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*J Immunol* published online 5 September 2012
http://www.jimmunol.org/content/early/2012/09/05/jimmunol.1200597

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Supplementary Material
http://www.jimmunol.org/content/suppl/2012/09/05/jimmunol.1200597.DC1

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T Cell Costimulation by TNFR Superfamily (TNFRSF)4 and TNFRSF25 in the Context of Vaccination

Taylor H. Schreiber, Dietlinde Wolf, Maria Bodero, Louis Gonzalez, and Eckhard R. Podack

TNFR superfamily (TNFRSF)4 (OX40, CD134) and TNFRSF25 are costimulatory receptors that influence CD4+ and CD8+ T cell responses to cognate Ag. Independently, these receptors have been described to stimulate overlapping functions, including enhanced proliferation and activation for both regulatory T cells (CD4+Foxp3+; Tregs) and conventional T cells (CD4+Foxp3− or CD8+Foxp3+; Tconv). To determine the relative functionality of TNFRSF4 and TNFRSF25 in T cell immunity, the activity of TNFRSF4 and TNFRSF25 agonistic Abs was compared in the context of both traditional protein/adjuvant (OVA/aluminum hydroxide) and CD8+-specific heat shock protein-based (gp96-Ig) vaccine approaches. These studies demonstrate that both TNFRSF4 and TNFRSF25 independently and additively costimulate vaccine-induced CD8+ T cell proliferation following both primary and secondary Ag challenge. In contrast, the activities of TNFRSF4 and TNFRSF25 were observed to be divergent in the costimulation of CD4+ T cell immunity. TNFRSF4 agonists were potent costimulators of OVA/aluminum hydroxide-induced CD4+ Tconv proliferation, but they only weakly costimulated Treg proliferation and IgG2a production, whereas TNFRSF25 agonists were strong costimulators of Treg proliferation, producers of IgG1, IgG2a, and IgG2b, and weak costimulators of CD4+ Tconv proliferation. Interestingly, Ag-specific cellular and humoral responses were uncoupled upon secondary immunization, which was dramatically affected by the presence of TNFRSF4 or TNFRSF25 costimulation. These studies highlight the overlapping but nonredundant activities of TNFRSF4 and TNFRSF25 in T cell immunity, which may guide the application of receptor agonistic agents as vaccine adjuvants for infectious disease and tumor immunity. The Journal of Immunology, 2012, 189: 000–000.
Materials and Methods

Mice and cell lines

Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Foxp3+RFP+ reporter mice on a B6 background (provided by Dr. Richard Flavell) (9) and OT-II and OT-I mice were bred in our animal facility. Mice were used at 6–12 wk age and were maintained in pathogen-free conditions at the University of Miami Animal Facilities. All animal use procedures were approved by the University of Miami Animal Care and Use Committee. 3T3-OVA-gp96-Ig vaccine cells were maintained and used as previously described (10, 11).

Reagents, Abs, and flow cytometry

Commercial Abs for use in flow cytometry, ELISA, and in vivo studies were purchased from BD Pharmingen, eBioscience, or BioLegend. The Armenian hamster IgG isotype control was bought from eBioscience. Armenian hamster hybridomas producing Abs to mouse TNFRSF25 (4C12, agonistic) were generated as described previously (12). Briefly, 4C12 and OX86 were produced in hollow-fiber bioreactors (Fibercell Systems, Frederick, MD) and purified from serum-free supernatants on a protein G column (GE Healthcare, Little Chalfont, U.K.). For flow cytometry analysis, single-cell suspensions were prepared from spleen and lymph nodes. Cells (10^6) were preblocked with anti-mouse CD16/CD32 and stained with different Ab combinations. Intracellular staining was performed according to standard procedures. Flow cytometric analysis was performed on a Becton Dickinson Fortessa instrument and DIVA or FlowJo software. Suspensions of OVA (crystallized chicken egg OVA grade V; Sigma-Aldrich, St. Louis, MO) and aluminum sulfate (Sigma-Aldrich) were prepared as previously described (12). For flow cytometry analysis, cells were stained using 96-well plates coated with 100 μg/ml OVA in pH 7.7 bicarbonate buffer (Sigma-Aldrich) and biotin-conjugated anti-mouse IgM, IgG1, IgG2a, or IgG2b isotype Abs for detection.

Adoptive transfer model

OT-I cells were isolated from total splenocytes of OT-I TCR transgenic mice using a CD8+ T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. OT-II cells were isolated from total splenocytes of OT-II TCR transgenic mice using a CD4+ T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. Cell enrichment purity was determined by flow cytometry (≥90% purity). OT-I (5 x 10^5) and OT-II (10^6) cells were then mixed and injected in a total volume of 100 μl HBSS per mouse by i.v. injection.

Statistical analysis

All graphing and statistical analyses were performed using the Applied Biosystems Prism program. Paired analysis was performed using a Student t test. Analysis of conditions with more than two conditions was performed using one-way ANOVA with a Tukey post hoc test. A p value of <0.05 was considered statistically significant.

Results

T cell activation by adjuvanted versus cross-presented Ag

Aluminum salts (alum) are well-known adjuvants that have been used in vaccine preparations for decades with, until very recently, limited understanding of how they contributed to enhanced immune responses. In order to determine the impact of adjuvanted Ag presentation on T cell responses, we developed a model to compare Ag presentation and T cell responses in OT-I and OT-II T cell transgenics. We hypothesized that presentation of Ag to OT-I cells in the context of Ag-pulsed APCs (cross-priming) would result in a T cell response comparable in magnitude and specificity to OT-I cells stimulated with Ag in vitro (adjuvanted). We examined whether Ag presentation by cross-priming generated sufficient Ag-pulsed APCs to compete with Ag-pulsed APCs presented by an Ag-pulsed, antigen-specific T cell line (FIR mice) at the time of adoptive transfer. Adoptive transfer with OT-I cells was performed as described in Fig. 1, followed by immunization with either 3T3-OVA-gp96-Ig (A) or OVA/alum (B) together with either IgG control Ab (100 μg, filled circles), TNFRSF25 agonistic Abs (clone 4C12, 20 μg, filled squares), TNFRSF4 agonistic Abs (clone OX86, 100 μg, filled triangles), or both 4C12 and OX86 Abs combined (filled inverted triangles). OT-I proliferation in the peripheral blood was measured by flow cytometry on the indicated days, and representative plots of Vβ5+ cells (pregated on CD3+CD8+ cells) are illustrated in (C) on day 5 for each condition. Data indicate means ± SEM for five or more mice in each of two or more independent experiments.
activation. The mechanism of alum as an adjuvant remains unclear, but most reports agree that alum functions as an innate trigger for dendritic cells and macrophages to produce inflammatory Th1 and Th2 type cytokines, contributing to activation of both CD4+ and CD8+ T cell immunity (13–19). Gp96 (gp94) is an endoplasmic reticulum chaperone and naturally occurring danger-associated molecular pattern released from damaged cells, which functions as a natural adjuvant for dendritic cells and macrophages and selectively delivers Ags for MHC I cross-presentation and cross-priming of CD8-mediated T cell immunity (20). Both alum and gp96 can stimulate Ag-specific T cell-mediated immunity by either precipitation or coexpression, respectively, with a selected Ag. For alums, the specific mechanism of innate activation and Ag delivery remains unclear for gp96.

Ag-specific activation of CD4+ and CD8+ T cell immunity using OVA complexed to alum (OVA/alum) was systematically compared with vaccination using a secretable form of gp96 (gp96-Ig) expressed in cis with OVA by 3T3 cells (3T3-OVA-gp96-Ig) (11). Vaccination with 3T3-OVA-gp96-Ig leads to specific activation of TCR transgenic (Vα2+Vβ6+) OVA-specific CD8+ T cells (OT-I) in vivo (10, 21, 22). C57BL/6 mice were adoptively transferred with a mixed population of TCR transgenic (Vα2+Vβ6+) OVA-specific CD8+ T cells (OT-I) in vivo and 106 cells/24 h or with 3T3-OVA-gp96-Ig (106 cells/24 h) or with these mice were vaccinated i.p. with either 3T3-OVA-gp96-Ig cells or (C) OVA/alum together with the indicated Abs. Representative flow cytometry plots of splenocytes analyzed on day 57 and pregated on CD3+ CD8+ cells are shown. The percentage of TCR Vα2+ Vβ6+ double-positive cells are overlaid on each plot. Data indicate means ± SEM for two independent experiments.

FIGURE 3. Tissue distribution and response of OT-I cells to secondary immunization. The indicated groups were treated as shown in Fig. 2, and the absolute numbers (left columns) and frequencies (right columns) of OT-I cells in the (A) spleen, (B) lymph nodes, or (C) peritoneal cavity determined on day 5 following primary immunization are shown. Certain groups treated as indicated in (A)–(C) were monitored until day 52 following the primary immunization, at which time they were treated with a secondary immunization with (D) 3T3-OVA-gp96-Ig or (E) OVA/alum together with the indicated Abs. Representative flow cytometry plots of splenocytes analyzed on day 57 and pregated on CD3+ CD8+ cells are shown. The percentage of TCR Vα2+ Vβ6+ double-positive cells are overlaid on each plot. Data indicate means ± SEM for two independent experiments.
Analyses of spleen, lymph nodes, and peritoneal cavity-infiltrating cells indicate that the observed effects of TNFRSF4 and TNFRSF25 costimulation on peripheral blood OT-I cells are systemic (Fig. 3). Both total numbers (left columns) and frequencies (right columns) of OT-I cells were determined in the spleen (Fig. 3A), pooled mesenteric and inguinal lymph nodes (Fig. 3B), and peritoneal cavity-infiltrating cells (Fig. 3C) 5 d after immunization with the indicated vaccine combination. This time point precedes the “peak” of OT-I expansion following immunization with OVA/alum but is at the time of peak expansion following immunization with 3T3-OVA-gp96. Analysis of peritoneal cells indicates that in mice immunized with 3T3-OVA-gp96, the observed enhancement of OT-I expansion by OX86 and 4C12 is already declining by day 5 at the site of immunization. In contrast, because the peak of OT-I expansion in the peripheral blood has not yet occurred for animals immunized with OVA/alum, it is not surprising that the costimulatory effect of OX86 and 4C12 is magnified in the peritoneal cavity on day 5. Taken together, these tissue analyses provide a consistent picture of OT-I costimulation across several tissues, which appears to originate at the site of immunization (peritoneal cavity), as previously shown by Oizumi et al. (10), and subsequently distributes systemically with slightly delayed kinetics and can be observed in the lymph nodes, spleen, and peripheral blood.

To determine the influence of TNFRSF4 and TNFRSF25 costimulation at the time of priming on the magnitude of the memory response, the frequencies of OT-I cells were followed in the peripheral blood of primed animals from day 38 to day 52. These data demonstrate that the “set point” for OT-I cells following contraction from the primary immunization is equal between all groups immunized with 3T3-OVA-gp96-Ig (2% of total CD8+ cells; Supplemental Fig. 2A). In contrast, mice immunized with OVA/alum exhibited dramatic differences in the resting frequencies of OT-I cells depending on whether they were additionally costimulated with 4C12 (5.17 ± 0.58% of total CD8+ cells), OX86 (8.16 ± 1.5% of total CD8+ cells), or the combination of 4C12 and OX86 (13.0 ± 2.7% of total CD8+ cells) as compared with IgG controls (1.56 ± 0.09% of total CD8+ cells; Supplemental Fig. 2A).

On day 52, primed mice received a secondary immunization with the vaccine/costimulator combination administered to each mouse at the time of priming. These data indicate that for animals immunized with 3T3-OVA-gp96-Ig, the OT-I proliferative response is weaker in the spleen on day 5 following the secondary immunization than observed on day 5 following the primary immunization (Fig. 3D). The addition of either 4C12 Ab or 4C12 in addition to OX86 Ab significantly improved the magnitude of OT-I proliferation at the time of the recall response. Similarly, secondary immunization with OVA/alum was observed to stimulate weaker OT-I proliferation unless combined with 4C12 Ab, and the effect of OX86 Ab was synergistic at the time of boosting with 4C12 Ab treatment (Fig. 3E).

Costimulation of vaccine-primed OT-II cells by TNFRSF4 and TNFRSF25

Because these experiments were performed in animals adoptively transferred with both OT-I and OT-II cells, the activation of each subset was monitored simultaneously within individual mice. In all animals vaccinated with 3T3-OVA-gp96-Ig, regardless of the addition of OX86 and/or 4C12 Abs, there was absolutely no increase in the frequency of OT-II cells out of total CD4+ cells at any of the time points analyzed (Fig. 4A). These findings are in agreement with previous reports demonstrating that the vaccine activity of gp96 functions as a powerful and exclusive Ag delivery system to the MHC I cross-presentation machinery of APCs and can function in the absence of CD4+ cells (8, 10, 11, 25).

Vaccination with OVA/alum stimulated efficient proliferation of OT-II cells, which was further costimulated by administration OX40 agonistic Abs (Fig. 4B). Specifically, stimulation of TNFRSF4 by OX86 significantly increased the rate, amplitude, and duration of OT-II cell proliferation in vivo. In contrast, administration of TNFRSF25 agonistic Abs did not stimulate a significant increase in OT-II cell proliferation as compared with administration of IgG control Abs. Addition of 4C12 Abs reduced the costimulatory effect of OX86 Abs to OT-II cell proliferation and is in stark contrast to the additive costimulatory effect of combined administration of OX86 and 4C12 to vaccine-primed OT-I cells. These data indicate that TNFRSF4 and TNFRSF25 differentially costimulate OVA/alum-primed OT-II effector T cells. Representative flow cytometry plots from day 5 after immunization are illustrated for each group (Fig. 4C). Similar to OT-I cells, no differences were observed in the expression pattern of CD44, CD62L, or CCR7 at 5 wk after immunization for any of the groups (data not shown). Also similar to OT-I cells, the systemic distribution pattern of OT-II cells was observed to reflect observations made in the peripheral blood when analyzed in the spleen (Fig. 5A), inguinal lymph nodes (Fig. 5B), and peritoneal cavity (Fig. 5C). As predicted by analysis of peripheral blood cells, OX86 appeared to provide the strongest costimulatory signal to OT-II cells systemically. Costimulation of OT-II cells in the peritoneal cavity...
was greatest by treatment with OX86 alone on day 5, but was already on the decline when combined with 4C12 (Fig. 5C). Similar to OT-I cells, these observations suggest that OT-II proliferation originates at the site of vaccination (within the peritoneal cavity) and that peak proliferation at this tissue site precedes the peak of expansion observed in the peripheral blood, which does not peak until day 8 in OX86-treated animals but which has already peaked in the peripheral blood when used in combination with 4C12 (compare Fig. 4B to Fig. 5C). The frequency of OT-II cells was monitored in the peripheral blood following contraction from the primary immunization from day 38 to day 52. Interestingly, these data indicate that, similar to OT-I cells, there were no differences in the set point of memory OT-II cells for any mice immunized with 3T3-OVA-gp96-Ig (Supplemental Fig. 2B). In mice immunized with OVA/alum, there were no differences in the set point of peripheral blood OT-II cells except for mice that received OX86 at the time of immunization (3.36 ± 0.69% of total CD4+ cells) as compared with IgG controls (0.43 ± 0.09% of total CD4+ cells). In mice boosted with 3T3-OVA-gp96-Ig, there was no primary proliferative response to immunization and there was also no proliferative response to secondary immunization (Fig. 5D). Interestingly, in mice that received the secondary immunization with OVA/alum, there was also no secondary proliferative response to immunization unless the immunization was combined with 4C12 Ab (1.5% of total CD4+ cells) or with OX86 Ab (6.8% of total CD4+ cells). In contrast to the synergetic effect of combined administration of 4C12 and OX86 Abs on the proliferative response of OT-I cells (Fig. 3D, 3E), no synergy was observed between 4C12 and OX86 on the proliferative response of OT-II cells upon secondary immunization (Fig. 5E).

Ag-specific serum Ig analysis

Serum was isolated from whole blood samples collected by cardiac puncture on day 5 following the primary (experimental day 5) or secondary (experiment day 57) immunization with the indicated treatment. The serum titers of OVA-specific IgM, IgG1, IgG2a, and IgG2b Abs were determined using ELISA assays. Following primary immunization with 3T3-OVA-gp96, OVA-specific Abs of all subtypes were below the limit of detection (data not shown). Low levels of IgG1, IgG2a, and IgG2b were detectable in mice immunized with OVA/alum, and levels of each of these Ig subtypes was increased by concurrent treatment with OX86 Ab (EC50 = 0.005, 0.035, and 0.021, respectively; Fig. 6, left column). TNFRSF25 stimulation with 4C12 Ab also increased the serum titer of OVA-specific IgG2b following primary immunization with OVA/alum (EC50 = 0.072). As predicted, the titers of OVA-specific IgM Ab were significantly higher than any of the IgG subtypes following primary immunization with either OVA/alum or 3T3-OVA-gp96 (Supplemental Fig. 3).

Secondary immunization with OVA/alum or 3T3-OVA-gp96 stimulated significantly higher serum titers of OVA-specific IgM, IgG1, IgG2a, and IgG2b than those observed following primary immunization, as expected. In OVA/alum-immunized mice, the costimulatory effect of TNFRSF4 and TNFRSF25 stimulation was statistically equivalent for all IgG subtypes examined (IgG1 EC50 = 361,674; IgG2a EC50 = 7,308; IgG2b EC50 = 11,167) and significantly increased the serum titer of IgG1 as compared with IgG Ab-treated controls (EC50 = 361,674 versus 35,933, p = 0.0039; Fig. 6, middle column). In 3T3-OVA-gp96-Ig–immunized mice, costimulation of TNFRSF25 led to a significant increase in the titer of IgG1 (EC50 = 7665 versus 100.5; p = 0.001) and a moderate increase in IgG2a (EC50 = 849.8) and IgG2b isotypes when compared with IgG Ab-treated controls (EC50 = 1806; Fig. 6, right column). Addition of OX86 did not further increase IgG1 or IgG2b concentrations following 3T3-OVA-gp96-Ig immunization with 4C12 Ab, but it did lead to an additive increase in the concentration of IgG2a Ab (EC50 = 5856 versus 849.8, p = 0.003).

**Differential costimulation of Tregs by TNFRSF4 and TNFRSF25**

Because both TNFRSF4 and TNFRSF25 have been described to costimulate Foxp3+ Tregs, the response of this CD4+ T cell subset was also analyzed. As previously reported, administration of 4C12 Abs stimulated the proliferation of CD4+ Tregs to 3- to 4-fold above
their resting frequency (Fig. 7) (7). The kinetics of expansion were slightly delayed in both vaccination groups as compared with naive mice, peaking on day 6 in the 3T3-OVA-gp96–immunized group at 30.72 ± 6.8% of all CD4+ cells (Fig. 7A) and on day 5 in the OVA/alum-immunized group at 30.55 ± 5.0% of all CD4+ cells (Fig. 7B). This proliferation occurred concurrently with the proliferation of OT-I cells in the context of 3T3-OVA-gp96-Ig vaccination (Figs. 2A, 7A) and together with proliferation of both OT-I and OT-II cells in OVA/alum-vaccinated mice (Figs. 2B, 4B, 7B). Administration of OX86 Abs led to minor proliferation of Tregs (3T3-OVA-gp96 immunization = 11.52 ± 1.1% of total CD4+ cells, OVA/alum immunization = 14.6 ± 3.0% of total CD4+ cells), which occurred regardless of the vaccination method and is similar to the response to OX86 without immunization (7). When OX86 and 4C12 were combined, increased proliferation to roughly additive levels was observed for Tregs: 37.35 ± 4.7% of all CD4+ cells and peaking on day 4 in the 3T3-OVA-gp96–immunized group (Fig. 7A) and to 44.47 ± 4.4% of all CD4+ cells and peaking on day 5 in the OVA/alum-immunized group (Fig. 7B). In the setting of OVA/alum vaccination, the combined influence of TNFRSF4 and TNFRSF25 stimulation led to Treg levels that peaked at nearly half of all CD4+ T cells (Fig. 7B), at a time when ~20% of the CD4+ T cells were also OT-II cells (compare Fig. 7B to Fig. 4B). Proliferation of Tregs to these levels concurrent with costimulation of Ag-specific OT-I and OT-II cells in the context of vaccination clearly indicates that absolute increases in Tregs are not predictive of immune suppression.

The distinctions of OVA/alum versus gp96-Ig chaperoned OVA as OT-I and OT-II cell stimulators both alone and in conjunction with TNFRSF4 and TNFRSF25 costimulation are summarized in Table I.

**Discussion**

Adjuvanted protein vaccines are a very common class of vaccines that typically lead to Ag presentation via the endosomal pathway to MHC II. We used OVA/alum as a model for this class of vaccine and analyzed the proliferation of Ag-specific CD4+ and CD8+ T cells following vaccination. These studies demonstrated that

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**Figure 6.** OVA-specific serum Ab analysis following primary and secondary immunization. Serum samples were collected from mice on day 5 following primary immunization (experimental day 5) and secondary immunization (experimental day 57) as outlined in Figs. 3 and 5. The OVA-specific Ab titers were determined for the indicated Ab isotypes using ELISA assays. Serially diluted titers of serum IgG1, IgG2a, and IgG2b are shown for the indicated groups on day 5 following primary immunization with OVA/alum (left column), secondary immunization with OVA/alum (middle column), or secondary immunization with 3T3-OVA-gp96-Ig (right column). Ab titers following primary immunization with 3T3-OVA-gp96-Ig were below the limit of detection and are not shown. Data indicate means ± SEM for each dilution of serum collected from two mice per group per condition.

**Figure 7.** Comparative costimulation of Tregs by TNFRSF4 and TNFRSF25 agonistic Abs. In FIR mice adoptively transferred with OT-I and OT-II cells and immunized as described in Figs. 1–3, the expansion of CD4+Foxp3+ Tregs (expressing the Foxp3-RFP transgene) were monitored by flow cytometry of peripheral blood cells following immunization with 3T3-OVA-gp96-Ig (A) or OVA/alum (B). Data indicate means ± SEM for five or more mice in each of two or more independent experiments.

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**Table I.** Summary of TNFRSF4 and TNFRSF25 adjuvant effects

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Summary of OT-I, OT-II, and Treg responses to immunization with adjuvanted (alum) versus cross-presented (gp96-Ig) OVA with and without costimulation by agonistic Abs to TNFRSF4 (OX86) and TNFRSF25 (4C12). Effects are arbitrarily scored as: –, no effect; +, mild effect; ++, moderate effect; and ++++, strong effect as compared to nonvaccinated controls following primary immunization.
OVA/alum vaccination leads to the proliferation of both CD4+ and CD8+ T cells, and that the Ag threshold for CD4+ cell activation is lower than that for CD8+ T cells. This finding confirms that Ag presentation of OVA-derived peptides occurs on both MHC I and MHC II. In contrast, vaccination with allogeneic cells expressing OVA and secreting gp96-Ig, which chaperones OVA-derived peptides, stimulated the exclusive proliferation of OT-I cells, indicating that Ag presentation was selective and exclusive to MHC I, likely via the Ag cross-presentation pathway. Additionally, an ~600-fold larger dose of aluminum salt-adjuvanted Ag was required to stimulate an equivalent OT-I response to gp96-Ig-chaperoned Ag assuming that 100% of OVA was chaperoned by gp96-Ig. If instead we assume that OVA competes equally for gp96-Ig binding with the estimated 10,000 other proteins actively produced by 3T3 cells and presented by MHC I, it is estimated that gp96-Ig-chaperoned peptides in the subpicogram range have equivalent CD8+ T cell-stimulating activity to alum-adjuvanted peptide in the microgram range. The two vaccine strategies are therefore distinct both in their efficiency of Ag presentation to MHC I (with gp96-Ig being far superior) and to MHC II (with OVA/alum being far superior).

Next, we compared the relative activity of TNFRSF4 and TNFRSF25 as costimulators of vaccine-primed effector T cells based on their published similarities in activity. These studies demonstrate that TNFRSF4 and TNFRSF25 are similar and additive in their ability to costimulate CD8+ T cells regardless of the vaccine approach used following primary immunization. However, the two receptors differ significantly in their capacity to costimulate effector and regulatory subsets of CD4+ T cells. TNFRSF25 is, as reported, an excellent stimulator of Treg proliferation (7), but it was not observed to alter the proliferation of OT-II cells primed by OVA/alum following either primary or secondary immunization. In contrast, TNFRSF4 was a weak stimulator of Treg proliferation but, as reported, a powerful costimulator of OT-II proliferation (26). The activity of TNFRSF4 and TNFRSF25 was not complementary in the case of effector OT-II cells, but addition of TNFRSF4 agonists further increased the proliferation of CD8+ OT-I cells and CD4+ Tregs stimulated by TNFRSF25 agonistic Abs. The unique activities of TNFRSF4 and TNFRSF25 agonists in T cell function support the conclusion that the downstream signaling pathways from these receptors are also distinct.

One of the most striking observations from these studies is that the Ag-specific cellular responses differ so dramatically from the Ag-specific Ab responses at both the primary and secondary immunization. As is widely known and included in immunology textbooks, the Ag-specific Ab response is dominated by IgM class Abs following primary immunization and class switches to predominantly IgG subtypes found at much higher titers in the serum following secondary immunization. Primary immunization with OVA/alum programs Ag-specific Ab responses that do not appear to be further augmented by costimulation of TNFRSF4 or TNFRSF25. In contrast, despite the lack of OT-II proliferation at either the primary or secondary immunization with 3T3-OVA-gp96-Ig, this strategy also led to high-titer Ab responses following secondary immunization that were preferentially class-switched to IgG1 and IgG2b, and to a lesser extent IgG2a, isotypes by addition of TNFRSF25 costimulation. In contrast to the increased Ab titer following secondary immunization, the proliferative OT-I and OT-II responses were diminished following secondary immunization unless combined with TNFRSF4 costimulation (increased OT-II proliferation) or TNFRSF25 costimulation (increased OT-I proliferation). These data indicate that the humoral and cellular Ag-specific immune responses are fundamentally uncoupled from one another at the time of secondary immunization. The side-by-side comparison of TNFRSF4 and TNFRSF25 agonists together with distinct vaccine approaches highlights the tremendous potential of combination vaccine strategies to selectively guide the activation and amplitude of specific T cell subsets and the dramatic influence these costimulators exert on memory T cell proliferation subsequent to secondary Ag challenge. The parallel observation that at a given point in time, the peripheral blood of an individual animal can simultaneously consist of 50% OT-I in the CD8+ subset as well as 50% Tregs and 20% OT-II in the CD4+ T cell subset challenges the idea that Tregs are immunosuppressive in an absolute sense, and it implies that proliferative and effector responses for each of these cell types can be differentially modulated by both Ag and costimulatory receptors at the time of priming and boosting.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure 1: Dose-dependent stimulation of OT-I and OT-II proliferation by ova/alum immunization. The total dose of ovalbumin administered in each ova/alum immunization was titrated as indicated on the x-axis, and the maximal percentage of OT-I (left panel) or OT-II (right panel) cells on the peak of the response for each cell type is indicated. These data indicate mean ± SEM for ≥ 3 mice.
Supplemental Figure 2: Memory OT-I and OT-II T cell frequencies following primary immunization. Mice immunized with the indicated vaccine/costimulatory antibody combinations as outlined in Figure 2 were monitored from day 30-52 for the frequency of peripheral blood A) OT-I and B) OT-II cells. Peripheral blood was drawn at 4 intervals over this time period and analyzed by flow cytometry as described in figures 2 and 4.
Supplemental Figure 3

Supplemental Figure 3: Serum IgM titer following primary and secondary immunization. Serum ova-specific IgM titers were measured by ELISA in samples collected on day 5 following primary immunization (experimental day 5) or secondary immunization (experimental day 57) with the indicated vaccine/costimulatory antibody combination.