**272.5**	P	1.6	161.9	P	**275.9**	P	2	**I**	**1.8**	**Bcl-x**	(1, 2)
160920_at	NM_007537	37.7	A	76.4	P	2.1	60.2	M	58.9	P	-1.1	NC	0.5	Bcl-2l2 (Bcl-w)	
93093_at	**NM_008562**	416.3	P	**366.5**	P	-1.4	581	P	**350.8**	P	-1.5	**NC**	**-1.5**	**Mcl-1**	(3)
99418_at	**NM_009754**	63.9	P	**50.1**	P	-1.1	60	P	**64.5**	P	1.1	**NC**	**0.0**	**Bcl2l11 (Bim)**	(4)
AFFX-MurFAS_at	**NM_007987**	32.3	P	**37.9**	P	1.4	24.7	P	**46.3**	P	1.2	**NC**	**1.3**	**Tnfrsf6 (Fas)**	(5)
92793_at	**NM_011609**	182.	P	**198.6**	P	-1.1	152	P	**168.3**	P	1.3	**NC**	**0.1**	**Tnfrsf1a (TNFR1)**	(6, 7)
94928_at	NM_011610	631.9	P	358.6	P	-1.7	699	P	275.2	P	-2.3	D	-2.0	Tnfrsf1b (TNFR2)	(6, 8)
103514_at	**NM_178589**	98.4	P	**35.4**	A	-2.3	58.6	P	**31.7**	A	-1.6	**NC**	**-2.0**	**Tnfrsf21 (DR6)**	(9)
97718_at	**NM_009843**	124.8	P	**113**	P	1.2	156	P	**165.6**	P	1.1	**NC**	**1.2**	**CTLA-4**	(10, 11)
98836_at	**NM_008798**	332.4	P	**190.5**	P	-1.3	271	P	**154.8**	P	-2.0	**D**	**-1.7**	**PD-1**	(10, 12)
97113_at	NM_010177	71.7	P	128.9	P	1.6	61	A	84.2	P	1.7	I	1.7	FasL	(5)
98282_at	NM_017480	531	P	416.2	P	-1.4	493	P	389.5	P	-1.2	D	-1.3	ICOS	(10)
99532_at	NM_009427	106.4	P	68.9	P	-1.6	154	P	41.2	P	-3.5	D	-2.6	Tob1	(13)

[a]TCR transgenic T cells were injected into antigen transgenic mice with or without anti-OX40. 3.5 days later, transgenic T cells were enriched and total RNA was purified for hybridization on an Affymetrix MG-74Av2 gene chip. This table shows selected genes whose expression was unchanged or changed less than 2.9-fold with anti-OX40 treatment. The table shows the hybridization signal, detection call (P for present and A for absent), and fold change for anti-OX40 and control rat IgG for these genes for two independent experiments. Also shown are the change call (NC for no change, I for increased, D for decreased), the gene or protein name, and references for some genes. Please refer to the materials and methods for a description of the data analysis.

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Chapter 4—Manuscript #3

OX40-Mediated Differentiation to Effector Function Requires IL-2 Receptor Signaling but not CD28, CD40, IL-12Rβ2, or T-bet

Cortny A. Williams, Susan E. Murray, Andrew D. Weinberg, and David C. Parker

*Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR 97239 †Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center, Portland, OR 97213

Summary

Antigen-specific CD4 T cells transferred into unirradiated antigen-bearing recipients proliferate, but survival and accumulation of proliferating cells is not extensive and the donor cells do not acquire effector functions. We previously showed that a single costimulatory signal delivered by an agonist antibody to OX40 (CD134) promotes accumulation of proliferating cells and promotes differentiation to effector CD4 T cells capable of secreting IFN-γ. In this study, we determined whether OX40 costimulation requires supporting costimulatory or differentiation signals to drive acquisition of effector T cell function. We report that OX40 engagement drives effector T cell differentiation in the absence of CD28 and CD40 signals. Two important regulators of Th1 differentiation, IL-12 receptor and T-bet, also are not required for acquisition of effector function in CD4 T cells responsive to OX40 stimulation. Finally, we show that CD25-deficient CD4 T cells produce little IFN-γ in the presence of OX40 costimulation compared to wild type, suggesting that IL-2 receptor signaling is required for efficient OX40-mediated differentiation to IFN-γ secretion.

Introduction

Effective CD4 T cell immunity requires recognition of cognate antigen followed by additional costimulatory signals. The classical costimulatory receptor, CD28, amplifies signals from the T cell receptor, which decreases the threshold for antigen specific activation, resulting in responsiveness to lower doses of antigen (23). CD28 signals lead to enhanced expression of transcription factors, antiapoptotic genes, cytokines, and cytokine receptors that lead to survival, differentiation, avoidance of anergy, and effector T cell function (287). A variety of other receptors, such as members of the tumor-necrosis factor receptor (TNFR) family, including 4-1BB, CD27, CD30, and OX40, also exhibit costimulatory function (15). Expression of these receptors and their ligands is selective in time and place and tightly controlled by antigenic stimulation and inflammatory or danger signals. While these costimulation pathways can reinforce outcomes initiated by CD28, they also have unique roles in survival and promote differentiation to effector cells that contribute to a specific immune response. For example, OX40 and OX40L are induced only after activation (81, 91), and OX40 signaling to CD4 T cells has been shown to directly enhance survival (80, 113, 114) and promote effector function by enhancing either Th1 or Th2 differentiation (1, 2, 164, 177, 195).

Adoptive transfer of antigen-specific CD4 T cells into unirradiated antigen-bearing recipients results in robust proliferation of donor cells and infiltration of tissues, but effector function is limited and recipients do not develop clinical signs of disease (1, 2, 57), presumably due to a lack of costimulation. Transfer of transgenic T cells into CD40 deficient antigen bearing recipients results in similar proliferation of donor cells,

suggesting that conventional costimulation is not limiting for T cell proliferation in this system (1). A single injection of agonist anti-OX40 provides a signal that induces accumulation of proliferating donor cells that acquire the ability to produce IFN-γ and cause disease (1, 2). In this model, donor T cells are unresponsive upon restimulation through the TCR, but produce IFN-γ upon stimulation distal to the TCR or through cytokine receptors (1). Agonist anti-OX40 treatment results in death of the recipient, presumably due to cytokine-producing effector T cells infiltrating non-lymphoid organs (1). OX40 also promotes differentiation early in T cell priming and produces larger changes in expression of cytokine and cytokine receptor genes than in survival genes (2). In other reports, OX40-driven differentiation results in enhanced secretion of IL-4 and IL-5 (165) and germinal center formation (89) in Th2 responses, and IL-2 and IFN-γ production in Th1 responses (1, 167). OX40 signaling has also been reported to reverse previously established peripheral tolerance (175).

OX40 costimulation could be directly orchestrating the acquisition of effector function, or could require supportive signaling from other costimulatory and cytokine receptors or other downstream regulators of differentiation to induce the effector T cell functions seen in each of these models. For example, OX40-driven differentiation could synergize with CD28 to promote activation of transcription factors, as suggested by a recent report (288), or OX40 could require interaction with supporting costimulatory pathways in APC such as CD40. Upon ligation with CD40L on T cells, CD40 enhances several costimulatory ligands on APC that interact with T cell costimulatory receptors, and consequently promote a positive feedback loop that drives differentiation (31). In a previous report, we showed that OX40-deficient donor cells gain effector function as

bystanders to wild type antigen-responsive cells stimulated with anti-OX40 (2). In this system, anti-OX40 was the only exogenous adjuvant provided to initiate costimulation, indicating that OX40 engagement led to activation of alternative pathways capable of driving differentiation of OX40-deficient T cells.

In this study, we use two previously published adoptive transfer systems (1, 2) to determine whether OX40 engagement promotes differentiation in the absence of costimulatory or cytokine receptors, or T-bet, a transcription factor promoting Th1 differentiation. We show that OX40 costimulation drives differentiation to IFN-γ-secreting effector T cells in the absence of CD28 or CD40 costimulation. We also show that OX40 induces acquisition of effector function in the absence of the IL-12 receptor and T-bet, two important regulators of Th1 differentiation. Finally, we examined the effect of OX40 costimulation in the absence of the IL-2 receptor alpha chain, CD25, and found that acquisition of effector function in this case requires functional IL-2 receptor signaling. Thus, while OX40 promotes differentiation of CD4 T cells in the absence of other costimulatory and differentiation pathways, IL-2 receptor signaling is essential for the development of effector CD4 T cells in response to OX40 costimulation.

Materials and Methods

Mice and Adoptive Transfers

Mice were housed under specific pathogen-free conditions at the Oregon Health & Science University animal facility. (B6.CD45.1 x bm12)F1 mice were made by crossing female B6.CD45.1 to B6.C-H2^{bm12}/KhEg mice, obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6J, CD28$^{-/-}$ (B6.129S2-CD28), IL-12Rβ2$^{-/-}$ (B6.129S1-Il12rb1), T-bet$^{-/-}$ (B6.129S6-Tbx21), and CD25$^{-/-}$ (B6.129S4-Il2ra) were also obtained from the Jackson Laboratory. Pigeon cytochrome C (PCC) specific AND TCR-transgenic, Rag-1-deficient mice, PCC-specific AD.10 TCR transgenic, and antigen transgenic mice expressing I-E^{k} plus covalently associated antigenic peptide on the C57BL/6 background sufficient or deficient in CD40 have been described previously (1). Donor splenocytes plus lymph nodes were pooled for each experiment and were prepared for intravenous injection as previously described (1). In some experiments, donor cells were labeled with 2 μM CFSE in 0.1% BSA in PBS for 10 minutes at 37°C and washed in Hanks Buffered Saline Solution (HBSS) with 2% serum. Cells were injected intravenously with 50 μg anti-OX40 or control IgG in HBSS without serum into unirradiated (B6.CD45.1 x bm12)F1 recipients or into antigen transgenic recipients.

Antibodies and Cytokines

PerCP anti-CD4 (RM4-5), biotin anti-CD25 (7D4), biotin anti-CD69 (FN50), allophycocyanin (APC) anti-IL-2 (JES6-5H4), PE anti-IL-17 (TC1-8H4.1), and labeled isotype controls were purchased from BD PharMingen (San Diego, CA). APC anti-IFN-γ (XMG1.2), PE or FITC anti-CD45.1 (A20), APC Streptavidin, PE goat anti-rat IgG and

appropriate isotype controls were purchased from eBiosciences (San Diego, CA). Biotin chicken anti-OX40 and isotype control were produced and purified for staining cells. Anti-OX40 antibody from clone OX86 (European Cell Culture Collection, Porton Down, UK) was produced and purified for i.v. injection. Rat IgG was purchased from Cappel, ICN Pharmaceuticals (Costa Mesa, CA). Anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), and recombinant mouse IL-2 were produced and purified for in vitro culture. Recombinant mouse IL-12 was purchased from Cell Sciences (Norwood, MA), recombinant mouse IL-18 and recombinant mouse IL-23 were purchased from R & D Systems (Minneapolis, MN).

Cell Culture and Flow Cytometry

Spleen cell suspensions were prepared for intracellular cytokine staining as previously described (1). In some experiments splenocytes were stimulated with 100 ng/mL IL-18 and 8 ng/mL IL-23 for five hours before intracellular cytokine staining. Labeled cells were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry, San Jose, CA) and analyzed using FlowJo (Tree Star, Inc., San Jose, CA). Th1 and Th2 cultures were set up using AD.10 splenocytes as described (289).

Quantitative PCR

10^6 AND TCR transgenic T cells were transferred with 50 μg anti-OX40 or control IgG into antigen transgenic recipients congenic for CD45.1. 3.5 days later, TCR transgenic T cells were isolated from recipient splenocytes by sorting CD45.1 negative CD4 positive cells to $\geq$ 92% purity on a FACSVantage flow cytometer (BD Immunocytochemistry, San Jose, CA). Total RNA was purified from Th1, Th2, and

sorted T cell populations as previously described (2). cDNA was made using the Stratascript First-Strand Synthesis System (Stratagene, La Jolla, CA). Quantitative real time PCR was performed on an ABI 7700 using SYBR Green Master Mix (Applied Biosystems, CA) and previously published primer sequences for T-bet and β-actin to measure DNA amplification (289). T-bet and β-actin C_T values were converted to standard curve values, and T-bet/β-actin ratios were analyzed as fold over baseline (Th2 cells). Th1 cells were used as a positive control.

Results

CD40 signaling to APC is not required for OX40 driven differentiation

To understand how OX40 signaling affects CD4 T cell differentiation, we sought to examine the requirement for other costimulatory signals during an immune response driven by an agonist antibody to OX40. CD40 engagement functions as a DC maturation stimulus, enhancing antigen presentation and inducing costimulatory ligand expression, which contributes to a positive feedback loop to promote donor CD4 T cell differentiation (31). Since DC in our model are not exogenously activated, we reasoned that if OX40 engagement increases CD40L expression, allowing CD40 signaling to initiate DC maturation, then the absence of CD40 on DC may prevent or dampen DC maturation, and subsequently affect the acquisition of effector function in CD4 T cells initiated by OX40. To test this hypothesis in vivo, we used our previously published model in which AND Rag1$^{-/-}$ TCR-transgenic T cells specific for a pigeon cytochrome C peptide are transferred into antigen-transgenic mice (1). We transferred 10^6 TCR-transgenic CD4 T cells in a spleen and lymph node suspension with anti-OX40 or control IgG into antigen-transgenic mice that are deficient in CD40 or not, and harvested spleens 5 days later. As seen in Fig. 1A and Table 1, all donor cells divide and dilute CFSE to background levels, but accumulation of donor CD4 T cells in spleen is diminished in CD40-deficient hosts compared to CD40$^{+/+}$ recipients. However, donor T cells from CD40$^{-/-}$ recipients that received OX40 costimulation accumulated 3-fold more donor cells compared to control IgG, and the donor cells had acquired effector function, as measured by CD25 expression and IFN-γ production (Fig. 1 and Table 1). Moreover, a higher percentage of donor CD4 T cells from CD40$^{-/-}$ recipients produced IFN-γ, especially

directly ex vivo, compared to $CD40^{+/+}$ recipients (40% vs. 15%), and were able to respond more robustly to restimulation in vitro with IL-12 and IL-18, a cytokine combination known to induce robust IFN-γ production (277), and PMA and ionomycin (Fig. 1B). These data show that OX40 costimulation induces differentiation to cytokine-producing effector CD4 T cells in the absence of CD40 signaling to APC. These data also suggest that signals to DC via CD40 may be important in enhancing CD4 T cell survival because in the absence of CD40, donor cell recovery from spleen was reduced.

IL-12 receptor and T-bet, part of the Th1 commitment pathway, are not required for OX40-mediated differentiation to IFN-γ production

IL-12 receptor signaling is important for commitment to Th1 effector cell differentiation (290). In a previous report, we showed that IL-12 receptor gene expression was enhanced in Th1 effector cells upon OX40 costimulation (2). Agonist anti-OX40 drives effector T cell development in a population of alloreactive B6 CD4 T cells transferred into unirradiated, (B6.CD45.1 x bm12)F1 recipients, which differ on one allele from MHC class II $I\text{-}A^b$ in the peptide binding domain (2). To test whether the IL-12 receptor influences the ability of anti-OX40 stimulated alloreactive donor cells to differentiate, we transferred 10^7 $IL\text{-}12R\beta2^{-/-}$ CD4 T cells in a spleen suspension with anti-OX40 or control IgG into (B6.CD45.1 x bm12)F1 recipients. Five days after donor cell transfer splenocytes were analyzed for activation markers and cytokine production. As shown in Fig. 2A, $IL\text{-}12R\beta2^{-/-}$ donor cell recovery and CD25 expression were similar to wild type, in the presence or absence of anti-OX40. Also shown in Fig. 2A, OX40 engagement promotes accumulation of $CD4^{hi}$ T cells, which also appear to be CD45.1 positive. This observation is not surprising since activated CD4 T cells have been shown

to acquire membrane proteins during T:APC interactions (291). IFN-γ production after in vitro restimulation with media alone, PMA and ionomycin, and IL-18 was also similar between experimental groups, although IL-12Rβ2$^{-/-}$ donor cells treated with anti-OX40 did not make as much IFN-γ after IL-12 and IL-18 stimulation, as expected when the IL-12 receptor is absent (Fig. 2B). These results show that although OX40 costimulation enhanced IL-12 receptor expression in wild type CD4 T cells (2), OX40 promoted differentiation of Th1 effector cells independent of IL-12 receptor signaling.

T-bet is a transcription factor that regulates IFN-γ gene transcription, and many reports have demonstrated the requirement for T-bet expression in Th1 effector cell differentiation (292, 293). Given that in our models, OX40 costimulation promotes antigen specific CD4 T cells to acquire a robust Th1 phenotype, we reasoned that OX40 signaling would enhance T-bet expression to drive Th1 differentiation. To test this hypothesis, we transferred 10^6 AND TCR transgenic T cells with anti-OX40 or control IgG into antigen transgenic recipients congenic for CD45.1. 3.5 days later, we purified TCR transgenic T cells from the spleen and extracted total RNA for quantitative PCR to measure levels of T-bet mRNA in comparison to in vitro cultured Th2 cells (baseline) and Th1 cells (positive control). As shown in Fig. 3A, OX40 costimulation induced T-bet mRNA expression more than 10-fold over control IgG treated donor cells to levels comparable to cultured Th1 cells.

The data in Fig. 3A show that T-bet expression is elevated as a result of OX40 costimulation, but does not address the importance of T-bet function in the OX40 signaling pathway. Therefore, we sought to test whether the effects of OX40 are dependent on this master regulator of Th1 differentiation, or if OX40 costimulation can

independently induce differentiation and IFN-γ effector cytokine production. We transferred 7.5 x 10^6 T-bet$^{-/-}$ CD4 T cells in a spleen and lymph node suspension with anti-OX40 or control IgG into (B6.CD45.1 x bm12)F1 recipients. Five days after transfer, recovery of donor T-bet$^{-/-}$ cells in spleen was similar to that of wild type donor cells, with control IgG or anti-OX40 (Fig. 3B first row, and Table 1). Compared to control IgG, anti-OX40 promotes an activated phenotype in T-bet$^{-/-}$ divided donor cells, with high expression of CD25 and OX40 (Fig. 3C). Interestingly, anti-OX40 induces greater expression of OX40 on T-bet$^{-/-}$ donor cells compared to T-bet$^{+/+}$ donors. These data show that despite a deficiency in T-bet, OX40 costimulation supports an activated phenotype in alloreactive CD4 T cells.

We next examined whether OX40 costimulation could induce effector cytokine production in T-bet$^{-/-}$ CD4 T cells. We measured IL-4, IL-10, TNF-α, IL-2, and IL-17 in addition to IFN-γ production after in vitro restimulation with PMA and ionomycin or media alone to detect OX40-mediated acquisition of effector cytokine production. Anti-OX40 promoted IFN-γ production in T-bet deficient alloreactive donor cells, although 4-fold fewer compared to wild type donors when stimulated with PMA and ionomycin (Fig. 3B, fourth row). Of note, T-bet$^{-/-}$ donor cells made IL-17 with or without OX40 signaling when stimulated with PMA and ionomycin (Fig. 3B). In addition, IL-23 and IL-18, a cytokine combination reported to induce IL-17 production in CD4 T cells (294), enhanced IL-17 production in T-bet$^{-/-}$ CD4 T cells, and particularly in donor cells from anti-OX40 treated mice (Fig. 3B, third row). No T-bet$^{+/+}$ donor CD4 T cells made IL-17 in response to IL-23 and IL-18, and very few produced IL-17 in response to PMA and ionomycin (Figure 3B). IL-12 and IL-18 stimulation prompted T-bet$^{+/+}$ divided donors to

make three times as much IFN-γ as with IL-23 and IL-18 (data not shown), while IL-12 and IL-18 promoted equal IFN-γ production from T-bet$^{-/-}$ divided donor cells as from cells stimulated with IL-23 and IL-18. T-bet$^{-/-}$ donor cells subjected to anti-OX40 made very little IL-2 compared to T-bet$^{+/+}$ donor cells (0.5% vs 13%), while both groups treated with control IgG made equal amounts of IL-2 (20%) when restimulated with PMA and ionomycin (data not shown). All other cytokines tested were not induced above background in any experimental group (data not shown). These data show that in the absence of T-bet, total OX40-mediated effector cytokine production is reduced compared to wild type, indicating that T-bet is required for optimal cytokine production. However, these data also suggest that in the absence of T-bet, OX40 supports the ability to acquire alternative effector functions, as noted by the additional responsiveness to IL-23 and IL-18, and production of IL-17 in addition to IFN-γ.

A signal through OX40 can overcome the defect in differentiation in CD28$^{-/-}$ CD4 T cells

Signals through constitutively expressed CD28 promote initial T cell activation and additional T cell effector function (287), while OX40 expression is delayed in T cell priming. To test the hypothesis that OX40 promotes differentiation in CD4 T cells in the absence of CD28, we transferred 7.5 x 10^6 CD4 T cells deficient in CD28 into (B6.CD45.1 x bm12)F1 recipients. Five days after T cell plus control IgG transfer, few CD28$^{-/-}$ donor cells were recovered from spleen, but some had proliferated, as measured by CFSE dilution (Fig. 4A). Wild type donor cells accumulated 15 to 20-fold more donor cells with or without OX40 costimulation, confirming that CD28 is important for progressive cell division and/or accumulation of divided cells (Fig. 4A and Table 1).

However, in the absence of CD28, OX40 enhanced accumulation of divided donor cells compared to control IgG, suggesting that OX40 signals contribute to optimal accumulation of divided donor cells independent of CD28. We also looked at T cell activation by measuring CD25 and OX40 expression. As seen in previous in vitro studies (95), OX40 is expressed on CD28-deficient divided donor cells, as well as on wild type (Fig. 4A, bottom row), and anti-OX40 enhanced CD25 expression in $CD28^{-/-}$ divided donor cells compared to control IgG (Fig. 4A, middle row). To determine if OX40 can promote effector cytokine production in the absence of CD28, we examined IFN-γ production from $CD28^{-/-}$ donor CD4 T cells exposed to anti-OX40. Day 5 splenocytes from each animal were restimulated in vitro for 5 hours with media alone, IL-12 and IL-18, or PMA and ionomycin. Although a higher percentage of wild type than $CD28^{-/-}$ donor cells from anti-OX40 treated mice are able to make IFN-γ upon in vitro restimulation, OX40 costimulation enhanced IFN-γ production by $CD28^{-/-}$ divided donor cells in response to IL-12 and IL-18 or PMA and ionomycin compared to $CD28^{-/-}$ donor cells plus control IgG (Fig. 4B). Although CD28 is required for optimal effector cytokine production, these data show that OX40 signaling supports the necessary machinery to acquire effector cytokine production without CD28 costimulation.

OX40 mediated differentiation is dependent on IL-2 receptor signaling

IL-2 has primarily been characterized as a T cell growth factor (34). However, many recent studies have found that IL-2 also plays a role in driving effector T cell differentiation (39-41, 295). In our systems, we note that OX40 engagement induces and maintains expression of the IL-2 receptor alpha chain, CD25, but the importance of CD25

expression in acquisition of effector function is unknown (1, 2). To test the importance of IL-2 receptor signaling on OX40-mediated effector cell development, we transferred 7.5 x 10^6 $CD25^{-/-}$ CD4 T cells from mice less than 8 weeks of age with anti-OX40 or control IgG into (B6.CD45.1 x bm12)F1 recipients, and five days later looked at donor CD4 T cell recovery in the spleen. Few $CD25^{-/-}$ donor cells were recovered, but some proliferated, as measured by CFSE dilution (Fig. 5A and Table 1). However, 10 to 20-fold more divided wild type donor cells accumulated with or without costimulation (Fig. 5A and Table 1), suggesting that CD25 is important for cell division or accumulation of dividing cells. Importantly, in the absence of CD25, OX40 still induced a 3-fold increase in accumulation of the divided donor cells compared to control IgG (Table 1), suggesting that costimulation via OX40 can rescue the accumulation defect to some extent, but not to wild type levels.

To determine if OX40 costimulation induces effector function in CD25 deficient CD4 T cells, we restimulated splenocytes from each group with media, IL-12 and IL-18, or PMA and ionomycin for 5 hours. Surprisingly, we found that although OX40 engagement of CD25 deficient donor T cells causes an increase in the percentage of IFN-γ^+ cells in unstimulated cultures and in response to IL-12 and IL-18, the percentage of IFN-γ^+ cells is 20-fold less than that of wild type donor cells from OX40 treated animals (Fig. 5A). $CD25^{-/-}$ donor cells from control IgG and anti-OX40 treated animals were equally able to make IFN-γ upon PMA and ionomycin stimulation and at the same low levels as wild type donor cells from control IgG treated animals (Fig. 5A). These data indicate that although OX40 signaling is able to provoke slight accumulation of effector

CD4 T cells independent of CD25 expression, IL-2 receptor signaling is essential for efficient differentiation to IFN-γ secretion in OX40-responsive CD4 T cells.

CD25 deficient T cells develop autoimmunity due to a defect in regulatory T cells (296, 297). To ensure that the observed donor CD4 T cell proliferation was antigen specific and not a byproduct of autoimmunity, we transferred $CD25^{-/-}$ cells into syngeneic B6 hosts with anti-OX40 or control IgG and looked at donor CD4 T cell recovery and differentiation five days later. As shown in Table 1, very few divided donor CD4 T cells accumulated in syngeneic hosts with either anti-OX40 or control IgG, confirming that the transfer of CD25 deficient CD4 T cells into (B6.CD45.1 x bm12)F1 recipients, reported above, resulted in an antigen specific immune response. In addition, the divided donor cells transferred into syngeneic hosts do not produce IFN-γ upon in vitro restimulation (data not shown). We measured the expression of OX40 on $CD25^{-/-}$ divided donor cells to ensure that the lack of differentiation reported above was not due to lack of the OX40 receptor on $CD25^{-/-}$ donor cells (data not shown). Taken together, these data show that OX40 signaling greatly enhances effector CD4 T cell effector function in an IL-2 receptor-dependent manner.

Discussion

Antigen-specific CD4 T cells transferred into unirradiated antigen-bearing recipients undergo massive proliferation, but survival of divided cells is limited and acquisition of effector function does not occur (1, 2, 57). Lack of differentiation in this case could be attributed to lack of costimulatory signals, as there is no exogenous adjuvant or danger signal provided in this system to bolster costimulation. The introduction of a single injection of anti-OX40, a costimulatory signal that promotes differentiation (1, 2), provides a model for understanding the mechanism of effector CD4 T cell development. In this study, we asked whether OX40 costimulation required other costimulatory or differentiation signals to drive acquisition of effector function. We found that OX40 costimulation promotes effector cytokine production in the absence of CD28 and CD40. OX40 signals also partially rescue a survival and/or accumulation defect in CD28 mutants, and can promote limited accumulation of donor T cells in $CD40^{-/-}$ recipients. We also found that OX40 drives acquisition of effector function in the absence of two important Th1 differentiation signals, IL-12 receptor and T-bet. Finally, we show that functional IL-2 receptor signaling is required for enhanced differentiation to IFN-γ secretion in donor T cells responsive to OX40 costimulation.

Antigen recognition and costimulation have been reported to promote T cell activation and proliferation independent of IL-2, but differentiation to effector function in these models was IL-2 dependent (39, 298, 299). Recently, two reports have used recombinant IL-2/anti-IL-2 immune complexes, which enhanced the potency of IL-2 for T cells in vivo, and showed the importance of IL-2 in promoting differentiation of effector T cells (40, 41). In our model, OX40 costimulation promoted limited

accumulation of CD25-deficient donor CD4 T cells, but very few of those cells acquired the ability to secrete IFN-γ. Although CD25 was not necessary for OX40 expression, it is possible that the lack of CD25 expression in CD4 T cells disconnects the OX40 signaling pathway that drives differentiation. OX40 activates NFκB (144), which can then interact with the NFκB-responsive elements in the IL-2 and CD25 promoters (151). Enhanced IL-2 receptor signaling induces other cytokine and cytokine receptor expression, including CD25, ultimately driving full effector cytokine production (271, 300). In this way, OX40 costimulation could require functional IL-2 receptor signaling to enhance effector cytokine and cytokine receptor expression to promote effector function. Alternatively, IL-2 receptor signaling could be important in establishing development of effector function early in T cell priming, and effects of OX40 engagement could depend upon early signals from the IL-2 receptor. In a previous report, we showed that antigen responsive CD4 T cells expressed high levels of CD25 within 24 hours after T cell transfer, but in the absence of OX40 costimulation, expression waned by 48 hours and few cells expressed CD25 at 72 and 120 hours. OX40 costimulation induced effector cytokine production beginning at 48 hours, and CD25 expression was maintained at high levels at all time points (2). Those experiments did not show that enhanced CD25 expression accounts for OX40 effects on acquisition of effector function, but data in this report clearly show that IL-2 receptor signaling is essential for enhanced effector function in CD4 T cells in response to OX40 engagement.

We found that OX40 ligation induces effector cytokine production in the absence of either CD28 on donor CD4 T cells or CD40 on recipient APC. These data support findings in a related model of skin graft rejection, in which CD28 and CD40L double-

deficient naïve or memory T cells were able to induce graft rejection, even in the presence of blocking antibodies to ICOS:ICOSL, 4-1BB:4-1BBL, or CD27:CD70. Furthermore, $OX40^{+}$ $CD4^{hi}$ T cells were found in the target organ, and blocking OX40:OX40L signaling improved acceptance of the graft (189, 301). Our results also show that when CD40 is deficient on recipient APC, OX40 is less effective at enhancing accumulation of donor cells but more effective at promoting IFN-γ secretion. These data suggest that OX40 does not independently enhance accumulation of donor cells in this model, and instead requires additional signals from DC licensing via CD40 engagement to restore accumulation of CD4 T cells. It is also possible that additional signals from endogenous activated T cells capable of responding to anti-OX40 could affect effector cytokine production and accumulation of donor CD4 T cells. However, we did not detect a difference in effector cytokine production owing to anti-OX40 from endogenous CD4 T cells in any experimental group (data not shown). Thus, the primary role of OX40 signaling may be to allow the expression of cytokines and cytokine receptors, which then both lead to further development of effector function and enhance survival.

Although we showed that anti-OX40 enhanced T-bet expression and Th1 effector cell development in a Th1-biased model, when IFN-γ was dampened in the absence of T-bet, OX40 engagement still promoted accumulation of cytokine-producing effector cells. OX40 signaling in the absence of T-bet enhanced IL-23 responsiveness measured by IL-17 production, as compared to the IL-12 responsiveness measured by IFN-γ production when T-bet was functional. Ectopic T-bet expression has been shown to abrogate IL-17 production in Th17 polarized cells (294). This suggests that a lack of IL-17 production from wild type CD4 T cells in this report could be due to the enhanced T-bet expression

in OX40-responsive CD4 T cells. However, a recent report shows that small interfering RNA targeted toward T-bet suppresses IL-23 receptor expression and subsequent IL-17 production (302). We show that in the genetic absence of T-bet, OX40 enhanced IL-23 responsiveness measured by IL-17 production, suggesting that OX40 signaling promotes additional pathways for effector cell differentiation.

Although the data in Figure 3 showed a small increase in IL-17 production upon in vitro stimulation, the majority of T-bet$^{-/-}$ divided donor cells did not produce IL-17, IFN-γ, or other measured cytokines, suggesting that T-bet expression is important for optimal effector cytokine production. Interestingly, recipients that received T-bet$^{-/-}$ donor cells plus anti-OX40 developed larger spleens, thickened intestines, and had excessive mucous in their eyes after 5 days, compared to recipients that received T-bet$^{+/+}$ donor cells and anti-OX40 (unpublished observations). These observations could indicate that in the absence of T-bet, anti-OX40 promoted sufficient IL-17 production in effector CD4 T cells to lead to exacerbated gross pathology compared to wild type. Alternatively, it is possible that OX40 signals upregulated other pro-inflammatory cytokines normally suppressed in the presence of T-bet, which may have acted alone or in synergy with the small amount of IFN-γ and IL-17 produced by T-bet$^{-/-}$ CD4 T cells to enhance disease progression.

In summary, we provide evidence that OX40-mediated differentiation to cytokine-producing effector CD4 T cells is not strictly dependent on costimulatory signals such as CD28 and CD40. In addition, although OX40 enhances regulators of Th1 differentiation such as IL-12Rβ2 and T-bet expression (2), OX40 signals still promote accumulation of cytokine producing effector T cells in their absence. It is possible that

OX40 directly signals through transcription factors to amplify the original effector cell developmental program, or indirectly through cytokine receptors such as IL-2R. Based on the data in this report, although cytokine receptors, transcription factors, and additional costimulation promote optimal OX40-mediated effector cell differentiation, it appears that OX40 signals act through cytokine receptors like IL-2R to support effector cell activities.

Table 1: *Donor $CD4^+$ Cell Recoveries from Spleen*[a]

Donor	Recipient	Donor $CD4^+$ Input ($x10^6$)	Divided Donor $CD4^+$ Recovered ± SEM ($x10^6$)	
			Anti-OX40	Control IgG
B6 (n=7)	F1	7.5	28.8 ± 6.1	3.12 ± 0.27
TCR Tg (n=6)	$CD40^{-/-}$	1	8.6 ± 2.9	2.8 ± 1.1
TCR Tg (n=6)	$CD40^{+/+}$	1	43.4 ± 5.9	7.2 ± 1.4
IL-12Rβ2$^{-/-}$ (n=6)	F1	10	18.9 ± 4.6	3.8 ± 0.32
B6 (n=6)	F1	10	19.3 ± 3.7	4.2 ± 0.6
T-bet$^{-/-}$ (n=6)	F1	7.5	49.9 ± 4.1	4.4 ± 0.42
$CD28^{-/-}$ (n=6)	F1	7.5	1.1 ± 0.4	0.19 ± 0.06
$CD25^{-/-}$ (n=6)	F1	7.5	1.1 ± 0.06	0.37 ± 0.06
$CD25^{-/-}$ (n=6)	B6	7.5	0.07± 0.01	0.03 ± 0.001

[a] $CD4^+$ T cells in a B6 or TCR Transgenic spleen cell suspension were transferred with anti-OX40 or control IgG i.v. into unirradiated (B6.Ly5.1 x bm12)F1 or antigen transgenic recipients, and spleens were harvested on day 5. The number of divided donor $CD4^+$ cells recovered was determined by multiplying the percent $CD4^+$ $CFSE^{lo}$ donor cells determined by flow cytometry by the total cells recovered from the spleen. n equals the number of animals in each treatment group, combining data from one to 4 experiments.

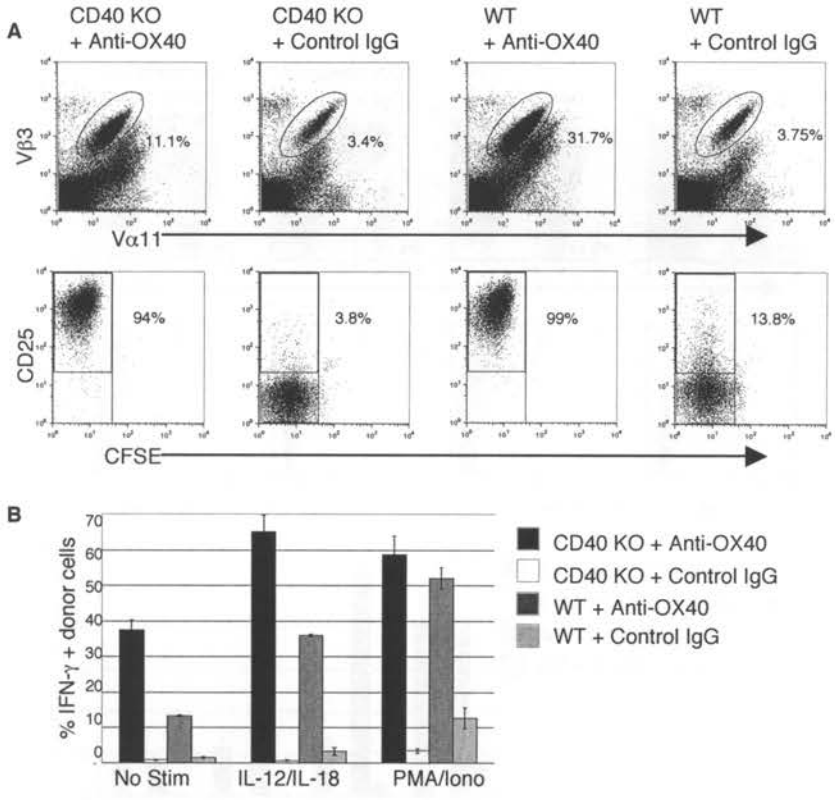


FIGURE 4-1. CD40 signaling is not required to support OX40 mediated acquisition of effector function. 10^6 TCR transgenic CD4 T cells from AND $Rag1^{-/-}$ spleen and lymph node suspension were transferred with 50 μg of anti-OX40 or control IgG into $CD40^{+/+}$ or $CD40^{-/-}$ antigen transgenic recipients, and spleens were harvested on day 5. *a*, Percent TCR transgenic T cells of total $CD40^{-/-}$ (left) or $CD40^{+/+}$ (right) recipient splenocytes is shown in top panels. CD25 expression on donor TCR transgenic cells is shown in the bottom panels. The number shows the percentage of $CFSE^{lo}$ divided donor cells that are also $CD25^{+}$. *b,* Bar diagrams show mean and standard error of the divided donor TCR transgenic T cells that are IFN-γ^{+} after restimulation in vitro for 5 hours with media only, IL-12 and IL-18 (IL-12/18), or PMA and ionomycin (PMA/iono) in two separate experiments, 6 animals total per group.

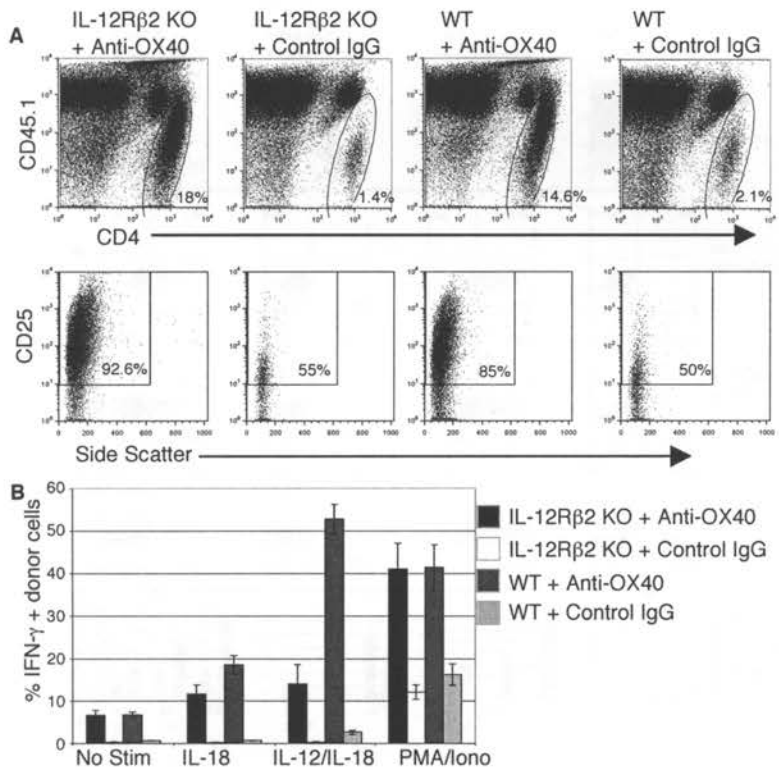


FIGURE 4-2. OX40 drives differentiation independent of IL-12 receptor signaling. 10^7 IL-12Rβ2$^{-/-}$ or B6 CD4$^+$ T cells in a spleen suspension were transferred with 50 μg anti-OX40 or control IgG into (B6.CD45.1 x bm12)F1 recipients for five days. *a*, Percent CD4$^+$ CD45.1$^-$ donor cells of total splenocytes is shown in the top panels. Donor cell size and CD25 expression are shown in the bottom panels. *b*, Bar diagrams show mean and standard error of the divided donor TCR transgenic T cells that are IFN-γ$^+$ after restimulation in vitro for 5 hours with media, 1 μg/mL IL-18, IL-12 and IL-18, or PMA and ionomycin in two separate experiments, 6 animals total per group.

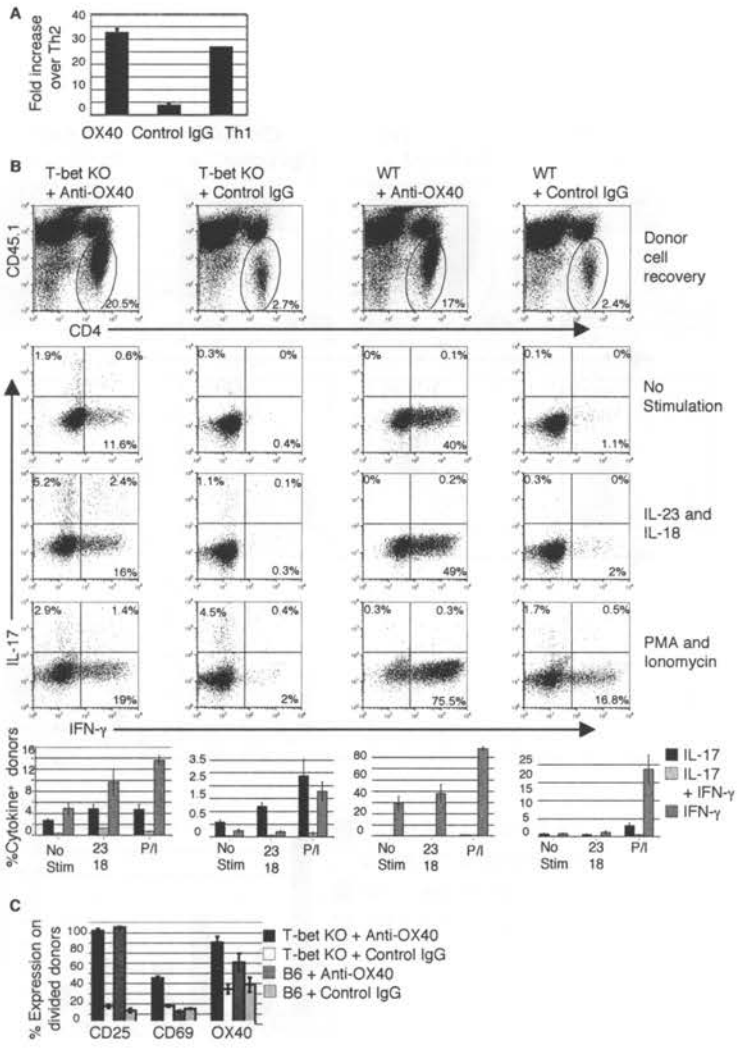


FIGURE 4-3. OX40 costimulation enhances T-bet expression, but T-bet is not required for OX40 driven acquisition of effector function. *a*, Quantitative analysis of the fold-change in T-bet expression from day 3.5 TCR transgenic T cells subjected to anti-OX40 or control IgG are compared to T-bet expression of in vitro cultured Th1 and Th2 cells. Quantitative PCR analysis and RNA isolation from recipient antigen transgenic spleens is described in the materials and methods. *b*, 7.5 x 10^6 T-bet$^{-/-}$ or WT donor cells were transferred with 50 μg anti-OX40 or control IgG into (B6.CD45.1 x bm12)F1 recipients and spleens were harvested 5 days later. Percent CD4$^+$ CD45.1$^-$ donor cell recovery is shown in the first row of panels. FACS plots show divided donor CD4$^+$ CD45.1$^-$ cells that are IL-17$^+$, IFN-γ$^+$, and IL-17$^+$ IFN-γ$^+$ double positive after restimulation in vitro for 5 hours with media (second row), IL-18 and IL-23 (third row), or PMA and ionomycin (fourth row), and bar diagrams below each column show mean and standard error of the divided donor cytokine production from two independent experiments, 6 animals total per group. *c*, The bar diagram shows the percentage of CFSE^{lo} divided donor cells that are also CD25$^+$ or OX40$^+$ from two experiments, 6 replicates per group.

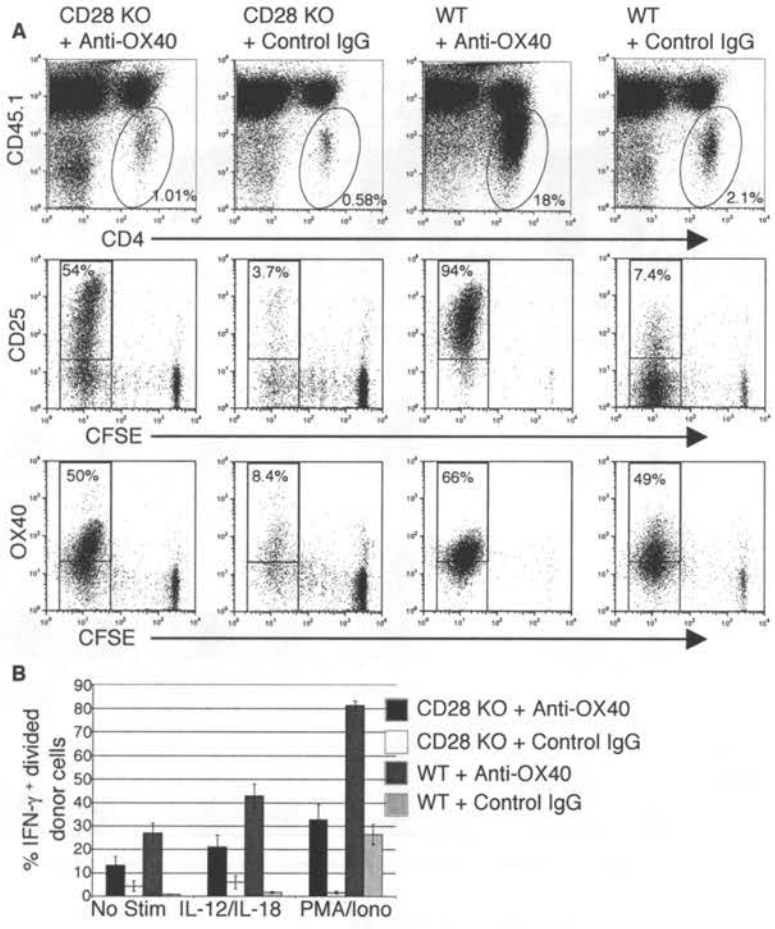


FIGURE 4-4. OX40 costimulation enhances proliferation and effector function of $CD28^{-/-}$ CD4 T cells. 7.5 x 10^6 CD4 T cells in a spleen and lymph node suspension from $CD28^{-/-}$ or B6 mice were transferred with 50 μg agonist anti-OX40 or control IgG into unirradiated (B6.CD45.1 x bm12)F1 recipients, and spleens were harvested 5 days later. *a*, Percent $CD4^+$ $CD45.1^-$ donor $CD28^{-/-}$ (left) or wild type (right) cells of total splenocytes are shown on the top panels. The middle and bottom rows represent the phenotype of $CD4^+$ $CD45.1^-$ donor cells. The number shows the percentage of $CFSE^{lo}$ divided donor cells that are also $CD25^+$ or $OX40^+$. *b,* Bar diagrams show mean and standard error of the divided donor CD4 T cells that are IFN-γ^+ after restimulation in vitro for 5 hours with media alone, IL-12 and IL-18 (IL-12/18), or PMA and ionomycin (PMA/iono) in two separate experiments, 6 animals total per group.

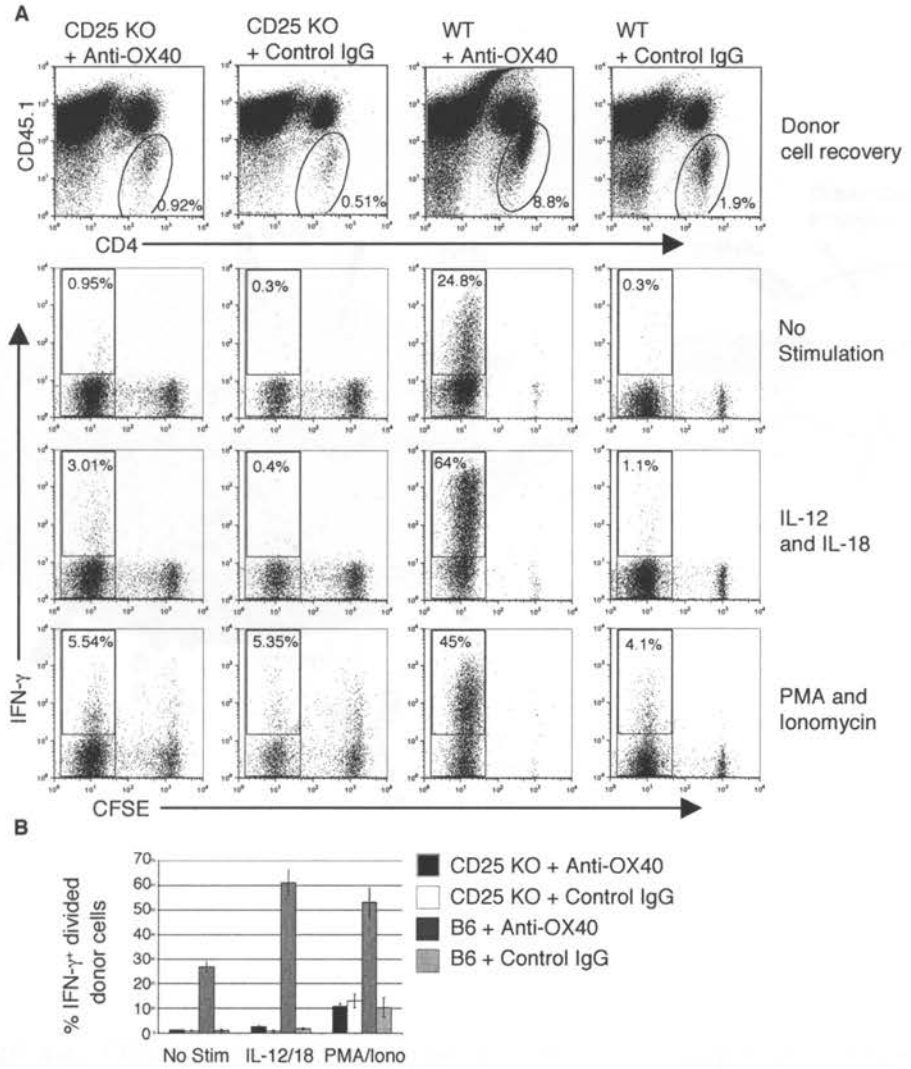


FIGURE 4-5. OX40 induced acquisition of effector function is dependent on IL-2 receptor signaling. 7.5 x 10^6 $CD25^{-/-}$ or WT donor cells were transferred with 50 μg anti-OX40 or control IgG into (CD45.1.B6 x bm12)F1 recipients, and spleens were harvested 5 days later. Percent $CD4^+$ $CD45.1^-$ donor cell recovery is shown in the first row of panels. FACS plots show divided donor $CD4^+$ $CD45.1^-$ cells that are IFN-γ^+ after restimulation in vitro for 5 hours with media (second row), IL-18 and IL-12 (third row), or PMA and ionomycin (fourth row), and the bar diagram in b shows mean and standard error of the divided donor IFN-γ production from two independent experiments, 6 animals total per group.

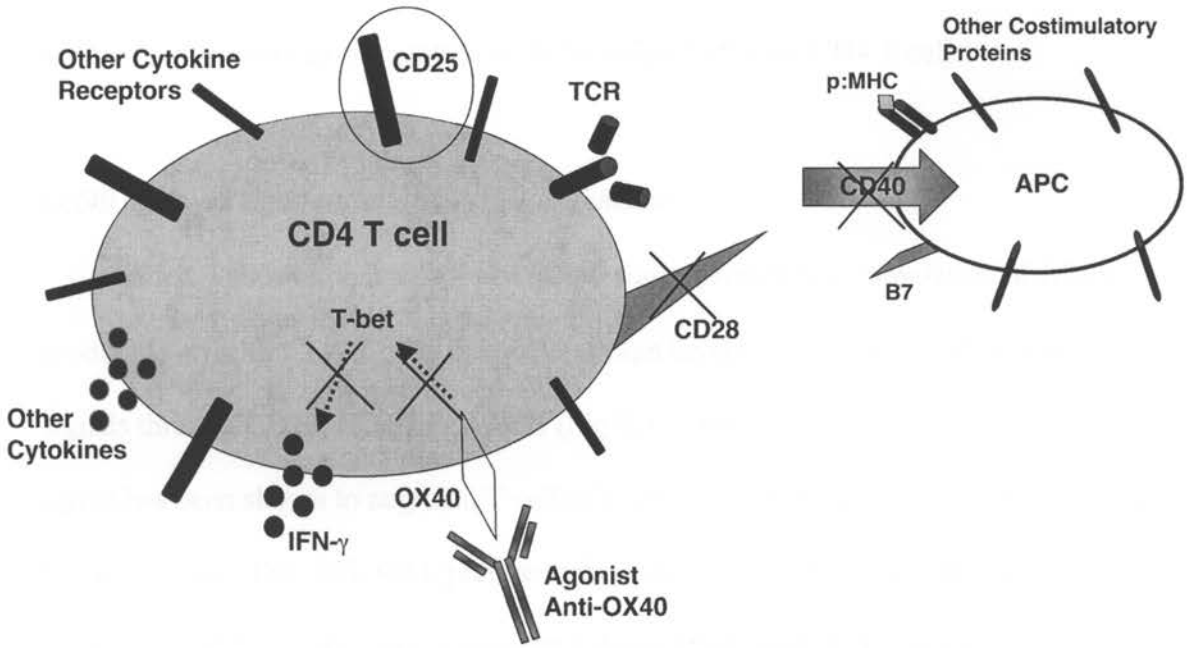


FIGURE 4-6. OX40 drives differentiation of CD4 T cells independent of other costimulatory signals but is dependent on IL-2 receptor signaling. OX40 promotes cytokine and cytokine receptor expression independent of CD28 signals to CD4 T cells that express OX40. OX40 also promotes effector cell development independent of APC maturation via CD40 signaling to the APC. OX40 enhances cytokine and cytokine receptor expression independent of T-bet expression, even though in the presence of T-bet, OX40 increases T-bet mRNA expression. However, OX40 does not efficiently promote cytokine and cytokine receptor expression independent of IL-2 receptor signaling.

Chapter 5—Summary and Conclusion

I began this work with the hypothesis that OX40 ligation drives differentiation of proliferating donor CD4 T cells under persistent antigen presentation. I have shown this to be true, and suggest that OX40 enhances expression of cytokines and cytokine receptors to enhance accumulation of differentiated effector CD4 T cells.

OX40 mediates differentiation to effector function

First, I showed that an agonist OX40 signal promotes accumulation of IFN-γ producing effector CD4 T cells that express high levels of CD25 better than agonist signals through CD28, CD40, or 4-1BB (Fig 9, Chapter 2). Although each costimulatory signal has been shown to augment T cell effector function in models of GVHD and organ transplantation (188, 303-305), preferential development of effector function in CD4 T cells mediated by OX40 signals suggests a direct effect on differentiation.

Although CD28 is the initial costimulatory signal, several studies indicate that CD28 signaling alone is not sufficient to foster a full immune response (80, 129, 273, 306), and thus it is not surprising that agonist CD28 signaling would be least effective in my system. While CD40 is expressed on activated CD4 T cells, signaling through CD40 primarily activates B cells and DC to provide subsequent costimulatory ligand expression and cytokine production to enhance T cell activation (31). In two separate experiments discussed in Chapter 4, I found that OX40 ligation induces differentiation independent of either CD28 on donor CD4 T cells or CD40 on recipient APC. These independent experiments indicate that OX40 does not rely on other costimulatory receptors to enhance accumulation of effector CD4 T cells, and in fact, OX40 may be the primary

costimulatory signal that regulates effector function under conditions of persistent antigen presentation in the absence of infection or adjuvants.

4-1BB and OX40 promote survival and differentiation in both CD4 and CD8 T cells (15), which may explain why agonist anti-4-1BB in my model produces effector CD4 T cells similar to those generated with anti-OX40. Although OX40 and 4-1BB are similar, they act independently and preferentially on CD4 and CD8 T cells, respectively. Double deficient $OX40L^{-/-}$ $4\text{-}1BBL^{-/-}$ challenged with influenza have reduced CD4 responses similar to OX40L single knockouts, and reduced CD8 responses similar to 4-1BBL single knockouts (307). In a separate study, OX40, 4-1BB, and CD27 are collectively responsible for generation of CD8 memory T cells during influenza infection (308). CD8 memory generation in these experiments could be due in part to effects of OX40 expression on CD4 T cells, which help CD8 T cell priming and secondary responses (308). OX40:OX40L engagement has been shown to induce B cell differentiation via signals through OX40L (96), and CD8 T cells, which also express OX40L (159), could be modulated similarly to B cells upon ligation through OX40 on CD4 T cells. OX40:OX40L signaling among purified T cells has been shown to promote Th2 responses (309), but under different antigen presentation conditions, may be able to promote CTL responses. These experiments point to an elaborate scheme in which the spatial and temporal expression of each TNFR family member supports a developing immune response. Additional studies further dissecting the specificity and complimentarity among TNFRs by investigating agonist antibody stimulation, antibody neutralization, and deficiencies in both transient and persistent antigen models should

provide a better understanding of how to manipulate these receptors for therapeutic interventions.

CD4 T cells responsive to OX40 produce IFN-γ direct ex vivo, and are highly sensitive to IL-12 and IL-18, producing more IFN-γ in the model systems that we analyzed. IL-12, IL-18, IL-23, and IFN-γ are all pro-inflammatory cytokines produced by the innate immune system that, in conjunction with TCR signaling, have been shown to promote robust development of IFN-γ production in CD4 T cells (310). TLR-activated DC can also induce substantial IFN-γ production independent of IL-12, IL-18, and IL-23 (310), suggesting collaboration or redundancy among TLR ligands and innate cytokines in fostering strong T cell responses. My experiments show that OX40 engagement enhances cytokines and cytokine receptors that in turn enhance effector cytokine production. Furthermore, OX40-deficient CD4 T cells can acquire effector cytokine production in the presence of $OX40^+$ antigen-specific CD4 T cells and anti-OX40. Anti-OX40 could diminish T regulatory cell function leading to OX40 independent T cell activation. Alternatively, OX40 responsive CD4 T cells could induce IFN-γ production in $OX40^-$ cells by secreting IFN-γ and other pro-inflammatory cytokines that induce IFN-γ production as mentioned above, or OX40 responsive cells could activate DC to enhance other costimulatory ligands to drive effector cytokine production independent of OX40 (Fig. 5-1). Recently, it was shown that IL-18 induces OX40L expression on APC that subsequently enhances clonal expansion and robust IFN-γ production in antigen-specific CD4 T cells, indicating that OX40 signaling could act as a bridge between innate and adaptive immune responses (311). Taken together, these experiments imply that OX40

encourages effector cell development by augmenting cytokine receptor signaling that results in pro-inflammatory cytokine production.

OX40 ligation also leads to tissue damage and death of the recipient. However, the role of OX40 in late-stage disease is not known. It is possible that OX40 mediates a pro-inflammatory cytokine feedback loop that drives TCR independent disease. I planned to neutralize antigen recognition early in T cell priming and after onset of late-stage disease to determine the role of TCR signaling in my system, but the neutralizing anti-MHC class II antibody did not prevent or dampen antigen recognition in vivo, since naïve T cell proliferation was similar to T cells transferred alone. However, given the strong response to cytokine stimulation in vitro, it is probable that OX40 enhances cytokine and cytokine receptor expression in vivo, which would induce a positive feedback loop that is independent of TCR signaling, similar to studies mentioned above. This implies that neutralization of IL-12 and IL-18 or other pro-inflammatory cytokines in the inflamed animals could reduce clinical signs of disease.

Interestingly, studies show that OX40 signaling is associated with pro-inflammatory T cell responses that result in pulmonary inflammation, skin graft rejection, and GVHD ((176, 178, 188, 189), and this thesis). TSLP-induced OX40L expression on DC specifically induces inflammatory Th2 cells (183). OX40 signaling also inhibits IL-10 producing type 1 regulatory T cells (312). Thus, it is possible that OX40 signals specifically induce pro-inflammatory effector functions that are independent of a specific Th1, Th2, or Th17 differentiation pathway.

Survival, Differentiation, and the IL-2 receptor

Previous reports on the mechanism of OX40 costimulation indicate that OX40 enhances survival by upregulating anti-apoptotic protein expression. Furthermore, ectopic expression of anti-apoptotic proteins in OX40-deficient CD4 T cells restores survival defects and confers effector cytokine production (80, 113, 114). However, I show that OX40 ligation induces effector cytokine production before changes in anti-apoptotic protein expression are detected. Additionally, I show that transgenic expression of Bcl-2 does not allow acquisition of effector function upon antigen stimulation alone, suggesting that enhanced survival does not directly lead to increased effector function. The initial outcome of OX40 costimulation is enhanced survival when antigen presentation is transient, while OX40 induces more robust inflammatory cytokine production under persistent antigen presentation. The difference in antigen presentation could affect the primary costimulatory requirements of the responding T cell, but in spite of this, the overall outcome of OX40 costimulation is accumulation of effector CD4 T cells under each condition of antigen stimulation.

IL-2 signaling promotes and maintains T cell growth by activating PI3K/AKT, ras/MAPK, and JAK/STAT signaling pathways, which lead to gene expression involved in proliferation and survival (313), and thus for many years IL-2 was characterized solely as a T cell growth factor. However, more recent studies have indicated that functional IL-2 signaling is essential for T cell differentiation to effector function in vivo. One study found that IL-2 signaling, among all other common-gamma chain cytokines, is specifically required for effector development in T cells from aged mice (314). In a non-obese diabetic model, IL-2 drives effector differentiation independent of cell division

(315). Recently, two independent models revealed that strong signals though IL-2R promote enhanced CD8 T cell proliferation, effector function, and secondary responses to antigen (40, 41). The importance of IL-2 in both survival and differentiation is also reflected in my experiments, in which I show that functional IL-2 receptor signaling is required to promote acquisition of effector cytokine production in addition to TCR plus other costimulatory signals.

To prove that OX40 signaling enhances cytokine and cytokine receptor expression to promote acquisition of effector function, future experiments involving neutralization of cytokines like IL-2, IL-12, or IL-18 in vivo or manipulating expression of cytokine receptors such as IL-2R are required. I attempted to neutralize IL-2 during T cell priming in the presence of anti-OX40. Administration of anti-IL-2 early in T cell priming (day 0 and 2) enhanced IFN-γ production compared to anti-OX40 alone, while IFN-γ production was not altered when anti-IL-2 was administered on day 2 and 4. I used the monoclonal anti-IL-2 antibody S4B6, which was recently shown to form an immune complex with IL-2 to provide a strong positive signal through the IL-2 receptor, which may in part explain my results (40). Boyman et. al. also show that other monoclonal anti-IL-2 antibodies can more effectively neutralize IL-2 signaling, such as JES6-1, which should be used in my system to determine the role of IL-2 signaling in OX40 mediated acquisition of effector function. Retroviral transduction of CD25 into donor T cells may result in enhanced effector function in the absence of anti-OX40, which would imply that OX40 promotes effector function by enhancing IL-2R expression. Alternatively, a CD25 deficient mouse with an inducible transgene that expresses CD25 would allow more accurate manipulation of CD25 expression on donor

cells. Controlling CD25 expression throughout a response to antigen and anti-OX40 may reveal that IL-2R signaling enhances IFN-γ production in the presence of OX40, again providing evidence that OX40 increases cytokine and cytokine receptor expression to promote effector cell development.

Perspectives

Although OX40 signaling clearly promotes acquisition of effector function before changes in survival are apparent, T cells in our models also undergo aggressive accumulation in response to antigen and OX40 costimulation. Furthermore, OX40 clearly enhances anti-apoptotic protein expression to regulate survival, clonal expansion, and enhance memory T cell populations in other systems. As I mentioned above, OX40 could enhance cytokine and cytokine receptor expression to promote downstream survival and differentiation. Alternatively, because TRAF adaptor proteins serve as platforms for multiple signaling cascades, OX40 engagement could involve multiple signaling outcomes. Dissection of the signaling pathways that OX40 regulates will be important for understanding how future OX40-specific therapies work.

OX40 recruits TRAF2 to initiate NFκB activation, but as discussed earlier, NFκB members can be divided into canonical or alterative pathways. The canonical pathway is important for survival gene, IL-2, and IL-2R expression (151), while the alternative pathway appears to be important in regulating gene expression of chemokines and cytokines involved in lymphoid organogenesis and possibly inflammation (152). Mechanistically, CD40 requires TRAF2 and TRAF3 for efficient processing of NFκB/p100 to p52 (155), and there is evidence that CD40 induces B cell activation and

effector function by activation of the alternative NFκB pathway through NIK (155). Thus, there is potential for OX40 modulation of T cell responses via the alternative NFκB pathway since OX40 shares common signaling pathways with CD40. Since NIK is an important activator of the alternative NFκB pathway, a future experiment to address the role of the alternative NFκB pathway in OX40 signaling is to transfer NIK deficient CD4 T cells on a B6 background into (B6.CD45.1 x bm12)F1 recipients with anti-OX40. If donor T cells exhibit defects in survival or differentiation, then OX40 may signal though NIK to activate the alternative NFκB pathway. However, I suspect that OX40 signaling is more complex. OX40 also recruits TRAF3 and TRAF5 to its cytoplasmic tail, regulating NFκB activation initiated by TRAF2. TRAF5 modulates Th2 responses (145) while TRAF3 is able to inhibit NFκB activation (142). A recent study has shown that the alternative NFκB pathway is induced later in T cell priming, and prevents canonical NFκB gene transcription (156). Therefore, more precise experiments looking at the phosphorylation states of IκK complexes and NFκB member locations in the cytoplasm and nucleus from initial T cell priming through late stage disease in our model would reveal how OX40-mediated NFκB regulation promotes accumulation of effector CD4 T cells. I envision that OX40 activation of the canonical NFκB pathway might occur first, but later in T cell priming, TRAF3 and TRAF5 may induce the alternative NFκB pathway that may inhibit the canonical pathway and induce pro-inflammatory cytokine expression. This sequence may best explain the results in this thesis, and spatial and temporal regulation of each NFκB pathway may occur during a single immune response.

T regulatory cells are able to suppress effector cells in vitro, and can also reduce the severity of established GVHD. T regulatory cells express OX40, but upon OX40 ligation, T regulatory suppressive function is inhibited (112, 316). In my transfer experiments, 10-15% of host CD4 T cells are $CD4^+CD25^+$ with control IgG, but anti-OX40 together with donor T cells induces CD25 expression on more than 50% of recipient CD4 T cells, yet these cells do not produce IFN-γ or IL-2 upon restimulation (unpublished observation). Although further characterization of these cells was not performed, it is possible that OX40 induces host T regulatory cell expansion. In preliminary studies in which I depleted $CD25^+$ T cells from antigen bearing recipients before transferring donor CD4 T cells plus anti-OX40, there was no change in proliferation or effector function compared to recipients that retained $CD25^+$ T regulatory cells. I did not determine the ability of T regulatory cells to suppress OX40-activated donor CD4 T cells by transferring expanded T regulatory cells with naïve donor cells plus anti-OX40. However, I suspect that host T regulatory cells in my system would behave similarly to T regulatory cells from another report, in which OX40 expanded T regulatory cells could not prevent IBD in the presence of OX40 activated T cells (112). I think that OX40 signaling provides a signal to effector T cells to resist the suppressive effects of T regulatory cells, similar to GITR (139), because OX40 signaling promotes robust effector function early in T cell priming, at an ideal time for T regulatory cells to suppress that function.

Finally, it is interesting that in the absence of anti-OX40 in my experiments, naïve CD4 T cells become tolerant. As shown in the appendix, antigen responsiveness is not recovered after rest in vitro or in vivo. Croft and colleagues showed that anti-OX40 can

reverse tolerance induced by peptide antigen immunization without adjuvant (175). In our system, I show that anti-OX40 delivered 3 days after tolerance induction enhances CD25 expression and promotes acquisition of effector function in 2 days, but responsiveness to antigen was not recovered direct ex vivo. Anti-OX40 given 5 days after tolerance induction enhances CD25 expression, but does not enhance effector cytokine production, as measured 2 days later. Thus, the tolerant state may become more irreversible with time, gene transcription could become more restricted by chromatin modifications, and costimulatory receptor expression may decline, and thus adjuvants such as anti-OX40 may take longer or may be unable to promote effector cytokine production over time. This observation indicates that there may be several stages of tolerance induction. In addition to hyporesponsiveness through the antigen receptor, perhaps cytokine signaling or responsiveness to costimulatory signals can also be inhibited as a downstream effect of inducing peripheral tolerance. Future experiments addressing the stages of peripheral tolerance and how tolerance can be reversed will be required to intervene effectively in transplantation, tumor immunology, chronic infection, and autoimmune disease.

OX40 as a therapeutic agent

The adjuvant effects of OX40 costimulation demonstrated in this thesis suggest that anti-OX40 could be used to boost anti-tumor immunity, while blocking OX40 signaling could impact autoimmune disease. Indeed, OX40 expression on tumor infiltrating lymphocytes in patients with cutaneous malignant melanoma correlate with patient survival and tumor progression (317), while OX40 expression on CD4 T cells from patients with myasthenia gravis suggests that OX40 might contribute to the

development of autoimmune disease (318). Several reports implicate the importance of OX40 signaling in translational research models as well. For example, OX40L over expression on tumor cells or on immunizing DC boost T cell anti-tumor immunity in mice (192, 193), while OX40L neutralization inhibits chemically induced autoimmunity in rats (319).

OX40 appears to be an ideal clinical target for boosting anti-tumor immunity because OX40 is primarily expressed on effector CD4 T cells found at the site of inflammation, and therefore, targeting OX40 should not interfere with other immune functions (274). However, data in this thesis indicate that systemic administration of anti-OX40 promotes effector CD4 T cells that subsequently induce effector function in other CD4 T cells independent of OX40 signaling (Fig. 5-1). These data suggest that careful local administration of anti-OX40 could reduce the incidence of bystander T cell activation, but some deleterious effects may be unavoidable. I also show that anti-OX40 promotes CD4 T cell activation and pro-inflammatory cytokine expression, which could augment tumor progression instead of boosting anti-tumor immunity. Activated T cells promote macrophages, endothelial cells, smooth muscle cells, and fibroblasts to produce pro-inflammatory cytokines, chemokines, and matrix metalloproteases, and induce vascular endothelial growth factor (VEGF) expression. This pro-inflammatory cascade promotes tissue destruction, cell hyperproliferation, and angiogenesis, which in turn promotes a premalignant environment for tumor progression (320). The microarray analysis in Chapter 3 showed an increase in chemokines, cytokines, and matrix metalloproteases that were likely expressed by contaminating APC and inflammatory cells in the anti-OX40 treated T cell preparation, and indicate that OX40-mediated T cell

activation could promote malignancy. However, with additional research on the mechanism of OX40 signaling and proper dosing and administration of agonist anti-OX40, a preferential boost in anti-tumor immunity could be achieved.

The data in this thesis also indicate that only OX40-bearing antigen-specific CD4 T cells can initiate antigen-specific effector function. Thus, blocking OX40 signaling in patients with autoimmune disease should be a specific means to reduce clinical signs of disease. Several studies in mouse models support this therapeutic application. For example, blocking OX40:OX40L in vivo reduced clinical signs of autoimmunity, but when treatment was discontinued, clinical signs of disease returned (105). Thus, a more permanent treatment for autoimmune disease would be to prevent OX40 signaling and specifically eliminate autoreactive T cells. Delivery of an OX40-specific immunotoxin that specifically kills OX40-bearing T cells ameliorated clinical signs of autoimmunity (169). Systemic delivery of the OX40 immunotoxin could be detrimental to the overall immune system of a patient receiving this type of therapy. Although autoreactive OX40-bearing cells would be eliminated, other cells combating infectious disease would also be eliminated. This could result in poor memory T cell pools that would combat another infection, or could allow outgrowth of a pathogen normally removed by $OX40^+$ effector cells. One report showed that drugs could be delivered in a liposome with anti-OX40 antibody, which prevented proliferation of auto-aggressive $OX40^+$ T cells (321). Thus, perhaps blocking OX40:OX40L signaling in combination with more specific compounds directed toward autoaggressive T cells would prevent the deleterious effects of therapies targeting all $OX40^+$ T cells.

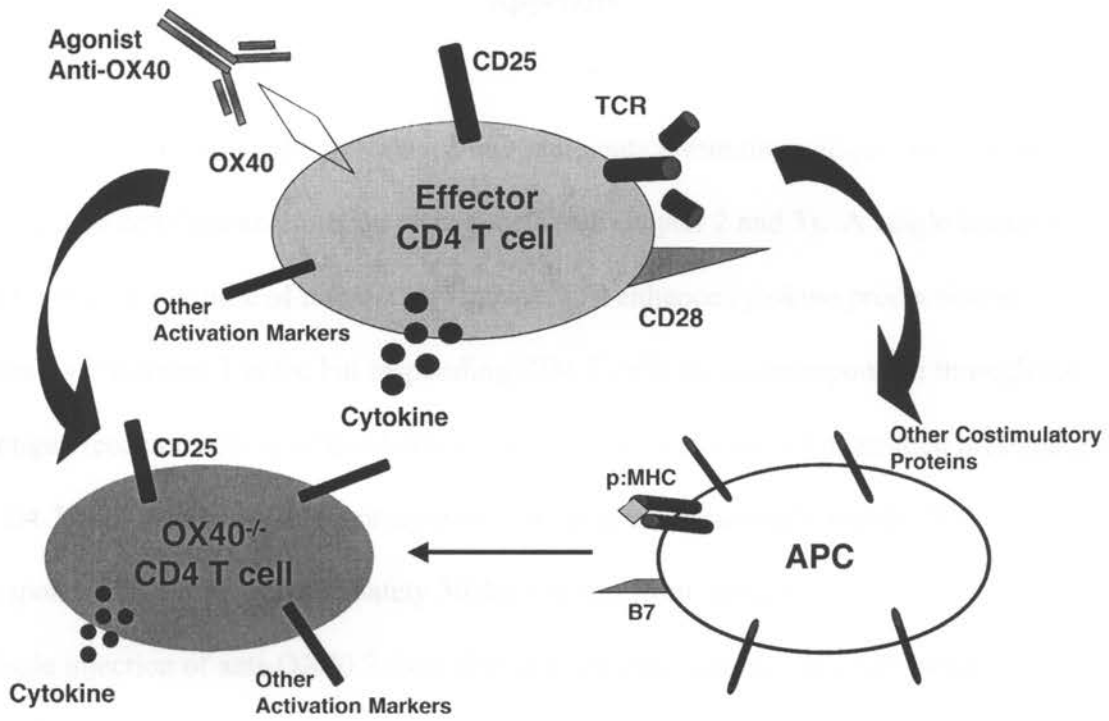


FIGURE 5-1. OX40 enhances effector function in other antigen specific CD4 T cells independent of OX40 signaling. In chapter 3 I showed that $OX40^{-/-}$ CD4 T cells acquired cytokine and cytokine receptor expression when $OX40^{+/+}$ CD4 T cells received a signal via anti-OX40. It is possible that OX40-activated CD4 T cells increase expression of activation markers like CD40L or cytokines that induce APC maturation and costimulatory ligand expression. The mature APC could subsequently activate $OX40^{-/-}$ T cells via other costimulatory ligands or cytokines. Alternatively, OX40-activated effector CD4 T cells could directly provide signals to $OX40^{-/-}$ CD4 T cells via cytokines or costimulatory ligand expression.

Appendix

Naïve CD4 T cells transferred into recipients presenting antigen on all MHC class II positive cells become tolerant after 5 days (see chapter 2 and 3). A single injection of anti-OX40 at the time of naïve T cell transfer can enhance cytokine production in otherwise tolerant T cells, but responding CD4 T cells are hyporesponsive through the antigen receptor with or without anti-OX40. In a transient model of antigen presentation, CD4 T cells also become hyporesponsive to antigen, but naturally regain TCR responsiveness after approximately 30 days of rest from antigen (51, 52). Interestingly, a single injection of anti-OX40 2 days after peptide immunization reverses tolerance and results in long-lived antigen dependent proliferation and IL-2 production (175). Under chronic antigen exposure, it is possible that the TCR on donor T cells is desensitized since the TCR is strongly down-modulated direct ex vivo at day 5 (see chapter 2 and appendix figure 1). Thus, hyporesponsiveness to antigen could be due to chronic antigen exposure, and with rest, antigen responsiveness could be regained if TCR surface expression is restored.

Splenocytes from day 5 anti-OX40 or control IgG treated animals, as set up in chapter 2, were cultured with anti-MHC class II antibody to block antigen recognition. After overnight rest, the TCR surface expression of both anti-OX40 and control IgG treated donor T cells were restored (appendix figure 1A). Interestingly, donor CD4 T cells subjected to anti-OX40 produced more IFN-γ after overnight rest without stimulation, but remained poorly responsive to antigen stimulation. Anti-OX40 treated T

cells also had enhanced responses to PMA and ionomycin and IL-12 and IL-18 stimulation compared to cells assayed directly ex vivo (appendix figure 1B). After 72 hours of in vitro culture with anti-MHC class II antibody, high levels of surface TCR were maintained, and antigen responsiveness was restored in anti-OX40 treated donor CD4 T cells. Responsiveness to PMA and ionomycin and IL-12 and IL-18 was also maintained in T cells from anti-OX40 treated mice compared to overnight rest. Control IgG treated T cells remained profoundly unresponsive to restimulation with antigen, PMA and ionomycin, and IL-12 and IL-18, despite normal TCR expression after overnight rest and at 72 hours (appendix figure 1). The in vitro cultures were maintained for 14 days, at which point the donor CD4 T cells in OX40 treated splenocytes maintained high levels of CD25 and IFN-γ production in response to antigen, while donor T cells in control IgG splenocytes retained unresponsiveness both proximal (peptide antigen stimulation) and distal (PMA and Ionomycin) to the TCR (data not shown).

I also hypothesized that TCR responsiveness can be recovered after rest in vivo. To test this hypothesis, I transferred purified CFSE-labeled CD4 T cells recovered from day 5 anti-OX40 or control IgG treated splenocytes into B6 $Rag1^{-/-}$ hosts. 14 days after T cell transfer, TCR transgenic donor cell recovery was greater for control IgG (1.88% of total cells) than anti-OX40 (0.79% of total cells) treated cells from the spleen (appendix figure 2A). A population that appears to be TCR low transgenic T cells shown in Fig. 2A are not CFSE positive or CFSE low. These cells also do not express CD25 or IFN-γ, and thus I suspect that these cells result from improper staining. All TCR transgenic CD4 T cells from anti-OX40 treated animals continued to proliferate in B6 $Rag1^{-/-}$ hosts, as measured by CFSE. T cells from control IgG treated animals were able to divide in vivo,

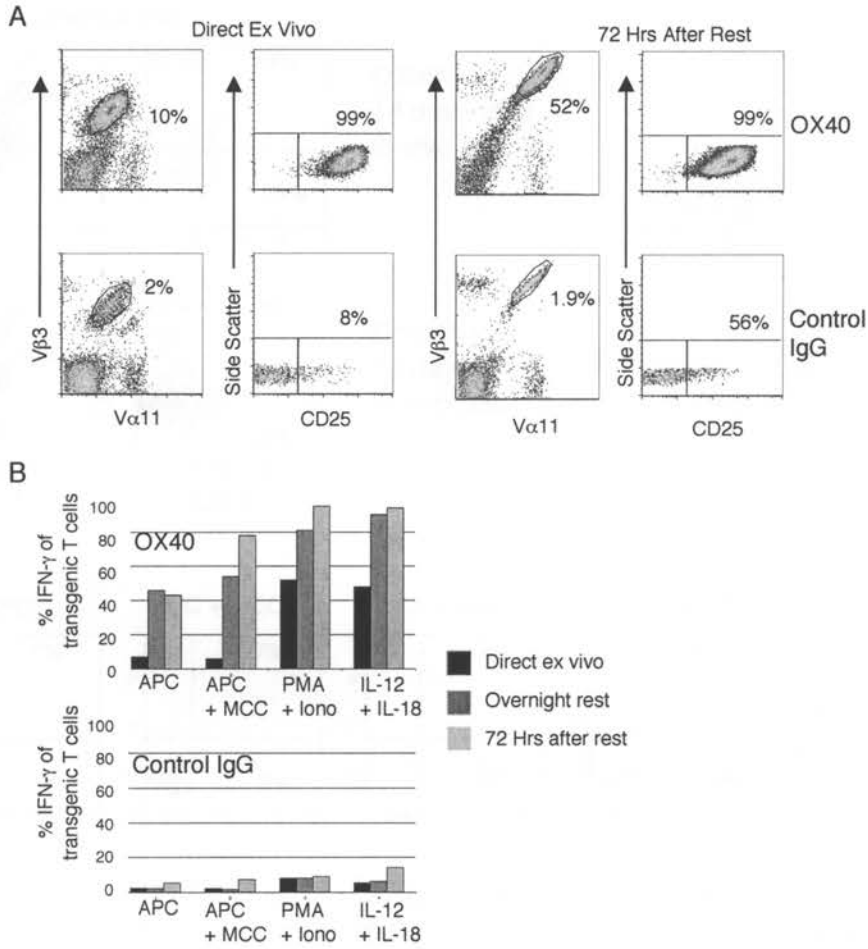
but a significant portion remained undivided. T cells from both anti-OX40 and control IgG treated animals may be dividing under homeostatic proliferation pressure, responding to residual antigen, or in the case of T cells from anti-OX40 treated animals, may be dividing in response to OX40 stimulation. These possibilities were not pursued during this project. CD25 expression on T cells from anti-OX40 treated animals was dramatically reduced after 14 days (99% to 29%), while CD25 expression on rested T cells from control IgG treated animals remained the same (appendix figure 2A).

To determine if 14 days of rest in vivo restores antigen dependent IFN-γ production, the day 14 splenocytes from each animal were restimulated with APC, peptide-pulsed APC, PMA and ionomycin, or IL-12 and IL-18 for 5 hours. Rested donor T cells from anti-OX40 treated animals recovered responsiveness through the TCR, as peptide stimulation induced 9% of TCR transgenic T cells to make IFN-γ compared to 3% with APC alone (appendix figure 2B). Anti-OX40 treated T cells also maintained responsiveness to PMA and ionomycin (76% IFN-γ^+) and IL-12 and IL-18 (36% IFN-γ^+). However, rested donor T cells from control IgG treated animals retained hyporesponsiveness through the antigen receptor, and made very little IFN-γ in response to either APC or peptide-pulsed APC. Rested control IgG treated T cells responded better to PMA and ionomycin (44% IFN-γ^+) compared to control IgG treated T cells recovered directly from antigen bearing recipients (10% IFN-γ^+). Interestingly, only control IgG treated T cells that divided in B6 Rag1$^{-/-}$ hosts made IFN-γ in response to IL-12 and IL-18 (appendix figure 2B). Perhaps homeostatic pressure to proliferate induced cytokine receptor expression and relieved some inhibition induced by peripheral

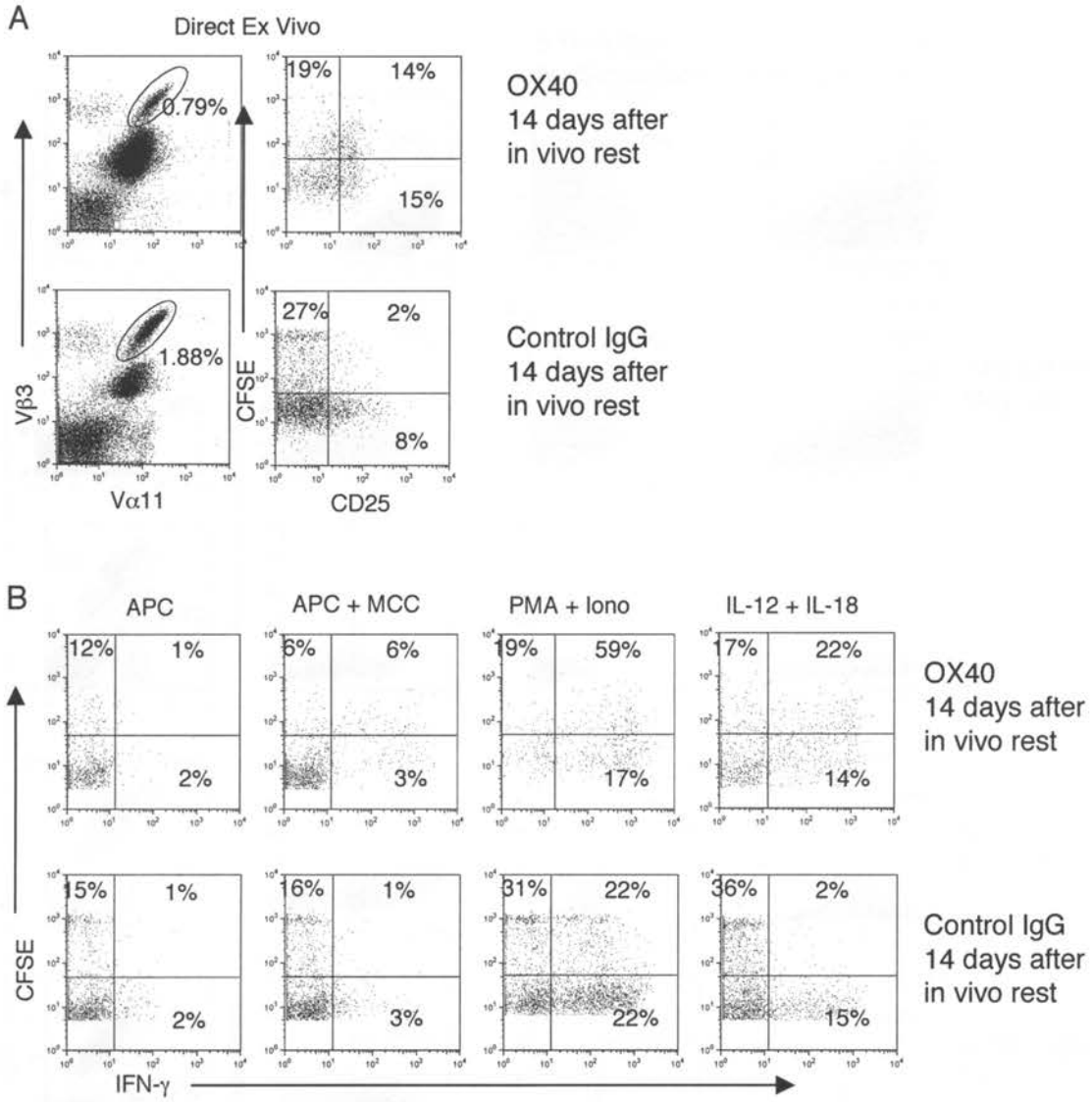
tolerance induction in antigen bearing hosts, allowing these cells to respond to IL-12 and IL-18 and PMA and ionomycin.

Since Croft and colleagues found that anti-OX40 could reverse previously established peripheral tolerance to transient antigen (175), I determined whether anti-OX40 could reverse established tolerance in our model of persistent antigen presentation. I transferred 1.5 x 10^6 AND TCR transgenic T cells into antigen bearing recipients on day 0. 3 days later, I administered anti-OX40 or control IgG i.v., and harvested splenocytes 2 days later. As shown in the second and third row of appendix figure 3, OX40 signaling induces accumulation of donor T cells and enhances CD25 expression compared to control IgG between days 3 and 5 after tolerance induction. In addition, OX40 promotes robust IFN-γ production upon PMA and ionomycin stimulation compared to control IgG.

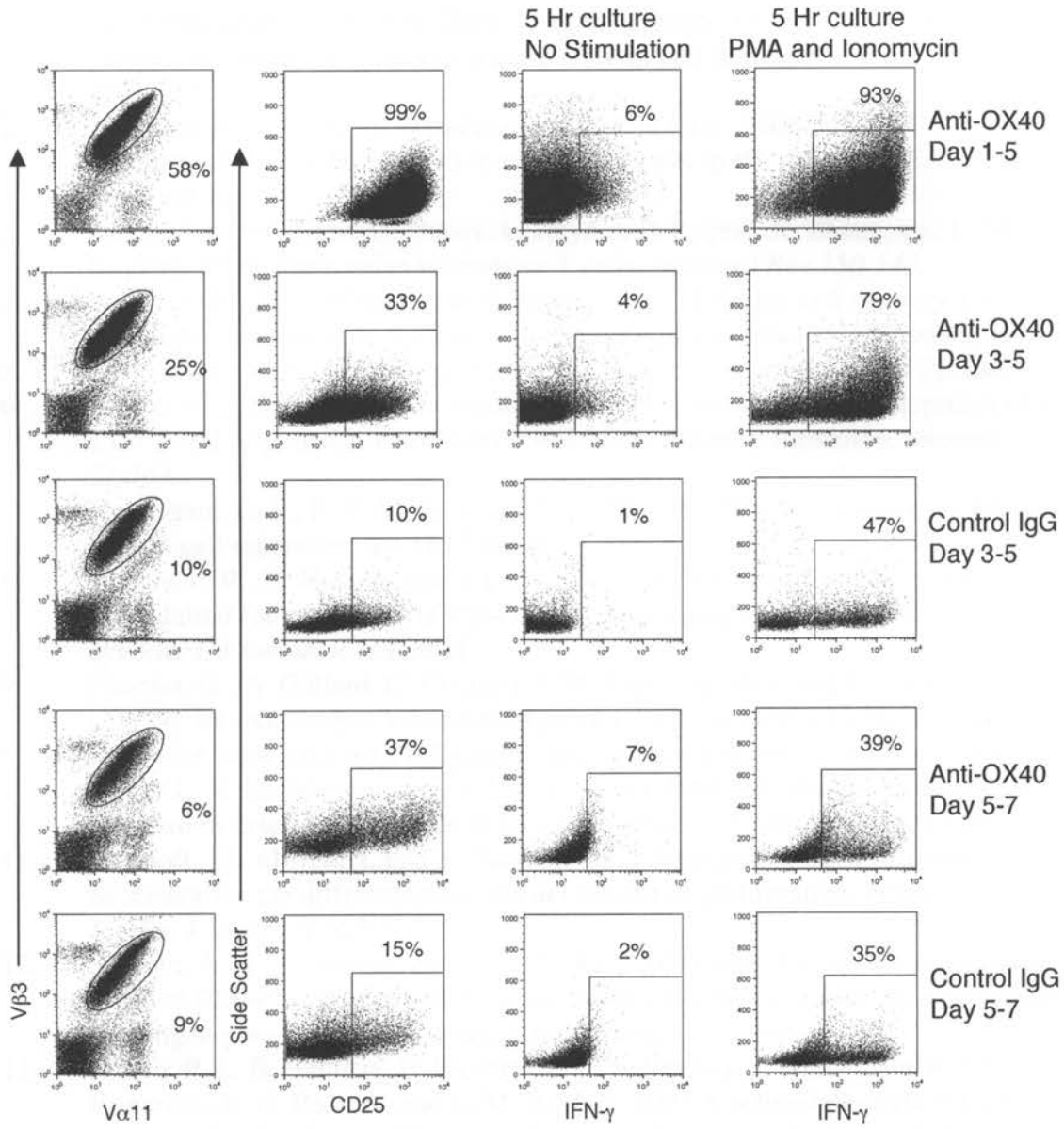
It is possible that peripheral tolerance is not fully established after 3 days compared to the profoundly tolerant donor T cells recovered after 5 days. Therefore, I administered anti-OX40 or control IgG 5 days after T cell transfer and harvested splenocytes 2 days later. As shown in the fourth and fifth rows of appendix figure 3, OX40 signaling does not induce accumulation of donor T cells (6% vs. 9%), but enhances CD25 expression (37% vs. 15%) compared to control IgG. OX40 does not enhance IFN-γ production upon PMA and ionomycin stimulation compared to control IgG. These experiments suggest that under continuous antigen stimulation without costimulation, peripheral tolerance becomes more permanent with time. Although OX40 signals were not able to enhance IFN-γ production after 2 days in day 5 tolerant T cells, OX40 did enhance CD25 expression. This observation implies that tolerance might be reversed with additional time.



Appendix Figure 1. In vitro rest restores TCR responsiveness in T cells from anti-OX40 treated mice. 10^6 AND $Rag1^{-/-}$ T cells were transferred into 102S recipients with 50 μg anti-OX40 or control IgG and 5 days later splenocytes were harvested. Total splenocytes were cultured at a concentration of 4 x 10^6 cells per mL with a 1:50 dilution of SAS purified 14-4-4S anti-I-E^k antibody, which inhibits AD.10 T cell blast proliferation to MCC pulsed splenic APC. A, Left column represents percent TCR transgenic T cells recovered from 102S recipients gated on live cells. The second column represents percent CD25 expression of TCR transgenic T cells. Right two columns represent percent TCR transgenic T cells and CD25 expression on T cells recovered 72 hours after rest in vitro. B, Splenocytes were cultured in vitro with APC, APC plus 1 mM MCC, PMA and ionomycin, or IL-12 and IL-18 as described in materials and methods of Chapter 2. Percent IFN-γ production of TCR transgenic T cells cells is shown for T cells direct ex vivo, cultured in vitro overnight, and after 72 hours rest. Data represent one experiment, 3 mice pooled into one in vitro culture.



Appendix Figure 2. In vivo rest restores TCR responsiveness in T cells from anti-OX40 treated mice. 10^6 AND Rag1$^{-/-}$ T cells were transferred into 102S recipients with 50 μg anti-OX40 or control IgG and 5 days later splenocytes were harvested. CD4 T cells were purified as described in chapter 2, labeled with CFSE, and 2.3 x 10^6 T cells were transferred into B6 Rag1$^{-/-}$ for 14 days. A, Left column represents percent TCR transgenic T cells recovered from B6 Rag1$^{-/-}$ gated on live cells. Right column represents TCR transgenic T cell division measured as CFSE dilution versus CD25. B, Splenocytes were cultured in vitro with APC, APC plus 1 mM MCC, PMA and ionomycin, or IL-12 and IL-18 as described in materials and methods of Chapter 2. Percent IFN-γ production of undivided and divided cells is shown gated on TCR transgenic T cells as shown in part A. Data represent one experiment, 3 mice per group.



Appendix Figure 3. OX40 reverses tolerance under persistent antigen presentation. 1.5 x 10^6 AND TCR transgenic T cells were transferred into 102S antigen transgenic recipients and 50 mg of anti-OX40 or control IgG were injected i.v. at day 1, 3, or 5, and splenocytes were harvested at day 5 or 2 days after anti-OX40 injection. The first column represents percent of total donor TCR transgenic T cells recovered, the second column shows CD25 expression on donor T cells directly ex vivo. Column 4 and 5 represent percent IFN-g production of donor T cells after 5 hour culture with no stimulation or PMA and Ionomycin, as followed in materials and methods in chapter 2. This figure represents three animals per group.

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