CD40 Activation Boosts T Cell Immunity In Vivo by Enhancing T Cell Clonal Expansion and Delaying Peripheral T Cell Deletion

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CD40 Activation Boosts T Cell Immunity In Vivo by Enhancing T Cell Clonal Expansion and Delaying Peripheral T Cell Deletion

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In this report, we show that activation of APC with an agonist anti-CD40 mAb profoundly alters the behavior of CD4 T cells in vivo. Stimulation of mice with anti-CD40 2 days before, but not 1 day after, administration of superantigen (SAg) enhanced CD4 and CD8 T cell clonal expansion by approximately threefold. Further, CD40 activation also delayed peripheral T cell deletion after activation. Dying, activated T cells were quantitated by detecting extracellular phosphatidylserine with concomitant staining for SAg-reactive T cells using a TCR Vβ-specific mAb. Upon close examination, it was shown that CD40 activation delayed the death of the activated T cells. Additionally, it was found that enhanced survival of CD4 T cells was equally dependent on APC expression of B7-1 and B7-2. This is in contrast to CD8 T cells, which did not depend as much on B7-1 as B7-2. Thus, CD40 activation indirectly promotes T cell growth and delays the death of SAgs-stimulated CD4 T cells in vivo. These data suggest that one way CD40 activation promotes a more robust immune response is by indirectly increasing the production of effector T cells and by keeping them alive for longer periods of time. The Journal of Immunology, 1999, 162: 2024–2034.

Although in an optimal T cell response clonal expansion is important for fighting pathogens, just as important is the ability to down-regulate the T cell response after the pathogen is cleared. T cell populations must decline in number or else autoimmunity and immunopathology can occur (14, 15). Some cells must survive, however, if T cell memory is to develop. Thus, a complex balance must be maintained between sustained immunity and tolerance in the immune system.

Efficient monitoring of the T cell response, both expansion and deletion, has been performed with the use of staphylococcal enterotoxin A (SEA)3. SEA is a SAg that binds MHC class II and selectively engages all TCRs that contain the Vβ3 chain (16–20). Injection of B10.Br mice with SEA promotes the clonal expansion of CD4 and CD8 T cell populations that bear Vβ3; however, these cells very soon afterward decrease in number to below normal un.injected levels (21, 22).

In this study, we set out to use the SEA model to investigate a very important molecule whose role in the immune system is only beginning to be made clear: CD40 (23). CD40 is a member of the TNF receptor family (24). It is expressed on all APC such as B cells (24), macrophages (25), and dendritic cells (26) and has also been reported on T cells (27, 28). CD40 ligand (CD40L, CD154, gp39) is a member of the TNF family as well (29, 30). It is expressed mainly on CD4 T cells (31) but is also found on CD8 T cells (31–33) as well as other cells such as eosinophils and NK cells (34, 35).

Ligation of CD40 has been shown to yield many effects on APC. Several groups have shown that CD40 ligation can enhance the costimulatory abilities of APC (36–38). In fact, other studies have shown that lack of CD40 ligation can actually promote T cell tolerance as a result of poor B7 expression (39, 40). Many groups have investigated the role this tolerance mechanism plays in autoimmunity and transplant rejection. For example, autoimmune conditions such as experimental allergic encephalomyelitis...
(41, 42), collagen-induced arthritis and insulin-dependent diabetes mellitus (43, 44) are augmented by CD40 activation. Other studies have found that stimulation of CD40 plays a role in graft transplant rejection (45-47).

Effects of CD40 activation on T cell activation have also been reported. For example, IL-12-dependent Th1 differentiation has been shown to be modulated by CD40 activation (38, 48). Most recently, CD40 ligation has been shown to circumvent the requirement for T helper cells in the priming of CTL (49-51). CD8 T cell responses are thus also affected by CD40 ligation. Observed effects include improved antitumor-killing abilities (52, 53) and memory CTL responses (53, 54). Little has been reported on death susceptibility of T cells after CD40 ligation; however, recent reports have shown that CD40 activation of B cells, monocytes, and dendritic cells can block apoptosis within these populations (55, 56).

To examine the effects of CD40 stimulation on T cell clonal expansion and deletion upon response to an Ag, an anti-CD40 agonistic mAb was used in conjunction with SEA injection. We found that activation of CD40 not only enhanced CD4 and CD8 T cell clonal expansion but also delayed, but did not prevent, their subsequent deletion. Thus, CD40 enhances an immune response in vivo by increasing the number of effector T cells and delaying their subsequent death.

Materials and Methods

Mice

Female B10.BR/SgSn and B10.A/Cr mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD), respectively, and maintained in our animal facility under specific pathogen-free conditions. In all experiments, mice between the ages of 6 and 12 wk were used.

Reagents, experimental protocols, and Abs

SEA was purchased from Toxin Technology (Madison, WI) and admin-istered to mice i.p. injections of 0.15 or 0.30 μg. The anti-CD40-producing hybridoma FGK45.5 was a kind gift from Dr. Ton Rolink (Basil Institute, Switzerland) (57). The Ab was purified from hybridoma supernatants over protein G columns (Pharmacia, Piscataway, NJ). As a control, rat IgG (Sigma, St. Louis, MO) was injected in doses equal to the anti-CD40.

In experiments designed to block interactions between CD28 and B7-1/B7-2, CTLA4Ig (58) or an isotype-matched control chimeric Ab, L6, was injected i.p. at 0.50 mg/injection. These were both kind gifts from Dr. Peter Linsley (Bristol Myers-Squibb, Seattle, WA). Alternatively, Abs directed against B7-1 (16-10A1; Ref. 59) or B7-2 (GL1; Ref. 60) were used at doses of 1 mg/injection. These hybridomas were obtained from ATCC (Manassas, VA), and the resulting Abs were purified separately from hybridoma supernatants over protein G columns (Pharmacia).

Anti-TCR Vβ3 (KJ25-607.7; Ref. 59) and anti-IEα (14.4.4; Ref. 61) were purified separately from hybridoma supernatants over protein G columns (Pharmacia). These Abs were FITC conjugated by us. FITC-conjugated anti-Vβ14, PE-conjugated anti-CD4, PE-conjugated streptavidin, and biotinylated anti-TCR Vβ3 were all purchased from PharMingen (San Diego, CA). PE-conjugated anti-B7-1, PE-conjugated anti-B7-2, PE-conjugated anti-macrophage Ab (F4/80), and PE-conjugated anti-CD45R (B220) were all purchased from Caltag (Burlingame, CA). Red 613-conjugated anti-CD4 and Red 613-conjugated anti-CD8 were purchased from Life Technologies (Carlsbad, CA).

Cell processing and flow cytometry

Spleens were removed and teased through nylon mesh (Falcon, Becton Dickinson, Franklin Lakes, NJ) and subjected to ammonium chloride to lyse red blood cells. Peripheral LN (inguinal, axillary, and bronchial) were teased into single cell suspensions and washed with balanced salt solution (BSS). T cells from spleen or LN populations were purified on nylon wool columns as described previously (60). Briefly, 3-cc syringes were filled with 0.12 to 0.15 g of washed and brushed nylon wool. The columns were prepared with warm BSS 5% FBS, after which the columns were loaded in a 0.5-ml volume and incubated for 30 min at 37°C. After draining 0.5 ml away, the columns were incubated an additional 30 min, followed by elution with BSS 5% FBS.

For two- and three-color staining, cells were incubated on ice with the primary Abs in the presence of 5% normal mouse serum, culture supernatant from hybridoma cells producing an anti-mouse Fc receptor mAb (24-G2; Ref. 61) and 10 μg/ml human γ-globulin (Sigma) to block non-specific binding. After a 30-min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide) with primary Abs, the cells were washed twice and analyzed by flow cytometry, or, if a second staining step was necessary, the incubation and wash procedures were repeated. Flow cytometry was conducted on an EPICS XL flow cytometer (Coulter Electronics, Miami, FL). Greater than 5000 viable cells were analyzed with WinList software (Verity Software House, Topsham, ME).

Histochemistry

The mesenteric LN and spleen from each mouse were fixed in PBS 4% paraformaldehyde. They were transferred into Omnifuge tissue cassettes (Fisher Scientific, Pittsburgh, PA) and washed with distilled water for 2–3 h and then soaked in 70% ethanol overnight. The tissues were embedded in paraffin after a 1-h wash in 85% ethanol, three 1-h washes in 95% ethanol, three 1-h washes in 100% ethanol, and three 1-h washes in xylene. Paraffin-embedded tissues were sectioned and baked onto Superfrost/Plus microscope slides (Fisher Scientific) overnight. Tissues were stained using the Apoptosis Detection System, Fluorescein from Promega (Madison, WI), which uses the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (62). Briefly, tissues were deparaffinized with organic solvents and permeabilized with proteinase K. The tissues were then incubated with TdT enzyme and a nucleotide mixture containing FITC-labeled dUTP. This was done in a humidified chamber for 1 h at 37°C. Tissues were then washed, counterstained with propidium iodide, and then sealed under a cover slip. Confocal images were captured at ×20 magnification using a Leica TCS 4D confocal microscope (Heidelberg, Germany) and combined using Adobe Photoshop software (Mountainview, CA).

Results

Activation of CD40 inhibits Ag-induced T cell deletion in lymph nodes and spleen

Initial studies sought to examine the effects CD40 activation would have on T cell populations in the presence of stimulating Ag. To examine this issue, mice were treated with SEA. SEA is a SAg that stimulates T cells bearing TCR Vβ3 chains (16); therefore, SAg-stimulated T cells can be directly analyzed after SEA treatment. Mice were injected either with SEA alone or with SEA and anti-CD40. As a control for the SEA/anti-CD40 group, a third group was injected with SEA and rat IgG. A final group was left un.injected (normal) as a negative control. Fourteen days after SEA injection, the peripheral (inguinal, axillary, and bronchial) LN and the mesenteric (mucosal) LN, as well as the spleen, were removed, and the T cells were isolated by nylon wool fractionation separately from each tissue. The T cells were stained for CD4 Vβ3 and CD8 Vβ3 expression and analyzed by flow cytometry.

The results show that the presence of Ag alone causes significant deletion of both CD4 and CD8 T cells bearing Vβ3 in each tissue examined 14 days after SEA (Fig. 1). Percentages of each T cell population were two- to fourfold lower in SEA-injected mice than in un injected controls. In contrast, injection of anti-CD40 in SEA-treated mice potently inhibited Ag-induced T cell deletion in every tissue examined. CD4 Vβ3 and CD8 Vβ3 percentages in these mice were generally as high as, if not higher than, those...
in the uninjected control, and in all cases higher than those in the mice injected with SEA alone.

To test whether T cell rescue from deletion was due to CD40 stimulation, and not to a nonspecific effect from the injected Ab, rat IgG was injected with SEA (Fig. 1). The results showed the same amount of T cell deletion that was observed with SEA alone, strongly suggesting that CD40 activation blocks SAg-induced T cell deletion. Percentages can be misleading due to migratory effects and bystander T cell death, so it was important to examine the absolute number of T cells in the various lymphoid organs. Data shown in Table I confirm that deletion was inhibited, since CD4 Vβ3 numbers are elevated in the anti-CD40- and SEA-treated mice compared with SEA alone. An additional control experiment was performed where anti-CD40 alone was injected. The results showed no differences in T cell percentages when compared with an uninjected control (data not shown). Furthermore, staining of the T cells from each tissue for TCR Vβ14 chains, a subset of T cells that does not respond to SEA, showed no significant difference in their percentages when compared with the uninjected control (data not shown).

### Optimization of anti-CD40 injection

To standardize future experiments with anti-CD40, the optimal conditions for anti-CD40 injection were determined. Experiments were set up in which B10.Br mice were injected with 1 mg of anti-CD40 at different times in relation to SEA. Anti-CD40 injections were performed at 5 days before SEA injection (day -5) and up to 1 day after (day +1). Eight days after SEA injection, T cells from the LN (Fig. 2) and spleen (not shown) were counted, stained for CD4 Vβ3 and CD8 Vβ3, and analyzed by flow cytometry.

### FIGURE 1. CD40 activation blocks Ag-specific T cell deletion in peripheral and mucosal LN and spleen populations. Two groups of B10.Br mice were injected with either 0.5 mg of anti-CD40 or rat IgG 24 h before, and concurrent with, injection of 0.15 μg of SEA. Two other groups received no injection or SEA only. Fourteen days after SEA injection, T cells were isolated from the inguinal, bronchial, axillary, and mesenteric LN, as well as the spleen. These cells were stained for CD4 Vβ3 (left panel) and CD8 Vβ3 (right panel) and analyzed by flow cytometry. These data represent the mean percentages ± SEM from three or four mice over two separate experiments combined.

### FIGURE 2. The optimal day for anti-CD40 injection is 2 days before SEA administration. Female B10.Br mice were injected with 0.15 μg of SEA at time 0. At various times relative to SEA injection, 1 mg of anti-CD40 was injected. Control groups were also set up that received no injections or SEA only. LN T cells were isolated 8 days after SEA injection and were stained and counted for CD4 Vβ3 (A and B) and CD8 Vβ3 (C and D). Each bar represents data from four or five mice, collected over five separate experiments, except the day -5 bar, which was collected from one mouse. Except for day -5, these data represent mean percentages, as determined by flow cytometry, and numbers ± SEM.

### Table I. Absolute numbers of CD4 Vβ3 T cells in various lymphoid tissues

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inguinal LN</th>
<th>Bronchial LN</th>
<th>Axillary LN</th>
<th>Mesenteric LN</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>1.7 ± 0.5*</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>5.8 ± 1.6</td>
<td>8.3 ± 1.5</td>
</tr>
<tr>
<td>SEA alone</td>
<td>0.8 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>2.7 ± 0.6</td>
<td>3.1 ± 1.4</td>
</tr>
<tr>
<td>SEA/αCD40</td>
<td>3.0 ± 1.2</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 1.2</td>
<td>8.1 ± 0.6</td>
<td>11.6 ± 5.0</td>
</tr>
<tr>
<td>SEA/rat IgG</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.9 ± 0.5</td>
<td>2.8 ± 1.0</td>
</tr>
</tbody>
</table>

* Represents SEM. These data are taken from the experiment shown in Figure 1.
T cell percentages declined after SEA injection in comparison with uninjected control mice (Fig. 2, A and C). Most injections of anti-CD40 were effective at preventing the deletion, regardless of the day injected relative to SEA. Deletion was most efficiently prevented when anti-CD40 was injected before SEA. For example, when anti-CD40 was injected on day +1, the percentage of CD4 \( V\beta3 \) T cells was 3.8 ± 0.9%; when anti-CD40 was injected on days −2, −3, and −4, however, this rose to 11.6 ± 2.4%, 9.0 ± 2.5%, and 9.1 ± 3.1%, respectively (Fig. 2A). The percentages of CD8 \( V\beta3 \) T cells were above normal in every case, except when anti-CD40 was injected 5 days before SEA (Fig. 2C). The CD4 \( V\beta3 \) percentages were near or above normal, except when anti-CD40 was given on day −5 and day +1. All SEA/anti-CD40 injections consistently led to greater \( V\beta3 \) T cell percentages than those observed with SEA alone. Based on percentages, day −2 was the best day for anti-CD40 injection. On this day, CD4 \( V\beta3 \) percentages rose to 11.6 ± 2.4%, almost six times the level observed in SEA alone-treated mice (2.1 ± 0.5%) (Fig. 2A). CD8 \( V\beta3 \) percentages rose from a 1.5 ± 0.6% population found in SEA injected mice to 9.4 ± 1.6% (Fig. 2C).

The total numbers of CD4 \( V\beta3 \) (Fig. 2B) and CD8 \( V\beta3 \) (Fig. 2D) T cells were calculated. Days −2, −3, and −4 had the highest total numbers of \( V\beta3 \) T cells after SEA/anti-CD40 treatment. Day −2 was still the best, providing counts of 9.4 × 10^5 ± 4.0 and 4.9 × 10^5 ± 1.9 CD4 and CD8 T cells bearing \( V\beta3 \), respectively (Fig. 2, B and D). Based on these results, day −2 was chosen as the standard day of injection of anti-CD40, since it gave the most consistently high percentages and numbers of both CD4 \( V\beta3 \) and CD8 \( V\beta3 \) T cells.

Once the timing of anti-CD40 injection was determined, the dose of anti-CD40 was titrated. One mg of anti-CD40 was injected into each mouse for the timing experiments. We tested whether lower doses would still be effective at preventing Ag-specific T cell deletion. Experiments were set up in B10.Br mice, which were injected with anti-CD40 two days before receiving SEA. Anti-CD40 was injected at 1 mg, 0.5 mg, 0.25 mg, or 0.125 mg. An uninjected control mouse and a mouse receiving SEA only were also included. Seven days after SEA injection, the LN and spleen T cells were isolated, counted, and stained for CD4, CD8, and \( V\beta3 \). T cell analysis was done by flow cytometry.

LN-dosing experiments are shown in Fig. 3, and spleen data are similar, but not shown. The resulting percentages show the deletion of CD4 \( V\beta3 \) (Fig. 3A) and CD8 \( V\beta3 \) (Fig. 3C) T cells in the LN upon injection of SEA alone. All doses of anti-CD40 were effective at preventing deletion, as shown by the percentages (Fig. 3, A and C) and numbers (Fig. 3, B and D). The highest dose tested (1 mg) was the most effective at generating high T cell numbers, but even an eightfold lower dose of anti-CD40 provided some T cell rescue. Both percentages and numbers show that the CD8 \( V\beta3 \) T cells were rescued more effectively by lower doses of anti-CD40 than were CD4 \( V\beta3 \) T cells. A dose of 0.25 mg yielded equivalent numbers of CD8 \( V\beta3 \) T cells as the 1-mg dose (5 × 10^5 ± 0.6) (Fig. 3D). In contrast, CD4 \( V\beta3 \) T cell deletion was less inhibited by lower doses of anti-CD40 (Fig. 3B) but was still very effective. Based on these data, 0.25 mg of anti-CD40 was chosen as the standard dose in future experiments.

**Activation of CD40 enhances the expansion and delays the deletion of Ag-specific T cells**

Since anti-CD40 inhibited the deletion of Ag-specific T cells exposed to SEA (Figs. 1–3), we next investigated whether anti-CD40 affected the expansion and long-term deletion of \( V\beta3 \) T cells by conducting a detailed time course. In this experiment, mice were injected with 0.25 mg of anti-CD40 or, as a control, 0.25 mg of rat IgG. Two days later, SEA was injected into each group. On days 2, 5, 7, 12, and 21 after SEA injection, LN and spleens from both groups of mice were obtained. T cells were purified from these tissues, counted, stained for CD4 \( V\beta3 \) and CD8 \( V\beta3 \), and analyzed by flow cytometry.

The control mice injected with SEA and rat IgG (squares) show some CD4 \( V\beta3 \) T cell expansion (day two), and significant deletion by day five (Fig. 4). Examination of numbers shows a small degree of expansion in the LN (Fig. 4C) but a greater than twofold increase in the spleen (Fig. 4D). The degree of expansion is quite variable depending on dose and batch to batch variation of SEA (our unpublished observations). After clonal expansion, T cell populations depleted quickly. By day 5, both percentages (Fig. 4, A and B) and numbers (Fig. 4, C and D) fell below normal and stayed there until day 21. The slight rise in T cell numbers at the end of the time course is most likely due to repopulation of deleted T cells from the thymus.

The idea that CD40 activation enhances T cell clonal expansion and prevents their deletion was tested in mice injected with anti-CD40 and SEA (diamonds). The percentages of CD4 \( V\beta3 \) T cells show a small decrease in the LN (Fig. 4A) and a large increase in the spleen (Fig. 4B) to about three times starting levels on day 2. By day 5, both tissues were showing T cell expansion to about three times control levels. T cell percentages stayed above the rat IgG control levels throughout the time course but did decline to control levels as day 21 approached. Based on numbers, the LN show about a fourfold expansion of the CD4 \( V\beta3 \) T cells by day 5, only to decline to near control levels by day 12 (Fig. 4C). Spleen numbers were not much greater than controls on day 2, but T cell deletion was greatly inhibited until day 21 (Fig. 4D).

The CD8 \( V\beta3 \) time course results closely resembled the CD4 \( V\beta3 \) data (Fig. 5). The percentages and numbers of CD8 \( V\beta3 \) LN
Stimulation of CD40 delays T cell death

In each of the previous experiments, it was shown that injection of anti-CD40 inhibited the Vβ3 T cell deletion characteristic of SEA stimulation. These data raise the possibility that CD40 activation delays the death of the SEA-stimulated T cells.

To determine whether T cell death was inhibited by CD40 activation, mice were treated as follows: injected with SEA alone, anti-CD40 alone, SEA and anti-CD40, or left as un.injected controls. On days 3 and 9 after SEA injection, the mesenteric LN and spleen from each mouse were fixed in PBS containing 4% paraformaldehyde and stained for apoptotic cells as described in Materials and Methods. Confocal microscopy was used to generate images of each stained tissue.

Fig. 6, A-D, shows LN results 3 days after SEA injection, while Fig. 6, E-H, shows the results from day 9. On day 3, SEA alone led to significant apoptosis (Fig. 6B), some of which was still observed 9 days later (Fig. 6F). Injection of anti-CD40 alone yielded low levels of apoptosis on both days 3 and 9 (Fig. 6, C and G, respectively). Injection of SEA and anti-CD40 led to low levels of death on day 3 (Fig. 6D) but enhanced apoptosis by day 9 (Fig. 6H). These data show that injection of anti-CD40 and SEA does delay cell death, but they do not indicate whether the dead cells are Ag-stimulated T cells or not.

To test whether the dying cells were Ag-stimulated T cells, the peripheral LNs from the mice used to generate the data in Fig. 6 were crushed, and the T cells were isolated and stained for CD4, Vβ3, Vβ14, and extracellular phosphatidylserine (PS). PS is displayed extracellularly on dying cells and can be detected by staining with its natural ligand, annexin V, and analyzed by flow cytometry (Fig. 7) (63).

On day 3, the percentage of CD4 Vβ3 T cells with extracellular PS was 19.3 ± 6.0% in mice injected with SEA alone (Fig. 7A); thus, significant death was occurring at this early time point in comparison with uninjected animals (8.5 ± 0.5%). Mice injected with anti-CD40 alone, or with SEA and anti-CD40, remained about 2.5-fold lower (6.8 ± 2.7% and 7.1 ± 2.8%, respectively). On day 9, death had more than doubled in SEA-injected mice to 40.2 ± 3.9%; however, it should be kept in mind that these mice contained far fewer Vβ3 T cells on day 9 than on day 3 (see time course data in Figs. 4 and 5). Mice injected with anti-CD40 alone showed only a slight increase (Fig. 7A). The percentage of death in mice injected with SEA and anti-CD40 after 9 days (39.1 ± 3.3%) was nearly equal to that in mice injected with SEA alone, rising about sixfold from day 3. The day 9 mice treated with SEA and anti-CD40 also contained a greater number of Vβ3 T cells than the SEA alone group (Figs. 4 and 5). As a control, CD4 Vβ14 T cells were also examined for extracellular PS expression (Fig. 7B), and no major changes above normal percentages were observed in any of the groups.

These data suggest that SAg-stimulated T cells begin to die early after injection with SEA and will continue along that path for days afterward. Activation of CD40 delayed the death of those SAg-specific T cells.
B7-1 and B7-2 play different roles in CD4 and CD8 T cell stimulation in the presence of SEA and CD40 activation

Since CD40 activation delayed SAg-specific T cell death, we sought to test the mechanism by which CD40 was acting. One possibility was that CD40 activation altered APCs. To examine this hypothesis, three separate experiments were performed in which one mouse was injected with anti-CD40 and another mouse was given rat IgG. Two days later, the LNs and spleens were isolated, and the cells were stained to identify B cells and macrophages. Expression of MHC class II, B7-1, and B7-2 on these cells was examined by flow cytometry.

Mean channel fluorescence (MCF) of MHC class II expression increased approximately fourfold on both macrophages and B cells obtained from the LN and spleen of mice injected with anti-CD40 compared with rat IgG (data not shown). Both B cells and macrophages also up-regulated B7-1 and B7-2 in the spleen (Fig. 8) as well as the LN (data not shown) of mice treated with anti-CD40 compared with mice treated with rat IgG. Specifically, splenic MHC class II$^+$ B220$^+$ B cell MCF of B7-1 (16.7) and B7-2 (18.0) increased by more than 2.5-fold over control cells (6.6 and 3.9, respectively). MCF of MHC class II$^+$ F4/80$^+$ macrophage expression of B7-1 (17.6) and B7-2 (22.0) in anti-CD40-treated mice increased by more than fivefold over control levels (3.5 and 3.9, respectively). These data are similar to those previously reported (64). Thus, one possibility was that CD40 activation was assisting T cell expansion by increasing expression of B7-1 and/or B7-2 on APC.

Based on Fig. 8 and previous reports (64, 65), we next tested the hypothesis that CD40 mediated T cell activation in vivo through B7. We first sought to inhibit the CD28-B7 interaction using CTLA4IG. One group of mice received SEA only; one received SEA, anti-CD40 and CTLA4IG; and another group received SEA, anti-CD40, and the control chimeric Ab L6. Five days after SEA injection, T cells from the LN and spleen of each mouse were isolated, counted, and stained for CD4 V83 and CD8 V83. The cells were analyzed by flow cytometry.
MHC class II. CTLA4Ig was injected with SEA and anti-CD40, however, CD4 in the LN and spleen, similar to that shown in Figs. 4 and 5. When suppression over normal levels in both CD4 and CD8 T cell populations (Table II). Injection of SEA, anti-CD40, and L6 led to significant expansion, and stained for CD4, CD8, and V\textsubscript{b}3. These results represent one experiment of three conducted. In each, three B10.A mice were used. One mouse was left untreated, one mouse was injected i.p. with 0.25 mg of rat IgG (dotted line), and one mouse was injected with 0.25 mg of anti-CD40 (solid line). Two days later, LN and spleens were analyzed by three-color flow cytometry. MHC class II\textsuperscript{+}B220\textsuperscript{-} B cells were stained for B7-1 (A) and B7-2 (C) expression, as were MHC class II\textsuperscript{+}F4/80\textsuperscript{+} macrophages (B and D). Only spleen data are shown here. Uninjected mice were comparable to rat IgG injected mice (data not shown).

Mice injected with SEA alone showed the expected decrease in CD4 V\textsubscript{b}3 and CD8 V\textsubscript{b}3 expansion in the LN and spleen (Table II). Injection of SEA, anti-CD40, and L6 led to significant expansion over normal levels in both CD4 and CD8 T cell populations in the LN and spleen, similar to that shown in Figs. 4 and 5. When CTLA4Ig was injected with SEA and anti-CD40, however, CD4 V\textsubscript{b}3 percentages were lowered in both tissues, although never to the level of mice injected with SEA alone. CD4 T cell populations in the spleen and LN decreased twofold, (22.1 ± 0.6% to 11.1 ± 0.6%, and 16.0 ± 0.9% to 7.9 ± 1.2%, respectively). CD8 V\textsubscript{b}3 percentages differed from the CD4 percentages in that they were only slightly inhibited by CTLA4Ig injection. Spleen percentages decreased from 17.8 ± 0.9% to 15.1 ± 1.4%, while the LN percentages dropped from 13.7 ± 0.6% to 11.7 ± 1.1% (Table II).

Total numbers of CD4 and CD8 V\textsubscript{b}3 T cells were also calculated (Table III). The splenic CD4 V\textsubscript{b}3 T cell numbers in SEA/anti-CD40/L6-injected mice (30.6 × 10\textsuperscript{5} ± 2.5) dropped to uninjected levels (8.9 × 10\textsuperscript{5} ± 0.4) when CTLA4Ig was given. CD8 V\textsubscript{b}3 T cell numbers did show more of a decline than the percentages did, especially in the spleen, falling from 22.3 × 10\textsuperscript{5} ± 1.6 in SEA/anti-CD40/L6-treated mice to 14.9 × 10\textsuperscript{5} ± 1.7 in mice treated with CTLA4Ig. The numbers of T cells, however, was still almost fourfold greater than uninjected or SEA-injected (4.2 × 10\textsuperscript{5} ± 0.3) mice (Table III). These data led us to hypothesize that costimulation through B7 was important for expansion, as others have shown (66–68); however, a role for B7-1 vs B7-2 had yet to be discerned in this model. To this end we injected antagonistic Abs to B7-1 and B7-2. Six groups of mice were set up as follows: one group received no injection, one group received SEA alone, and one group received SEA and anti-CD40. The remaining three groups all received SEA and anti-CD40 but also received anti-B7-1, anti-B7-2, or both Abs, respectively. Five days after SEA injection, the LN and spleens were removed from each mouse. T cells were isolated, enumerated, and stained for CD4, CD8, and V\textsubscript{b}3. Analysis of the stained cells was done by flow cytometry.

Both percentages and numbers of CD4 V\textsubscript{b}3 T cells showed the expected deletion of SEA-activated T cells and the expected enhanced expansion of those V\textsubscript{b}3 T cells upon CD40 stimulation, both in the LN (Fig. 9 A and C) and spleen (Fig. 9, B and D). Injection of anti-B7-1 led to a modest drop in both percentages and numbers, while injection of anti-B7-2 led to a slightly greater decrease. When both anti-B7-1 and anti-B7-2 were injected, CD4 V\textsubscript{b}3 T cell populations dropped below uninjected control levels, close to SEA-injected levels. For example, in the LN-uninjected mice there were 2.7 × 10\textsuperscript{5} ± 0.2 CD4 V\textsubscript{b}3 T cells, while in mice treated with both Abs there were 2.0 × 10\textsuperscript{5} ± 0.3 T cells.

Perhaps the most interesting results were found with the CD8 V\textsubscript{b}3 subpopulation (Fig. 10). The decrease and increase of CD8 percentages and numbers upon SEA injection and SEA/anti-CD40 injection, respectively, were still noted in both the LN (Fig. 10, A and C) and spleen (Fig. 10, B and D). Injection of anti-B7-1 did little to inhibit anti-CD40-mediated T cell expansion. Anti-B7-2

![B cells](image1.png) ![Macrophages](image2.png)

**FIGURE 8.** CD40 activation enhances expression of B7-1 and B7-2 on B cells and macrophages. These results represent one experiment of three conducted. In each, three B10.A mice were used. One mouse was left untreated, one mouse was injected i.p. with 0.25 mg of rat IgG (dotted line), and one mouse was injected with 0.25 mg of anti-CD40 (solid line). Two days later, LN and spleens were analyzed by three-color flow cytometry. MHC class II\textsuperscript{+}B220\textsuperscript{-} B cells were stained for B7-1 (A) and B7-2 (C) expression, as were MHC class II\textsuperscript{+}F4/80\textsuperscript{+} macrophages (B and D). Only spleen data are shown here. Uninjected mice were comparable to rat IgG injected mice (data not shown).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of CD4 V\textsubscript{b}3 T Cells</th>
<th>Percentage of CD8 V\textsubscript{b}3 T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>LN</td>
</tr>
<tr>
<td>Uninjected</td>
<td>6.1 ± 0.3\textsuperscript{a}</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>SEA only</td>
<td>3.0 ± 0.6</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>SEA/α-CD40/L6</td>
<td>22.1 ± 0.6</td>
<td>16.0 ± 0.9</td>
</tr>
<tr>
<td>SEA/α-CD40/CTLA4Ig</td>
<td>11.1 ± 0.6</td>
<td>7.9 ± 1.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Twenty B10.A mice were set up as follows. Five mice were left as untreated controls. Three mice were given 0.15 μg of SEA. Six mice were injected with 0.5 mg of α-CD40 on day −2 and 0.5 mg of the control chimeric Ab L6.2 h before receiving an injection of SEA at time 0. The remaining six mice were injected with 0.5 mg of α-CD40 on day −2 and 0.5 mg of CTLA4Ig 2 h before SEA injection at time 0. Five days after SEA injection, T cells from the LN and spleen were isolated, stained for CD4 V\textsubscript{b}3 and CD8 V\textsubscript{b}3, and analyzed by flow cytometry. The data represent mean percentages ± SEM from two combined experiments.

\textsuperscript{a} Represents SEM.
consistently dropped the percentages and numbers, suggesting that it was the dominant molecule for CD8 Vβ3 T cell clonal expansion. These data show that B7-1 costimulation in the context of SAg and CD40 activation is less important for CD8 T cells than it is for CD4 T cells. Additionally, the increased drop in CD8 T cell expansion observed when blocking B7-1 and B7-2 may be due to the low levels of CD4 T cell help available.

Discussion

In this study, we set out to understand how SAg-induced T cell immunity is affected by ligation of CD40. Our results showed that activation of CD40, in the presence of SAg, enhanced CD4 and CD8 SEA-specific T cell clonal expansion. We further showed that CD40 activation delays SAg-induced peripheral T cell death.

Initial timing experiments provided many important clues as to the mechanism by which CD40 activation enhances a T cell response. The timing data show that stimulation of CD40 before SAg exposure creates the conditions for an optimal T cell response to foreign SAg (Fig. 2). Conversely, CD40 activation does not enhance SAg-induced T cell clonal expansion when it occurs after SAg exposure. One explanation for the latter result is that the T cells have already interacted with SAg/MHC and, thus, APC are unable to costimulate activated T cells outside the context of SAg in vivo. Therefore, it is likely that stimulating CD40 before Ag injection helps create a better costimulatory environment on APC, as evidenced by the enhanced expression of B7-1 and B7-2 on B

Table III. Total number of SEA-reactive T cells that CTLA4Ig inhibits in the presence of α-CD40 and SEA*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD4 Vβ3 T Cells ($\times 10^{5}$)</th>
<th>CD8 Vβ3 T Cells ($\times 10^{5}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>LN</td>
</tr>
<tr>
<td>Uninjected</td>
<td>8.9 ± 0.4^a</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>SEA only</td>
<td>5.1 ± 0.9</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>SEA/α-CD40/L6</td>
<td>30.6 ± 2.5</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>SEA/α-CD40/CTLA4-Ig</td>
<td>8.7 ± 0.8</td>
<td>2.5 ± 0.4</td>
</tr>
</tbody>
</table>

* The data are from the experiment in Table I and represent mean numbers ± SEM from two combined experiments involving a total of 20 mice.

Discussion

In this study, we set out to understand how SAg-induced T cell immunity is affected by ligation of CD40. Our results showed that activation of CD40, in the presence of SAg, enhanced CD4 and CD8 SEA-specific T cell clonal expansion. We further showed that CD40 activation delays SAg-induced peripheral T cell death.

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cells and macrophages (Fig. 8) as well as increased MHC class II expression (data not shown). These primed APC can enhance T cell stimulation. We have, however, recently found B cells to be unessential for the increased T cell expansion in this model, since a similar response occurs in B cell knockout mice (our unpublished observations).

Without stimulation of CD40, the SEA-activated T cells clonally expand to peak levels within 2 days and delete to below starting levels after about day 5 (Figs. 4 and 5). Activation through CD40 in the presence of SAg, however, enhances the clonal expansion observed on day 2 and delays the time it takes for the T cell populations to delete to control levels to about 21 days. Additionally, we show that low doses of anti-CD40 worked very well at enhancing clonal expansion and delaying SAg-specific T cell deletion (Fig. 3). Collectively, the results in this study show that acute activation of CD40 is sufficient to enhance T cell clonal expansion in peripheral and mucosal lymphoid tissue.

One of the most intriguing effects of CD40 activation was its ability to delay SAg-specific T cell deletion. We hypothesized that the observed deletion was due to death. Deletion during negative selection in the thymus has recently been shown to be due to apoptosis (69). Likewise, in the periphery, reports have shown that the observed deletion was due to death. Deletion during negative selection in peripheral and mucosal lymphoid tissue.

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was involved, and the response of CD8 T cells was not addressed in those studies. Moreover, it was unclear whether the amount of CD28 ligation was sufficient. This is an important point since it is known that APC up-regulate B7 after activation. Thus, activated APC may be able to deliver a qualitatively and quantitatively different signal to cognate T cells. For example, it is possible that ligation of CD28 in response to SAg alone was minimal in the absence of APC activation. In the present study we tested this idea more directly by activating APC with a potent agonist mAb specific for CD40. Under these circumstances it is clear that APC activation was profound, and, based on the data shown in Figs. 4, 9 and 10, CD28 was ligated significantly over that when SEA alone was injected. Perhaps surprisingly, this treatment was still unable to break tolerance even though clonal expansion was far greater when CD40 was activated. Therefore, CD28 ligation, be it minimal or presumably maximal, with TCR stimulation in vivo does not promote long-term T cell survival but only enhances expansion and delays subsequent death in this model.

These data raise the question of what can block T cell deletion. As shown previously, coinjection of bacterial LPS is capable of inhibiting Ag-induced deletion (87). This response was shown to occur independently of CD28 ligation but was profoundly dependent on TNF-α production (66, 87). In contrast, the CD40 mAb response shown here drives CD4 responses almost entirely through CD28 ligation. One possibility is that CD40 activation does not induce as much TNF-α as LPS. Also it is possible that a different pattern of cytokines is produced when comparing the two types of responses. Ultimately, it may be that the combination of TNF-α and CD40 activation may synergize to yield an optimal response. For example, CD40 may drive the clonal expansion phase, and TNF-α may prevent death by delivering a survival signal to the activated cells. These ideas are currently being tested by our laboratory. Nevertheless, these data suggest that vaccine development should rely not only on CD28 ligation or APC activation through CD40, but also on treatments that prime for long term T cell survival.

Acknowledgments

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