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COMPARISON OF OX40L AND CD70 IN THE PROMOTION OF CD4+ T CELL RESPONSES

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Abstract

The TNFSF members CD70 and OX40L have both been reported to be important for CD4+ T cell expansion and differentiation. However, the relative contribution of these co-stimulatory signals in driving CD4+ T cell responses has not been addressed. Here, we find that OX40L is a more important determinant than CD70 of the primary CD4+ T cell response to multiple immunization regimens. Despite the ability of a combined TLR and CD40 agonist (TLR/CD40) stimulus to provoke appreciable expression of both CD70 and OX40L on CD8+ DCs, resulting CD4+ T cell responses were substantially reduced by antibody blockade of OX40L, and to a lesser degree CD70. In contrast, the CD8+ T cell responses to combined TLR/CD40 immunization were exclusively dependent on CD70. These requirements for CD4+ and CD8+ T cell activation were not limited to the use of combined TLR/CD40 immunization, as vaccinia virus challenge elicited primarily OX40L-dependent CD4 responses and exclusively CD70-dependent CD8+ T cell responses. Attenuation of CD4+ T cell priming induced by OX40L blockade was independent of signaling through the IL12 receptor, but was reduced further by co-blockade of CD70. Thus costimulation by CD70 or OX40L appears to be necessary for primary CD4+ T cell responses to multiple forms of immunization, and each may make independent contributions to CD4+ T cell priming.

Introduction

CD4+ T cells are important in controlling CMV and other chronic infections (1–6), and are involved in protection from *Mycobacterium tuberculosis* (7–9). They are a major target of human immunodeficiency virus infection and their loss is diagnostic of the acquired immunocompromised state. Additionally, CD4+ T cells are important for formation of CD8+ T cell memory populations (10), and they may be important for CD8+ T cell priming to self-antigen, a prerequisite for effective tumor immunity (11). Furthermore, they have been found to be important for distinct types of B cell responses (12, 13). Thus there is an ongoing need to understand the processes governing CD4+ T cell activation, expansion, and memory formation in order to comprehend their role in immune responses and to develop clinically useful, CD4+ T cell-directed vaccines.

Modern vaccines are based on one of two principal platforms: Either subunit based, or based on killed or attenuated pathogens. Of these, killed and attenuated pathogens provide an intrinsic adjuvanticity based around inherent molecular activators of innate immunity. Generally speaking, these vaccines bear intrinsic ligands for pattern recognition receptors,

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such as the Toll-like receptor (TLR) family, which have the capacity to mobilize innate immune cells (14). Unfortunately, effective vaccines formulated around killed or attenuated pathogens can be difficult to derive, they are limited by problems with storage and transport, and attenuated vaccines are complicated by the capacity to revert to virulent form. Whereas subunit-based vaccines are arguably safer than those formulated around killed or attenuated pathogens, all such vaccines in use today in the US utilize alum as an adjuvant, which is generally recognized as inadequate for generating cellular immune responses (15, 16). Unfortunately, efforts to molecularly engineer subunit vaccines using TLR agonists have also been disappointing (17).

Ideally, it would be possible to manipulate individual molecular pathways governing adaptive immune responses in order to program a particular outcome. To this end, there are many potential costimulatory markers expressed by activated antigen-presenting cells (APC), including those of the Ig superfamily and those of the tumor necrosis factor superfamily (TNFSF), which may have the capacity to promote or inhibit T cell responses. To date, the molecular processes governing expression of these markers by antigen presenting cells, and the precise impact of one costimulatory marker or another on T cell responses, are still unclear. We have chosen to focus on the role of TNFSF signaling in CD4+ T cell priming in an attempt to resolve the relative contribution of different TNFSF ligands for CD4+ T cell immune responses. While it may be expected that CD4-specific TNFSF signals might exist, or that different patterns of TNF ligand signaling may convey information on the nature of threats to the adaptive immune system, there is a great deal of overlap in the literature regarding TNFSF dependency of CD4+ T cells (18–27). In particular, receptor-ligand interactions such as CD27-CD70 (20–22) and OX40-OX40L (23–27) are both reported to be important for CD4+ T cell priming, but we could find no studies directly assessing the relative contributions of CD70 and OX40L for CD4+ T cell responses in a given model system. We became interested in the details of how CD4+ T cells may be codependent on two TNFSF members. We reasoned that a better understanding of the relevant signals conveyed to CD4+ T cells by priming APC could accelerate the development of alternative vaccine platforms.

We have previously shown that combined TLR/CD40 stimulus promotes exponential CD8+ T cell responses in mice. These responses are obligately dependent on the TNF ligand superfamily member CD70 (28, 29) and independent of CD4+ T cells (17). However we had not investigated what impact this combined TLR/CD40 stimulus would have on CD4+ T cells; to what degree it would promote their exponential expansion, whether it would promote cytokine production or memory formation, and upon what costimulatory pathways it might depend. We demonstrate here that antigen-specific CD4+ T cells are synergistically induced by combined TLR/CD40 stimulus relative to either agonist alone. Induced CD4+ T cells produce IFNγ and can mount potent anamnestic responses following a single vaccination. Furthermore, we found that costimulation for CD4+ T cell priming was dependent on both CD70 and OX40L. We did not find a role for IL12 signaling in this system. We observed that simultaneous blockade of both OX40L and CD70 reduced responses below that of single blockade, indicating either TNFSF member can play a role in CD4+ T cell priming. Using a bone-marrow chimera approach, we observed that complete priming of CD4+ T cells required expression of CD40 on innate cells only. We then observed that splenic CD8+ DCs detectably coexpressed both CD70 and OX40L in response to combined TLR/CD40 stimulus, but not single agonists alone. To validate our results, we found that the costimulatory requirements of CD4+ and CD8+ responses to combined TLR/CD40 immunization were the same as those for responses to vaccinia virus challenge, such that CD4+ T cell priming depended upon OX40L and CD70, but CD8+ priming depended only upon CD70. Intriguingly, CD4+ T cell responses to vaccinia virus were also dependent on CD40-CD40L interactions, suggesting some physiologic overlap between our combined

agonist system and vaccinia infection. Thus, CD4+ T cell priming is characterized by a degree of TNF costimulation plasticity, which may facilitate their role as helpers for multiple arms of the immune response.

Materials and Methods

MICE AND INFECTIONS

Six- to eight-week old female C57BL/6 mice were obtained through the National Cancer Institute or Harlan Laboratories. Mice deficient in CD40 (B6.129P2-*Cd40*^{tm1Kik}/J), IL12Rβ2 (B6.129S1-*IL12rb2*^{tm1Jm}/J), and CD11c-DTR mice (B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J) were obtained from Jackson Labs. Mice deficient for the Rag1 gene (RagKO, B6.129S7-Rag1^{tm1Mom}/J) were a gift of Dr. Phillippa Marrack, National Jewish Health. Vaccinia virus expressing 2W1S was developed by Thomas Mitchell as described (30). Virus was titered and propagated using BSC-1 cells. Post-infectious BSC-1 cells and supernatant (stock) were stored at −80°C and were thawed and sonicated immediately prior to injection. Mice were injected intraperitoneally with 1–2×10^7 pfu of virus in 200μL of stock diluted in PBS. Mice were housed at the Biological Resource Center at National Jewish Health. The Institutional Animal Care and Use Committee at National Jewish Health approved all animal procedures.

IMMUNIZATIONS, DEPLETIONS, AND ANTIBODY BLOCKADE

Unless indicated otherwise, mice were immunized intraperitoneally with 500μg of whole chicken ovalbumin protein (Sigma-Aldrich, St. Louis, MO), 100μg of 2W1S peptide (EAWGALANWAVDSA, custom synthesized by Pi Proteomics, Huntsville, AL), 50μg of CD40 agonist antibody (clone FGK4.5, Bio X Cell, West Lebanon, NH), and 50μg poly-Inosine:Cytosine (polyI:C, Amersham/GE Healthcare, Piscataway, NJ). All vaccinations were prepared by mixing each component together in PBS, and injected in 200μL. PolyI:C was stored in frozen aliquots in PBS at −20°C and was reconstituted prior to injection by melting at 56°C for 10 minutes and then allowing the solution to cool to room temperature in order to limit concatamerization. As indicated, 50μg of Pam-3-Cys-K4 (Pam-3-Cys, InvivoGen, San Diego, CA) were occasionally substituted for polyI:C. All reagents were found to contain minimal LPS content by Limulus Amoebocyte Assay (Lonza, Walkersville, MD). CD11c-DTR mice were depleted of CD11c+ cells by subcutaneous injection of 100ng diphtheria toxin (Sigma-Aldrich, St. Louis, MO) two days prior to immunization. Blocking antibodies for CD70 (FR70), OX40L (RM134L), 41BBL (TKS-1), and CD30L (RM153), were obtained from Bio X cell (West Lebanon, NH). TNF blockade for immunization was facilitated by intraperitoneal injection of 250μg of blocking antibody in PBS on days −1 and 0. On day 0, blocking antibodies were mixed with prepared vaccines and both were delivered in a single injection. For pathogen challenge, TNF blockade was facilitated by intraperitoneal injection of 250μg on days −1, 0, and 2.

FLOW CYTOMETRY

For T cell assays, mice were sacrificed 7 days following immunization, peripheral blood was harvested from the abdominal aorta, and spleens were harvested and minced with forceps in HBSS containing 5mM EDTA and 2% heat-inactivated fetal calf serum (FCS). Single cell suspensions were made by passing minced spleens through nylon mesh filters. Red blood cells were lysed in peripheral blood samples using ACK lysis solution (BioSource, Rockville, MD). All samples were resuspended in RPMI 1640 medium containing 2.5% FCS, 2-mercaptoethanol, L-glutamine, non-essential amino acids, HEPES, sodium pyruvate, penicillin, and streptomycin. Cells were stained with phycoerytherin-labeled, tetramerized MHC molecules bearing antigens of interest. Drosophila S2 cells transfected with 2W1S-IA^b monomers were a gift of Marc Jenkins at the University of Minnesota (31). Secreted 2W1S-IA^b was harvested from supernatants as described (32), but did not require additional

biotinylation due to co-transfection of the BirA enzyme (James Moon and Marc Jenkins). K^b reagents were purified as described (33) and were loaded with OVA (SIINFEKL) or B8R (TSYKFESV) antigens prior to staining. Cells were coincubated with tetramer for 1 hour at 37°C prior to staining with surface antibodies. Surface antibodies were purchased from BioLegend (San Diego, CA), or eBioscience (San Diego, CA). For cytokine assays, cells were incubated for 3.5 hours at 37°C with 6μg/mL brefeldin A in complete media as above. Cells were restimulated with 10μg/mL 2W1S and SIINFEKL or B8R peptides, and following the incubation period cells were fixed, permeablized, and stained for intracellular cytokines as described (34). Dendritic cells were isolated from spleens in EHAA media (Invitrogen) containing DNAse (Worthington, Lakewood, NJ) and Collagenase D (Roche Diagnostics, Indianapolis, IN) as described (33). All cells were stained in FACS buffer containing 10% supernatant from 2.4G2 cell culture (B cell hybridoma blocking Fcγ receptors).

MIXED BONE-MARROW CHIMERAS

Recipient mice were lethally irradiated with 900 rads and were grafted via the tail vein with 4×10^6 total T cell-depleted donor bone marrow cells in 200μL of PBS. Most mixed bone marrow chimeras were grafted at a ratio of 1:1, however Rag1-deficient bone marrow was enriched relative to competitor bone marrow 3:1 to enhance engraftment. Chimeric mice were given a diet containing Septra (trimethoprim/sulfamethoxazole, Teklad) for 6 weeks following irradiation and rested a minimum of 12 weeks before being used in experiments.

IN VITRO CULTURE

Spleen cells were harvested as described above and cultured unfractionated at 1×10^6 cells/mL in 24-well, flat-bottom plates. Cells were stimulated with 0.5μg/mL anti-CD3ε (clone 2C11, BioLegend, San Diego, CA) in complete RPMI medium supplemented as above and with 10% FCS. At indicated timepoints, cells were harvested by washing and stained as above.

EXPERIMENTAL AND STATISTICAL ANALYSIS

Spleen cells were quantified on a Vi-Cell cell viability analyzer (Beckman-Coulter). Cytometry samples were acquired on a CyAn ADP (Dako Cytomation) using Summit acquisition software. Samples were analyzed using FloJo software (Tree Star, Inc. Ashland, OR). In most cases, results from FloJo analysis were imported into Prism (GraphPad, La Jolla, CA) and pairwise statistical analyses were made between samples using the Students' t-Test. *In vivo* experiments used in this manuscript were completed independently at least twice with a minimum three individuals per group. *In vitro* experiments were completed independently at least three times.

Results

CD4+ T CELLS, LIKE CD8+ T CELLS, RESPOND SYNERGISTICALLY TO COMBINED TLR/CD40 STIMULUS

Our previous results demonstrated that immunizing mice with antigen combined with Toll-like receptor agonists (TLR) and an agonistic antibody to murine CD40 (clone FGK4.5), evoked a massive expansion of antigen-specific CD8+ T cells (17, 28, 29). In this setting, antigen specificity was assessed by using MHC-I tetramers and revealed that combined TLR and CD40 stimulus (TLR/CD40) promoted expansion of splenic CD8+ T cells 5–10-fold greater than immunization with either TLR or CD40 agonist alone (17, 28, 29). This effect was apparent for all TLR agonists tested as well as several other stimulators of innate immunity (29), indicating that it was a generalizable trait of pattern-recognition receptors.

We became curious as to whether the synergism between TLR agonists and CD40 agonists were restricted to CD8+ T cells, or whether they could be extended to other cell types of the adaptive immune system. Thus we chose to assess CD4+ T cell immune responses in the setting of TLR/CD40 immunization.

We injected C57BL/6 mice with the MHC-II IA^b-binding peptide 2W1S (EAWGALANWAVDSA) and whole chicken ovalbumin protein (35), alone or in combination with TLR agonist (polyI:C for TLR3/mda5 or Pam-3-Cys for TLR1/2) and/or anti-CD40 antibody. After 7 days, which was found to correspond to the peak of the primary immune response (not shown), mice were sacrificed and spleen and peripheral blood were harvested. FACS analysis of MHC class I and class II tetramer staining on splenic and peripheral blood cells revealed that both CD4+ T cells and CD8+ T cells developed a population of $CD44^{high}$ antigen-specific responders with all adjuvants (Figure 1, A and B). However, in the case of TLR/CD40 immunization, elicited populations of CD4+ T cells far outnumbered those elicited by either adjuvant alone (Figure 1, C), and were numerically about 10-fold higher in the spleen than any other adjuvant. Thus TLR/CD40 stimulation synergizes to induce the potent expansion of antigen-specific CD4+ T cells in the same fashion as for CD8+ T cells, in that the T cell expansion by combined agonists is exponentially greater than either agonist alone.

To determine whether TLR/CD40 immunization produced functional CD4+ T cells, we immunized mice as above and restimulated splenocytes 7 days later with 2W1S peptide in the presence of Brefeldin A to test for intracellular cytokines. Combined TLR/CD40 stimulation elicited a large population of CD4+ T cells producing IFNγ in response to 2W1S restimulation by intracellular cytokine staining (Figure 1, D), which was proportional to the size of the tetramer-stained pool. Thus TLR/CD40 immunization elicits a presumptive T-helper-type-1 (Th1) type of primary CD4+ T cell response. Many of these cells were polyfunctional for the cytokines IL2 and TNFα (Supplemental Figure 1), which has been shown to correlate with helper T cell function in certain infections (3, 5, 8, 9, 36). Furthermore, CD4+ T cells elicited by a single injection of combined TLR/CD40 stimulus were long-lived and could mount anamnestic responses to vaccinia virus challenge, regardless of whether primed with Pam-3-Cys/CD40 or polyI:C/CD40 (Figure 1, E). Recall responses were similarly characterized by IFNγ production (data not shown). Thus, combined TLR/CD40 stimulus is a potent inducer of Th1-type, CD4+ T cell differentiation. We became interested in understanding how TLR/CD40 might exert such potent CD4+ T cell priming, with the hope that we might better understand the systems governing CD4+ T cell immunity.

CD40 EXPRESSION BY CD4+ T CELLS IS NOT NECESSARY FOR THEIR RESPONSE TO COMBINED TLR/CD40 IMMUNIZATION

CD4+ T cells are reported to express CD40 (37), which is a member of the TNF superfamily. Given our use of an exogenous CD40 stimulus, we wanted to rule out a role for direct stimulation of CD4+ T cells through CD40 in influencing the response of CD4+ T cells. Indeed, a recent publication suggested that the use of polyI:C can induce CD40L expression on DCs leading to direct stimulation of CD8+ T cells, presumably though their expression of CD40 (38). To address this issue, we generated bone-marrow chimeras by reconstituting lethally-irradiated CD40-deficient mice with congenically-marked CD45.1 wild-type (WT) B6 and CD45.2 CD40-deficient (CD40KO) bone marrow mixed at a 1:1 ratio (Figure 2, A). Our prediction was that if direct CD40 stimulation of CD4+ T cells by the CD40 agonist was required to produce the exponential T cell expansion we observed following combined TLR/CD40 immunization, then we should be able to see a defect in the response of CD40KO CD4+ T cells when compared to those that are wild-type for CD40. Reconstituted mice were immunized with 2W1S, OVA, CD40 agonist, and polyI:C or

Pam-3-Cys. The CD8 and CD4+ T cell responses were determined by tetramer stain and the relative responses of the antigen specific T cells derived from WT and CD40KO backgrounds compared using the CD45 congenic marker. We observed that the responding T cells had equal representation from CD40KO and B6 compartments (Figure 2, B). This was true for the antigen specific CD8+ T cells as well (data not shown). These data indicate that CD40 is not required on responding T cells for their expansion in response to combined TLR/CD40 immunization, demonstrating that the CD40 agonist does not have a direct costimulatory effect on CD4+ T cells.

CD4+ AND CD8+ T CELL RESPONSES TO COMBINED TLR/CD40 DEPEND ON DENDRITIC CELLS

We next hypothesized that combined TLR and CD40 stimulus could support CD4+ T cell priming through indirect effects on antigen presenting cells. It is generally well established that primary CD4+ and CD8+ T cell responses are dependent upon antigen presentation by dendritic cells (DCs), and several studies show that priming of CD4+ and CD8+ T cells might depend on different dendritic cell subsets (39, 40). However, naive B cells also express significant levels of CD40 (41), and both CD4+ (42) and CD8+ (43) T cells can enter B cell follicles. As our immunization utilized an agonistic CD40 antibody and a pre-processed CD4+ T cell epitope, this presented the very real possibility that we might be endowing B cells with the capacity to present antigen to naïve CD4+ and/or CD8+ T cells. As CD40 was not necessary on CD4+ T cells for TLR/CD40 responses to priming, we reasoned that CD40 expression would be necessary on the required APC. We therefore generated mixed bone marrow chimeras using wild-type B6.SJL bone marrow or recombinase-activating gene (RAG)-deficient (RagKO) bone marrow mixed with CD40KO bone marrow. In some cases, CD40KO bone marrow alone was used as a control (Figure 2, A). We chose CD40KO mice as recipients because we could be assured that no remnant T cells could express CD40 in these mice. In addition, the use of CD40KO hosts as bone marrow recipients would determine whether hematopoietic expression of CD40 was required for eliciting the magnitude of the T cell responses observed in response to combined TLR/CD40 immunization. Using this system, we were able to isolate CD40 to expression only on innate immune cells derived from RagKO bone marrow. We found that, consistent with a role for CD40 in combined TLR/CD40 immunization, CD40KO chimeric controls mounted very poor responses to immunization in both the CD8+ and CD4+ T cell subsets (Figure 2, C and D). However, both B6.SJL and RagKO mixed chimeric mice mounted effective responses to the combined stimulus. Thus, CD40 is sufficient on innate, bone marrow-derived cells for effective CD4+ and CD8+ T cell immunization in this system (Figure 2, C and D).

This result would seem to implicate DCs or myeloid cells as the principal APC in the combined TLR/CD40 model system. To clarify the identity of the required APC we utilized the CD11c-DTR transgenic mouse, in which the DCs express the diphtheria toxin receptor and are selectively depleted following injection of diphtheria toxin. We found that a low (100ng), subcutaneous, dose of diphtheria toxin given two days prior to immunization effectively depleted DC (data not shown). CD11c-DTR mice or littermate controls were selectively depleted of DC and immunized with antigen and combined TLR/CD40 as described previously. We found that both CD4+ and CD8+ responses were attenuated in DC-depleted mice (Supplemental Figure 2). As dendritic cells are innate bone marrow-derived cells, we concluded that classical DC are likely to be the primary APC for T cells following combined TLR/CD40 immunization.

CD4+ T CELLS ARE SENSITIVE TO BLOCKADE BY EITHER OX40L OR CD70 DURING PRIMARY IMMUNE RESPONSES

Our previous work indicated that another member of the tumor necrosis factor superfamily (TNFSF), CD70, mediated CD8+ T cell responses to combined TLR/CD40 stimulus (28). To determine whether CD70 or other TNFSF members play a role in CD4+ T cell responses to TLR/CD40, we administered blocking antibodies to the TNF family ligands CD70, OX40L, 4-1BBL, and CD30L, and assessed the T cell response 7 days later. Consistent with previous data (28), we found that CD8+ T cells were sensitive to CD70 blockade in response to either combined polyI:C/CD40 (Figure 3, A) or Pam-3-Cys/CD40 (Figure 3, B) immunization. Notably, this pattern was consistent whether whole OVA protein or SIINFEKL peptide was used as an antigen (not shown). In contrast to CD8+ T cells however, we found that CD4+ T cells were sensitive to either CD70 or OX40L blockade for polyI:C/CD40 (Figure 3, C), whereas they were only detectably sensitive to OX40L blockade in the case of Pam-3-Cys/CD40 (Figure 3, D). The reduction in CD4+ T cell responses following CD70 blockade of polyI:C/CD40 primed mice was consistently more modest than that provoked by OX40L blockade. Elicitation of IFNγ-producing CD4+ T cells was similarly impaired by TNFSF blockade (Supplemental Figure 3, A and B). The loss of antigen specific CD4+ T cells in TNF ligand-blocked mice was not due to the depletion of antigen presenting cells as we (Supplemental Figure 4) and other authors have demonstrated (28, 44). We conclude from these studies that while CD4+ and CD8+ T cell responses have overlapping and divergent costimulatory requirements, the optimal response of either T cell subset is dependent on members of TNF ligand superfamily. Furthermore, we find in our system that CD4+ T cell responses are dependent upon CD70 and OX40L, but less so CD30L or 41BBL.

CD4+ T CELL PRIMING BY POLYI:C/CD40 DOES NOT REQUIRE IL12, BUT MAY BE PROMOTED BY EITHER CD70 OR OX40L

Our findings were somewhat surprising given the results of a recent publication (21), which suggested that CD4+ T cell responses to combined polyI:C/CD40 were dependent either on IL-12 or CD70, depending on which DC subset was presenting antigen to the responding T cells. Th1 priming by DEC205+ DCs required CD70 costimulation while T cell priming by 33D1+ DCs required IL12 (21). We therefore examined more directly the difference between these published results and our demonstration of the importance of OX40L. We reasoned that the published results predict that the elimination of both IL12 and CD70-mediated signaling should eliminate the CD4+ T cell response to combined polyI:C/CD40 immunization, as 33D1+ DCs and DEC205+ DCs constitute the majority of classical DC subsets.

We immunized wild-type or IL12β2 receptor-deficient (IL12β2RKO) mice with antigen and polyI:C/CD40, with or without CD70 or OX40L blockade. IL12β2RKO mice lack the capacity to signal through IL12, but retain normal IL23 signaling. We found that in the absence of IL12 signals, CD4+ T cell proliferative (Figure 4, A) and IFNγ responses (Figure 4, B) were modestly, but not significantly reduced. The number of IFNγ+ CD4+ T cells was proportional to the number of 2W1S-IA^b tetramer+ cells in the absence of IL12β2R, suggesting that loss of IL12 signaling did not result in a major change in CD4+ T cell differentiation. These data were consistent with other experiments using IL12 β1 receptor deficient mice, as well as mice administered the IL12p40 blocking antibody, C17.8 (not shown). Furthermore, we found that CD70 blockade did not combine with IL12β2R deficiency to eliminate CD4+ T cell responses. While CD70 blockade had an impact on CD4+ T cell responses in wild-type mice, CD70 blockade had little to no impact on CD4+ T cell responses in IL12β2RKOs (Figure 4, A and B). In contrast to CD70 blockade, we found that OX40L blockade consistently and reproducibly reduced CD4+ T cell proliferation

(Figure 4, A) and IFNγ production (Figure 4, B) in both wild-type and IL12β2RKO mice. Our failure to find a CD70-dependent response in IL12β2RKO mice by CD4+ T cells was not due to insufficient blockade by the antibody, as CD70 blockade reduced CD8+ T cell responses in both wild-type and IL12β2RKO mice (Supplemental Figure 5, A and B). These data are consistent with the interpretation that CD70 and IL-12, rather than being separate and independent means of eliciting CD4+ T cells responses, are actually separate components of the same pathway; a conclusion supported by other authors (22). Our data suggest that CD70 and OX40L may act in semiredundant pathways for CD4+ T cell priming, as CD70 blockade was insubstantial in the IL12-deficient setting, but OX40L blockade inhibited CD4+ T cell responses in both IL12-sufficient and -deficient animals.

To determine whether this was indeed the case, we blocked mice with both anti-CD70 and anti-OX40L. Our results did not show a statistically significant additive effect to dual blockade of CD70 and OX40L for CD4+ T cell expansion (Figure 4, C). However, combined CD70 and OX40L blockade did produce a statistically significant effect on CD4+ T cell IFNγ production (Figure 4, D). We conclude from these data that, while the expansion of CD4+ T cells driven by TLR/CD40 is largely dependent upon OX40L interactions, CD70 can play a detectable role in the absence of OX40L. This suggests CD70 and OX40L may operate along parallel, but semiredundant, pathways for CD4+ T cell costimulation. Therefore, either CD70 or OX40L may be used to promote CD4+ T cell priming.

ONLY COMBINED TLR/CD40 PROMOTES CD70 AND OX40L EXPRESSION ON CD8+ DENDRITIC CELLS

Our previous data indicated that combined TLR/CD40 immunization uniquely elicited a significant increase in CD70 expression on lymphoid resident DC subsets *in vivo* (28). While we had also previously observed an elevation in DC OX40L expression, our observation of the dependency of the CD4+ T cell response on OX40L compelled us to take a closer look at the expression of this TNF ligand family member. We therefore harvested spleens at different time points following immunization with combinations of TLR and CD40 agonists, and analyzed the surface expression of different costimulatory molecules on dendritic cells by FACS.

Detectable CD70 expression was found primarily on CD8+ DC (Figure 5, A and B) and only under conditions of combined polyI:C/CD40 immunization (Figure 5, B). This confirmed that high CD70 expression was unique to combined TLR/CD40 agonist administration, and that CD8+ DC activation positively correlated with synergistic T cell activation we find with combined agonists. The kinetics of OX40L expression were similar to those observed for CD70 expression (Figure 5, A and B). CD8+ DC expressed the most OX40L (Figure 5, A and B) and expression was most significant when combined polyI:C/CD40 was used for immunization (Figure 5, B). Interestingly, we found that CD70 and OX40L were coexpressed on the same cells within the CD8+ DC subset (Figure 5, A). Thus, coordinated CD70 and OX40L expression on the same DCs correlate with the effects of combined TLR/CD40 immunization. We also found that these same DC can express high levels of MHC-II with OX40L (not shown). CD8+ DC are normally associated with cross-presentation (45) and CD8+ T cell activation (46). Our finding that a population within this DC subset coexpressed OX40L and MHC-II suggests that they may be the dominant APC for promoting helper T cell responses to combined TLR/CD40 immunization. Thus, while we could not rule out a role for activation by other APCs, our data suggest that similar CD8+ DC could mediate the effects of combined TLR/CD40 immunization for both CD4+ and CD8+ T cells.

OX40 AND CD27 ARE DIFFERENTIALLY REGULATED ON CD4+ AND CD8+ T CELLS *IN VITRO* AND *IN VIVO*

As CD4+ and CD8+ T cells may both utilize CD8+ DCs for priming, and CD8+ DCs express both CD70 and OX40L, it was curious that CD8+ T cells were exclusively CD70 dependent (Figure 3, A and B). To explain how CD8+ T cells could be insensitive to OX40L signals, we stimulated unfractionated spleen cells isolated from naïve mice for 24 hours in culture with anti-CD3 and stained for CD27 and OX40, the receptors for CD70 and OX40L, respectively. We found that unstimulated CD4+ and CD8+ T cells expressed appreciable CD27 (Figure 5, C, top, gray line), and that following 24h of culture, both CD4+ and CD8+ T cells upregulated CD27 (Figure 5, C, top, black line). In contrast, unstimulated CD4+ and CD8+ T cells expressed low levels of surface OX40 (Figure 5, C, bottom, gray line), and only CD4+ T cells appreciably upregulated OX40 following 24 hours of culture (Figure 5, C, bottom, black line). These data suggested that the insensitivity of the CD8+ T cell response to OX40L expressing DCs during priming may be due to their lack of OX40 expression.

To validate this result, we immunized mice with OVA, polyI:C, and CD40 agonist and harvested splenocytes at different timepoints following activation. By gating on CD44+ CD4+ and CD8+ T cells, we examined OX40 and CD27 expression *in vivo*. Similar to our results *in vitro*, we found that CD4+ and CD8+ T cells expressed appreciable CD27 at the time of immunization (Figure 5, D). In contrast, only CD4+ T cells expressed appreciable OX40, and this was not fully expressed on activated ($CD44^{high}$) cells until 24 hours of stimulus (Figure 5, E). Thus, CD4+and CD8+ T cells have different requirements for TNFSF costimulation in part because of their different expression patterns for TNF receptors.

CD4+ IMMUNITY TO VACCINIA DEMONSTRATES A DEPENDENCE ON CD40, AS WELL AS AN OVERLAPPING DEPENDENCE ON CD70 AND OX40L

We next wished to assess whether the important role for CD70 and OX40L in the CD4+ T cell response was unique to combined TLR/CD40 immunization or was common to primary immune responses elicited by other means. We therefore enlisted a model pathogen – recombinant vaccinia virus expressing 2W1S antigen (VV-2W1S). VV-2W1S also expresses the immunodominant vaccinia virus antigen B8R, which is presented on MHC K^b (47). Mice were challenged with the recombinant vaccinia virus and sacrificed 8 days later, which corresponded to the peak of CD4+ and CD8+ T cell responses in peripheral blood (not shown). Representative mice were concomitantly injected with 250μg of blocking antibodies to CD70, OX40L, 4-1BBL, or both CD70 and OX40L combined, on days −1, 0, and +2 relative to immunization.

We found that responses to VV-2W1S infection were comparable to responses to combined TLR/CD40, such that CD8+ T cell responses were dependent on CD70 (Figure 6, A). CD4+ T cell responses were dependent on both OX40L and CD70, with a greater dependence on OX40L (Figure 6, B). Of note, combining CD70 and OX40L antibodies noticeably reduced CD4+, but not CD8+ T cell responses below that of single blocking agents alone. This suggested that, for vaccinia, as for combined TLR/CD40 stimulus, the effects of CD70 and OX40L on CD4+ T cell responses were parallel and somewhat redundant with each other.

Consistency between vaccinia virus infection and polyI:C/CD40 immunization was not limited to TNFSF costimulation. We found that CD4+ T cells responding to vaccinia virus demonstrated a requirement for CD40-CD40L signals to promote optimal priming. Blockade of CD40L (CD154) with the MR1 antibody during vaccinia infection precipitously reduced CD8+ and CD4+ T cell responses (Figure 6, C and D). Furthermore, CD4+ T cell

proliferation and IFNγ production was also attenuated in CD40KO mice (data not shown), suggesting that CD4+ T cell responses to vaccinia are CD40-CD40L dependent.

In contrast to combined TLR/CD40 immunization and vaccinia infection, we found that CD4+ T cell response to *Listeria monocytogenes* was uniquely dependent on CD70 and we could find no role for OX40L (Supplemental Figure 5, B). We also found a greatly reduced requirement for CD40-CD40L signals for CD4+ T cell responses to *Listeria* (Supplemental Figure 5, D). Thus, the use of CD27 or OX40 by CD4+ T cells is highly context-dependent, and the role for different TNF receptors in primary CD4+ T cell immunity may vary depending on the mode of immunization. These data again underscore an intrinsic plasticity of CD4+ T cells for different forms of costimulation, and reaffirm the generalizable importance of the TNF ligand superfamily in mediating potent cellular immunity in response to diverse stimulus.

Discussion

We have demonstrated a method for inducing large populations of antigen-specific, IFNγ-producing, CD4+ T cells through the coordinate activation of TLR and CD40 pathways. We have also shown that single injection of this vaccine is sufficient to produce long-lived memory CD4+ T cells, and that the effects of this vaccine on CD4+ T cells are dependent on the TNF ligands CD70 and OX40L. CD4+ T cells elicited by combined TLR/CD40 were polyfunctional for multiple Th1 cytokines, including IFNγ and TNFα. Furthermore, we have shown that combined TLR/CD40 vaccination uniquely promotes expression of CD70 and OX40L on CD8+ DC, and that DC are necessary for productive vaccination. Although not shown, we found that CD8+ DC can express both TNF ligands in conjunction with MHC-II. Furthermore, we demonstrated that the role of OX40L or CD70 for CD4+or CD8+ T cells correlates with preferential receptor expression upon priming.

We find that the synergistic effect of combining TLR agonists with a CD40 agonist is independent of whether polyI:C or Pam-3-Cys used for CD4+ T cells, and regardless of whether polyI:C or Pam-3-Cys is used as a TLR agonist, TLR/CD40 promoted CD4+ memory T cell formation. In this case, we have tested both polyI:C, which activates TLR3, RigI, and MDA5 (48) but not MyD88, and Pam-3-Cys, which activates MyD88 through TLR1/2 (49). Although not shown, we have also tested agonists for TLR7 and found them to be consistent with the results shown here. Our demonstration that CD4+ T cell responses to Pam-3-Cys- or polyI:C-based immunizations have a major dependence on OX40L suggests the role of OX40-OX40L interactions in CD4+ immunity may be generalizable to other TLR agonists in concert with CD40. However, the role of OX40L in CD4+ T cell immunity is clearly not exclusive, as CD70 can also be found to play at least some role in our system (21) as well as a dominant role in *Listeria* infection.

The discrepancy in TNFSF costimulation for CD4+ T cells between vaccinia infection and *Listeria* infection correlate with a role for CD40-CD40L interactions in each response. Vaccinia-provoked CD4+ T cell priming was highly CD40L-dependent, and was also reduced in CD40KO mice, whereas *Listeria*-provoked CD4+ T cell responses were not significantly reduced by CD40L blockade. A search for the cause of the discrepancy must therefore focus on the role of CD40. Given both recent and established publications suggesting the necessity of CD40 expression in T cells for their effective activation (37, 50), it is of interest to note our demonstration that both CD4+and CD8+ T cell responses to combined TLR/CD40 immunization do not require T cell expression of CD40. Rather, CD40 expression on innate cells was sufficient to observe synergy between TLR and CD40 in mediating CD4+ and CD8+ T cell expansion. Our demonstration that partial reconstitution of a CD40−/− host with Rag-deficient bone marrow can rescue the T cell

response effectively eliminates a role for CD40 on B cells. The direct depletion of CD11c+ cells in the diphtheria toxin transgenic mouse and the corresponding reduced immune response confirms the key role of DCs in TLR/CD40 priming (21, 28, 39). Thus our data indicate that DC CD40 expression is critical for observing the magnitude of CD4+ and CD8+ T cell expansion elicited by combined TLR/CD40 immunization.

Combined TLR/CD40 elicits appreciable expression of CD70 and OX40L predominantly on CD8+ DCs, and blockade of these molecules reduces the magnitude of the immune response. While we are cautious about over-interpreting our results, these data suggest that CD8+ DC could play a key role in combined TLR/CD40 vaccination. Furthermore, a minimal role for IL12 signals in our system argues against a major role for 33D1+/CD11b+ DC subsets (21). However, we did not exhaustively rule out a role for other APCs, and we remain open to the likelihood that different APCs may play a role in CD4+ T cell priming under other conditions. A recent publication found that migrating dendritic cells within the draining lymph node of HSV-infected skin could uniformly activate CD4+ T cells, but only CD103+ dendritic cells could cross-present to CD8+ T cells (40). These data suggest that, whereas CD8+ T cell stimulation may derive from a narrow repertoire of APC, CD4+ T cell responses may be initiated by a broad variety of cells. Thus the reliance of CD4+ T cell responses on one or another cytokine or TNF family member could be, in part, dependent on the dominant APC migrating into priming sites following infection or immunization, and upon the differentiation state of that APC. Various APC may or may not be capable of cross-presentation and may express select TNF superfamily member ligands, which could focus CD4+ T cell costimulation on one of several TNF pathways. We believe this may also help explain the differences between our results with vaccinia viral infection and *Listeria* infection, where we could not otherwise detect obvious discrepancies in OX40 or CD27 expression on CD4+ T cells, or OX40L or CD70 expression on APC.

Indeed, regulation of TNFSF receptors also appears to be a dynamic process, as we have shown OX40 is limited on naïve cells but increases in an antigen-nonspecific fashion on CD4+ T cells with combined TLR/CD40 stimulus. Our data underscore the fact that CD4+ and CD8+ T cells operate under different rules for costimulation. We are not the first to suggest that CD4+ T cells are dependent on OX40-OX40L signals (23, 27, 51), while CD8+ T cells are preferentially dependent on CD27-CD70 (52–55). However, we believe we are among the first authors to simultaneously assess the effects of OX40L and CD70 blockade on CD4+ and CD8+ T cells in a common host. We confirm that CD4+ T cells are sensitive to CD70 blockade, as several authors have shown (20–22) and that the roles of OX40L and CD70 in CD4+ T cell priming partially overlap. However our data suggest that the dynamic regulation of OX40 may play an important role in this process. Thus, we can envision a model where TNF signaling pathways used by CD4+ T cells depend on activation and migration of diverse APC and the timing and regulation of TNF receptors on naïve CD4+ T cells.

Previous data using Balb/c mice with combined polyI:C and CD40 agonists with a dendritic-cell directed immunization showed that DEC205+ DC required CD70 to directly stimulate CD4+ T cells in the absence of IL-12 (21). The authors found that IL-12 signals were necessary for priming by 33D1+ DCs, while CD70 was required by DEC205 DCs (21). These authors used an *in vitro* stimulation approach using naïve transgenic CD4+ T cells paired with 24-hour activated DCs to demonstrate CD70 dependence, but did not explore a role for other TNF ligands (21). Given these data, we initially predicted that the absence of CD70 signals in IL-12β2RKO mice would eliminate CD4+ T cell activation, as CD8+ DEC205+ DC and CD11b+, 33D1+ DCs make up the bulk of classical DC subsets. However we found that CD70 blockade had a modest to no effect in IL-12-deficient mice, consistent with a model proposed by other authors, (22) in which IL-12 acts directly on

responding CD4+ T cells in a CD27-dependent manner. We found that OX40L blockade dominantly reduced responses, even in IL-12β2RKOs. Thus, our data build upon the work of previous authors (22) by showing that OX40L plays an IL12-independent role for CD4+ T cell priming *in vivo* in C57BL/6 mice.

We also find that naïve CD4+ T cells express very little OX40 *de novo*, and upregulate it *in vivo* in response to combined TLR/CD40 stimulus in an apparently antigen-independent fashion. This result may also provide a means to reconcile our observation of a role for OX40L where previous authors found a role only for CD70 (21) in mediating CD4+ T cell expansion following combined polyI:C/CD40 immunization. The data supporting an exclusive role for CD70 used DCs that had been stimulated by polyI:C/CD40 *in vivo* for 24 hours paired with T cells from unstimulated, transgenic mice. We found that unstimulated CD4+ T cells express CD27 but have very little OX40. In contrast, CD4+ T cells, in an antigen nonspecific fashion, upregulate OX40 as early as 8 hours after stimulus, but do not reach peak OX40 expression until 24 hours after immunization (Figure 5, E). Thus, naïve CD4+ T cells paired with activated DCs will have the ability to sense CD70 but not OX40L. The inability of naïve cells to quickly upregulate OX40 may have been a confounder of previous results (21).

However, another intriguing possibility is that nonclassical DC subsets could also play a role in polyI:C/CD40 priming when free antigen is used. The fact that our data for OX40L and CD70 are recapitulated in vaccinia viral infection confirms the physiological relevanace of our findings, suggesting that the differences between our results and those of other authors are probably due to differences in priming conditions, mouse models, or interpretation.

We have previously demonstrated the utility of the combined TLR/CD40 agonist approach for generating large numbers of antigen-specific polyfunctional CD8+ T cells (17, 28), and we now extend these findings to CD4+ T cells. The fact that substantial anamnestic responses may be generated by single injections of combined agonists should have considerable clinical utility. Additionally, our work demonstrates the potential of dendritic cell engagement without the requirement of coupling the antigen to targeting antibodies. The combination of two molecular agonists is a considerable improvement over live- or attenuated-pathogen-based vaccines, which can contribute to morbidity in immunocompromised individuals. Furthermore, while there are inherent dose-limiting toxicities to injection of TLR agonists alone (56) or CD40 agonists alone (57), the available data indicate that the synergistic effect of combining both agonists actually creates a wider therapeutic window by both reducing the toxicity of either agent (58) and by decreasing the effective dose of either agonist. This is convenient, as we noted only combined agonists promoted robust induction of the potent CD70 and OX40L costimulatory molecules on DC subsets. We hope that the availability of a vigorous, Th-1-skewing immunization regimen will prompt further exploration of its therapeutic uses in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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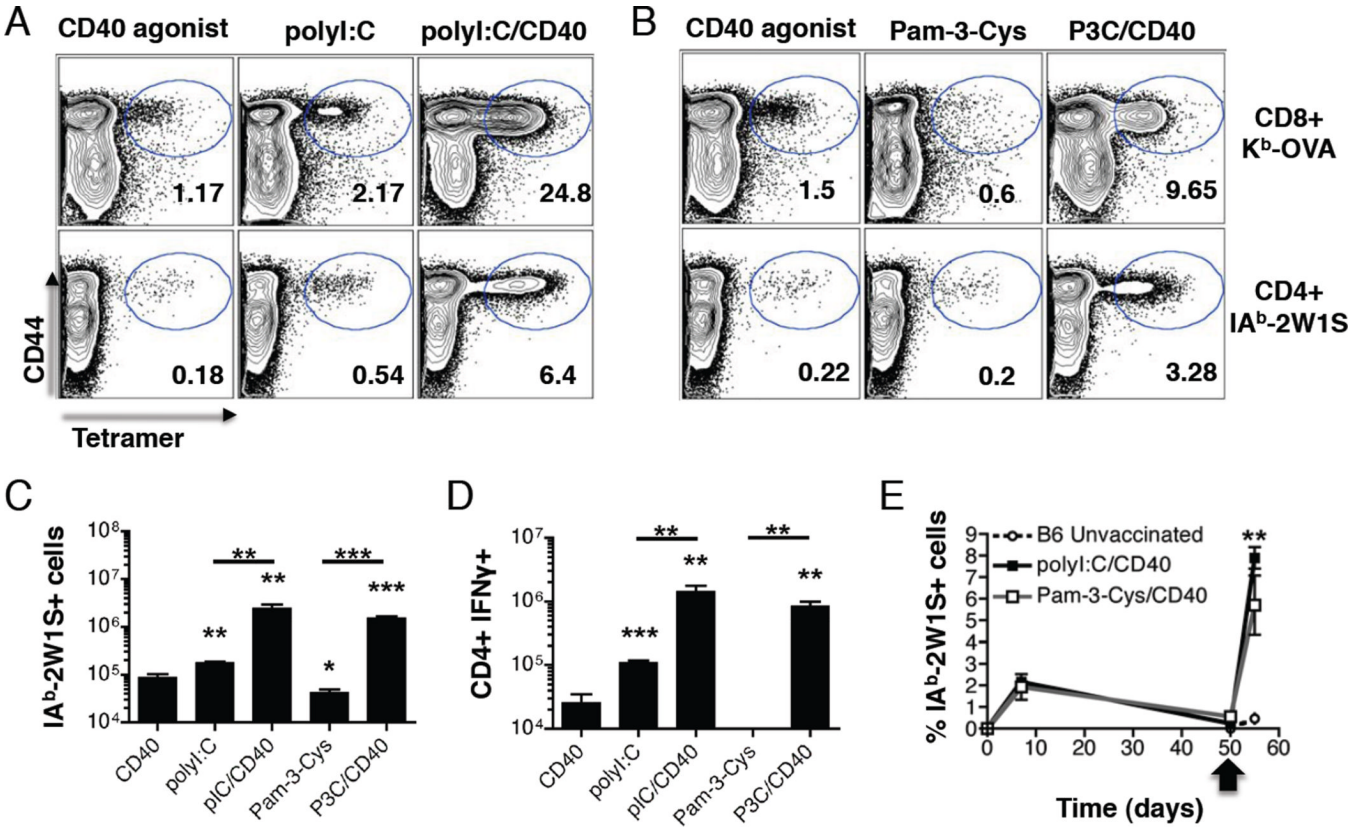

Figure 1.

Combined TLR and CD40 agonists promote synergistic immune responses for CD4+ T cells. Mice were immunized i.p. with OVA protein and 2W1S peptide with 50μg CD40 agonist alone (FGK4.5), 50μg TLR agonist alone, or both agonists combined, and spleens were harvested 7 days later. Shown are representative FACS plots of tetramer stains for single and combined adjuvants with either polyI:C (A) or Pam-3-Cys (B) as the TLR agonist. OVA-K^b-tetramer-specific CD8+ T cell responses are shown in the top row of each figure and 2W1S-IA^b-tetramer-specific CD4+ T cell responses are at bottom. 2W1S-specific CD4+ T cells per spleen are quantified for each treatment (C), as are the number of CD4+ T cells staining positive for intracellular IFNγ (D). Statistics represent pairwise comparisons between all samples and CD40 agonist alone (CD4), except as indicated by the bars, where ($p<0.01$ (**), $p<0.005$ (***)), calculated by Students' t Test. CD4+ T cells primed by combined TLR and CD40 promote robust secondary responses (E). Shown are peripheral blood 2W1S tetramer-specific CD4+ T cells over time. Mice were immunized on day 0 and challenged with recombinant 2W1S-expressing vaccinia on day 50. Statistics represent comparisons with naïve mice, and $p<0.01$ (**) for both groups as assessed by Students' t Test. Error bars represent the standard error of the mean. Data are representative of at least two independent experiments containing three or more mice per group.

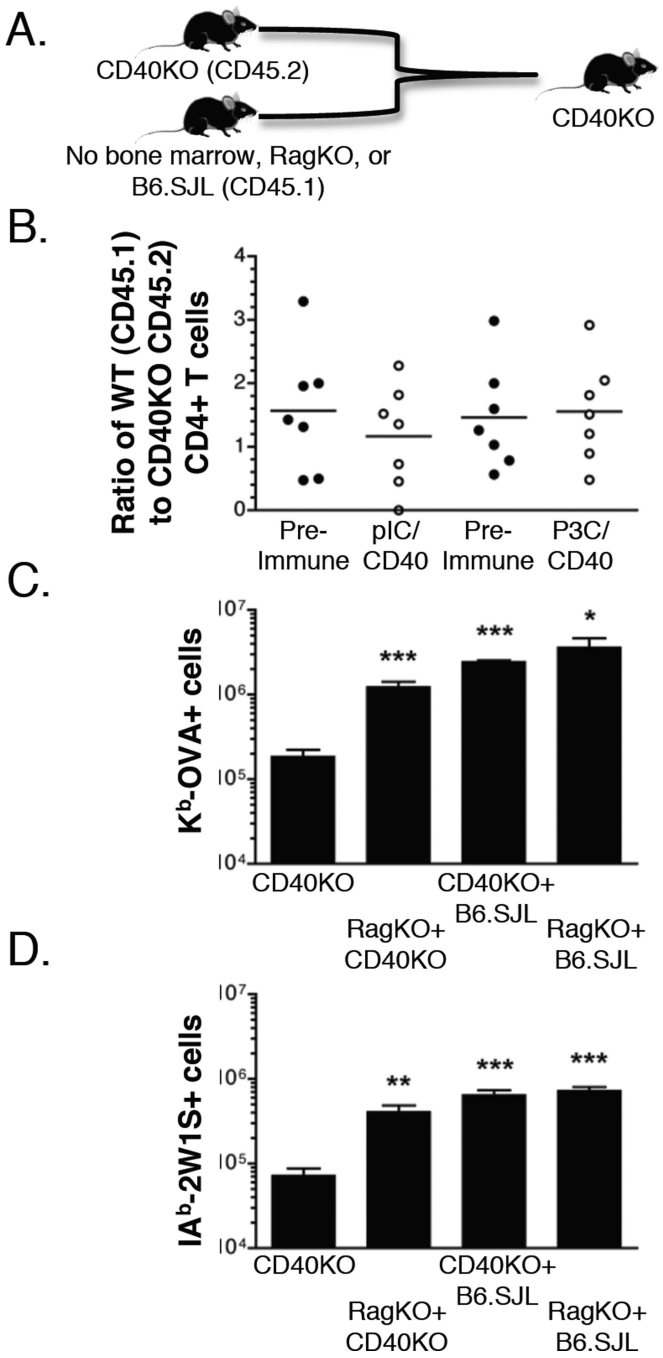

Figure 2.

The synergistic effect of combined polyI:C/CD40 is independent of T cell CD40, but is dependent on innate CD40 expression. Bone marrow was harvested from CD40 deficient (CD40KO), congenically-marked CD45.1+ CD40-sufficient (B6.SJL), or Rag-deficient but CD40-sufficient (RAGKO) mice and transplanted into CD40 deficient recipients (A). (B) CD4+ T cells do not require surface CD40 in order to respond to combined TLR/CD40 stimulus. Bone marrow from CD40 deficient (CD40KO, CD45.2) and sufficient (B6.SJL, CD45.1) mice was mixed 1:1 and injected into lethally irradiated CD40KO CD45.2 recipients. In analysis, cells were gated on CD45.1 expression and 2W1S-specific responses were measured in each group. For pre-immune samples, (filled circles) the ratio of WT

(CD45.1+) to CD40KO (CD45.1−) CD4+ T cells is shown. For immunized samples, (open circles) the ratio of WT (CD45.1+) to CD40KO (CD45.1−) 2W1S-IA^b tetramer staining is shown. (C and D) CD40KO recipient mice were lethally irradiated and grafted with CD40KO bone marrow, RagKO bone marrow mixed 3:1 with CD40KO bone marrow, B6.SJL bone marrow mixed 1:1 with CD40KO bone marrow, or RagKO bone marrow mixed 3:1 with B6.SJL bone marrow. All chimeras were grafted with 4×10^6 total T cell-depleted bone marrow cells. Mice were rested >12 weeks prior to immunization with polyI:C/CD40, and antigen-specific responses by CD8+ T cells (C) or CD4+ T cells (D) were quantified. Statistics were calculated by Students' t Test and are relative to CD40KO-grafted controls ($p<0.05$ (*), $p<0.01$ (**), $p<0.005$ (***)).

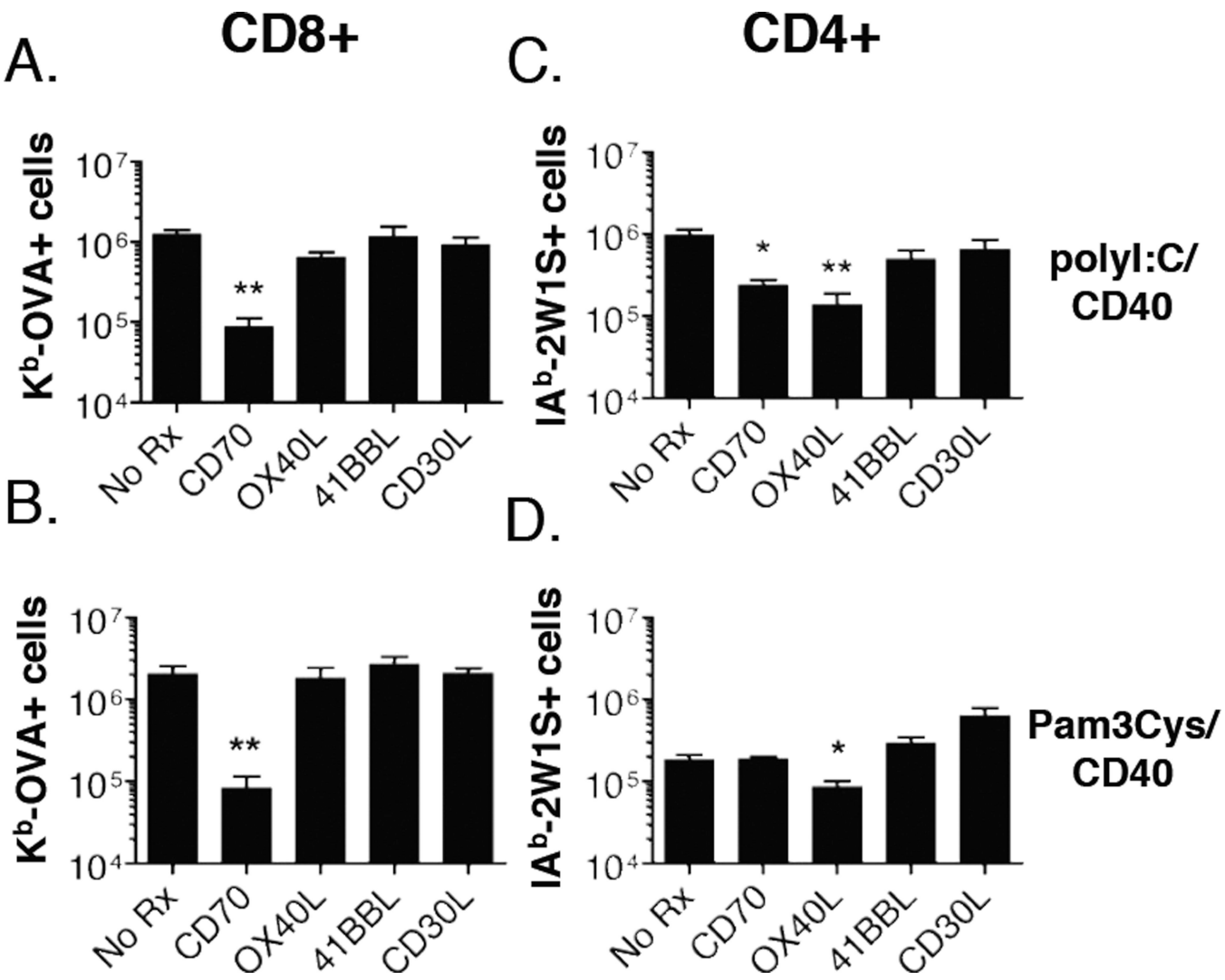

Figure 3.

T cell proliferation in response to combined TLR/CD40 stimulus is dependent on TNF superfamily members. Mice were administered blocking antibodies for several different TNF family members as shown IP on days −1 and 0 relative to immunization. Spleens were harvested 7 days after immunization and CD8+ T cell responses to polyI:C/CD40 (A) or Pam-3-Cys/CD40 (B) were quantified in the presence of the blocking antibodies. Likewise, the number of antigen-specific CD4+ T cells for polyI:C/CD40 (C) or Pam-3-Cys/CD40 (D) were quantified in the same mice. All statistics represent comparisons to unblocked controls (No Rx) and were calculated by Students' t Test with $p<0.05$ (*), $p<0.01$ (**), and $p<0.005$ (***). Data are representative of at least two independent experiments containing 3 or more mice per group. Error bars represent the standard error of the mean.

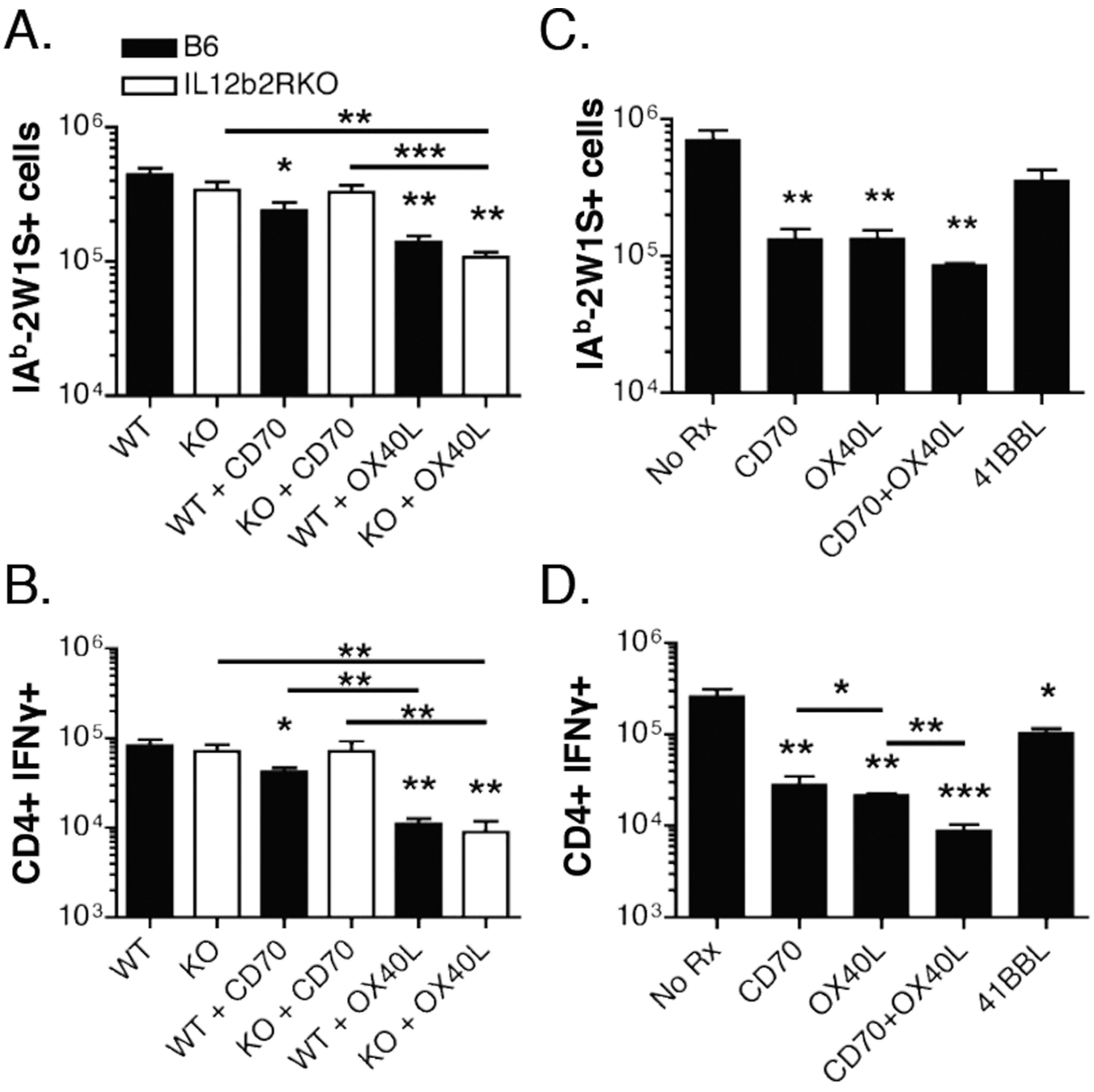

Figure 4.

Compared to the role of OX40L in CD4+ T cell responses to polyI:C/CD40, IL12 and CD70 are dispensable. (A and B) IL12 deficiency has a modest effect on polyI:C/CD40 immunization, but OX40L blockade reduces the response significantly. Wild-type B6 or IL12β2R-deficient (KO) mice were administered blocking antibodies for different TNF family members as shown IP on days −1 and 0 relative to immunization. Spleens were harvested 7 days after immunization and splenic 2W1S-specific CD4+ T cell tetramer (A) and cytokine (B) responses to polyI:C/CD40 were quantified in the presence of the blocking antibodies. (C and D) Combined blockade of CD70 and OX40L reduces CD4+ T cell responses more than single blockade. B6 mice were immunized as in A and B. Mice were

harvested 7 days after immunization and 2W1S-specific CD4+ T cell tetramer (C) and cytokine (D) responses in the spleen were quantified. Error bars represent standard error of the mean. Data are representative of at least two independent experiments with at least three mice per group. All statistics represent comparisons to unblocked controls (No Rx), unless indicated otherwise, and were calculated by Students' t Test with $p<0.05$ (*), $p<0.01$ (**), and $p<0.005$ (***).

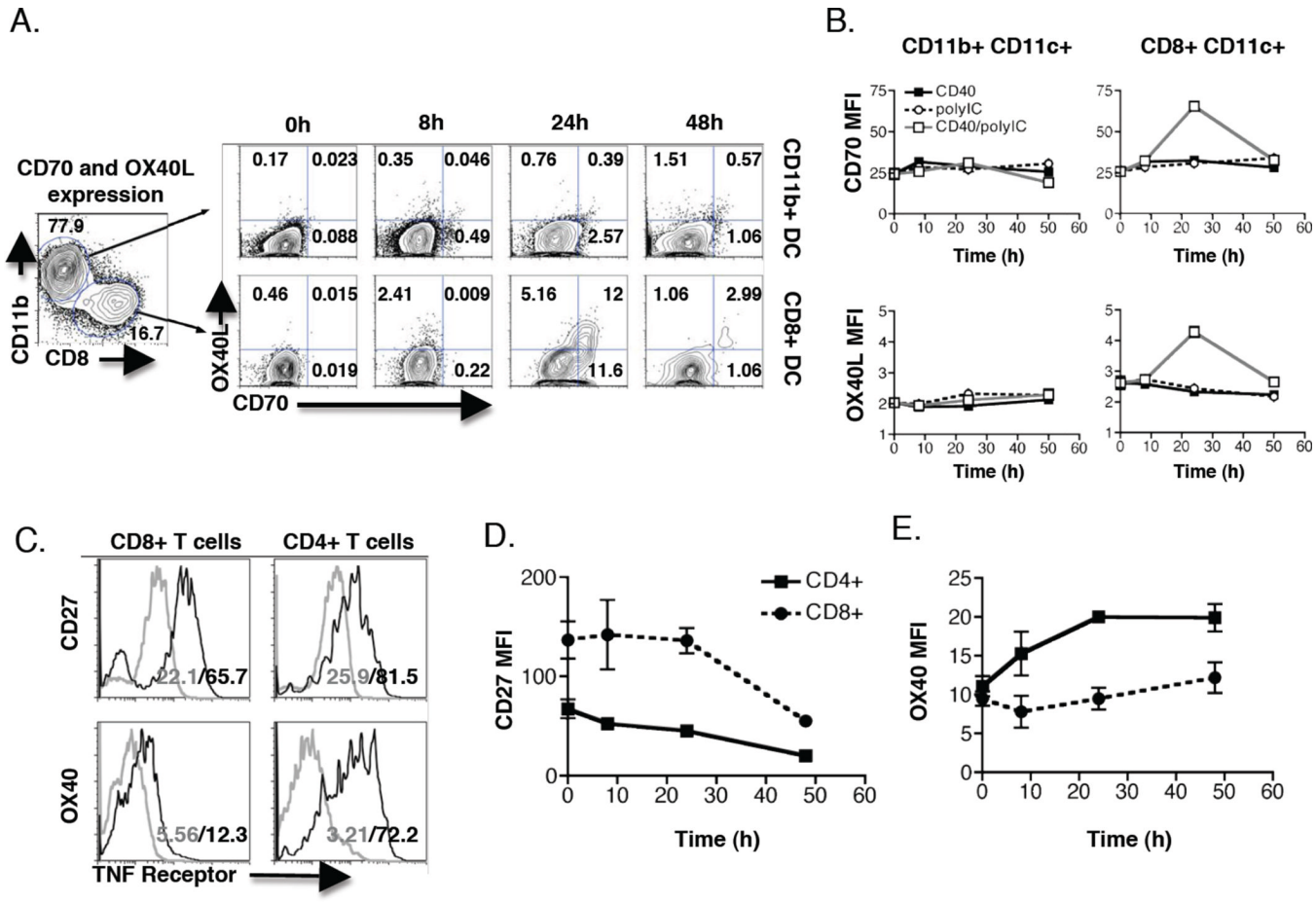

Figure 5.

CD8+ dendritic cells uniquely upregulate OX40L and CD70 in response to combined polyI:C/CD40 but not to either agonist individually. CD4+ T cells upregulate OX40 late after stimulation *in vitro* or *in vivo*. (A) Representative FACS plots for the kinetics of TNF ligand expression by CD8+ or CD11b+, CD11c+ CD19− CD3− cells (DCs). DCs were isolated from mice at various timepoints following combined polyI:C/CD40 immunization. Shown is the gating strategy for separating CD8+ DCs from CD11b+ DCs. (B) Expression of CD70 (top) or OX40L (bottom) by CD11b (left) or CD8+ (right) DCs in response to various agonist combinations. Mice were immunized i.p. at the outset and spleens were harvested at the indicated timepoints. (C–E) CD4+ T cells express OX40 in immunized, but not naïve mice, whereas CD4+ T cells from naïve mice express CD27 and only upregulate it modestly upon activation. (C) Representative FACS plots showing that activated CD8+ and CD4+ T cells both upregulate CD27 upon activation *in* vitro with 0.5μg/mL of anti-CD3 (2C11) after 24 hours (C, top). Naïve *ex vivo* profiles are in gray and activated profiles are in black. Shown are numerical values for MFI for 'naïve/activated' cells. Whereas CD4+ T cells massively upregulate OX40 in response to CD3 stimulus, CD8+ T cells only modestly change expression (C, bottom). (D and E) Mice were immunized intraperitoneally at t=0 with polyI:C/CD40, harvested at the indicated timepoints, and cells stained for CD44 and either CD27 (D) or OX40 (E). Shown are data gated on either CD4+ or CD8+ and $CD44^{high}$. Error bars represent standard error of the mean. Data are representative of at least two independent experiments with at least three mice per group, except C, which is representative of four independent experiments.

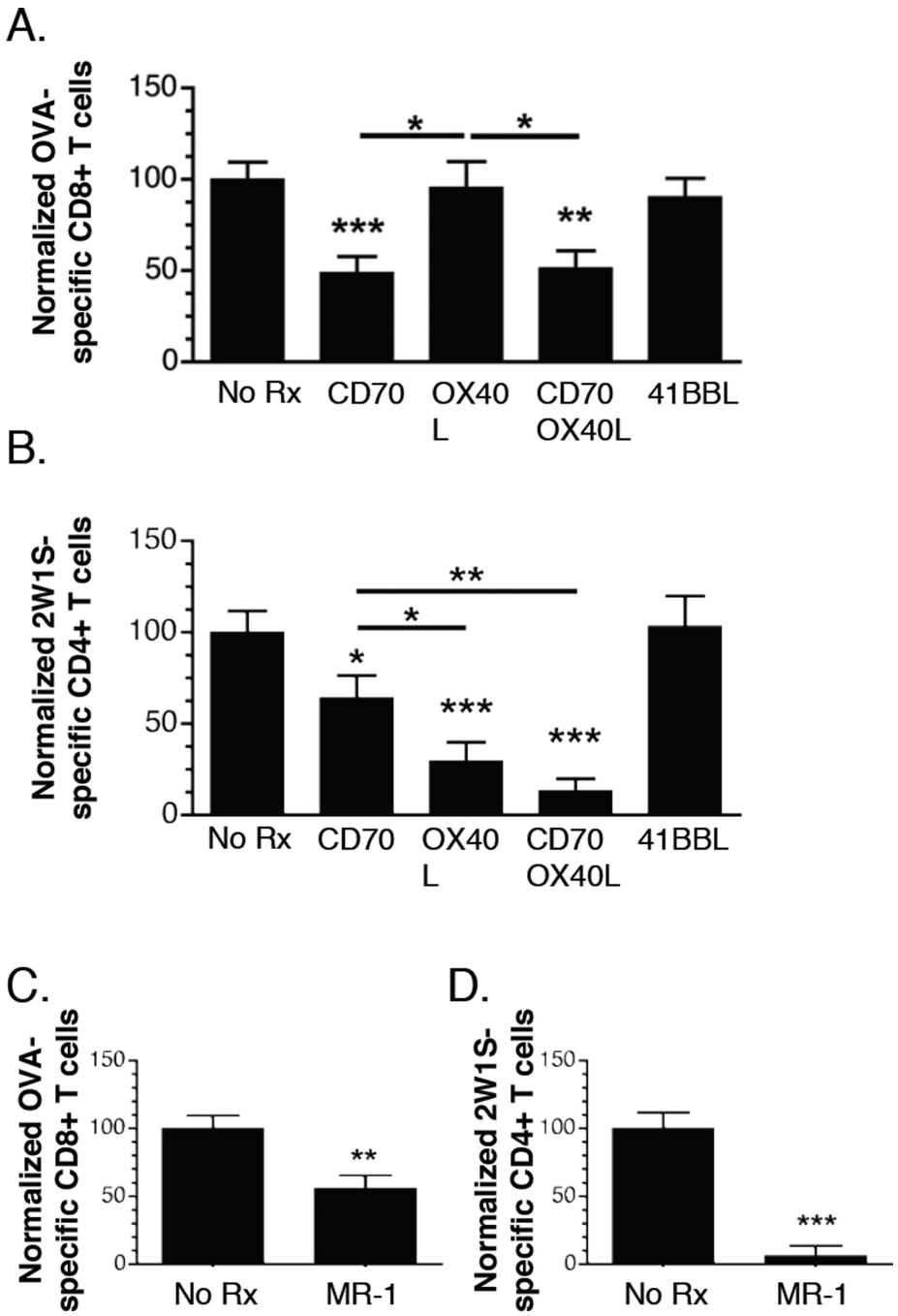

Figure 6.

Responses to vaccinia virus infection by CD4+ and CD8+ T cells depend on TNF ligands similar to combined TLR/CD40 immunization. (A) CD8+ T cell responses to vaccinia depend on CD70, whereas (B) CD4+ T cell responses to vaccinia depend dominantly on OX40L with a secondary dependence on CD70. Mice were infected with 2×10^7 pfu of recombinant vaccinia i.p. 8 days prior to harvest and administered 250μg of blocking antibodies against the indicated TNFSF members i.p. on days −1, 0, and +2 relative to immunization. (C and D) CD8+ (C) and CD4+ (D) T cell responses to vaccinia virus, like TLR/CD40 stimulus, depend on CD40-CD40L signals. Mice were infected with 2×10^7 pfu of recombinant vaccinia i.p. 8 days prior to harvest and administered the CD40L-blocking

antibody MR-1 (anti-CD154), 250μg i.p. on days −1, 0, and +2 relative to immunization. Shown are combined data for total splenic antigen-specific T cell numbers normalized to the control (No Rx) response for three independent experiments containing at least three mice per group in each experiment. Mean control responses are: (A and C) 2.37e6 cells/spleen and (B and D) 1.03e5 cells/spleen. Statistics are p values for the indicated group compared to controls, except where indicated, with $p<0.05$ (*), $p<0.01$ (**), and $p<0.005$ (***). All error bars represent standard error of the mean.