Requirement for a Complex Array of Costimulators in the Negative Selection of Autoreactive Thymocytes In Vivo

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Requirement for a Complex Array of Costimulators in the Negative Selection of Autoreactive Thymocytes In Vivo

Ruixia Li and Dawne M. Page

Autoreactive thymocytes can be deleted at an immature stage of their development by Ag-induced apoptosis or negative selection. In addition to Ag, negative selection also requires costimulatory signals from APC. We recently used a fetal thymus organ culture system to show that CD5, CD28, and TNF cooperatively regulate deletion of autoreactive thymocytes. Although these experiments provided strong evidence for the action of several costimulators in negative selection, we wished to demonstrate a role for these molecules in a physiologically natural model where thymocytes are deleted in vivo by endogenously expressed Ags. Accordingly, we examined thymocyte deletion in costimulator-null mice in three models of autoantigen-induced negative selection. We compared CD5−/− CD28−/− mice to CD40L−/− mice, which exhibited a profound block in negative selection in all three systems. Surprisingly, only one of the three models revealed a requirement for the CD5 and CD28 costimulators in autoantigen-induced deletion. These results suggest that an extraordinarily complex array of costimulators is involved in negative selection. We predict that different sets of costimulators will be required depending on the timing of negative selection, the Ag, the signal strength, the APC, and whether Ag presentation occurs on class I or class II MHC molecules. The Journal of Immunology, 2001, 166:6050–6057.

The TCR for Ag possesses an innate ability to recognize MHC molecules that are programmed into the TCR at the level of nucleotide sequence (1–4). However, mature T cells do not respond to self-MHC molecules, but instead recognize Ag as peptides bound to self-MHC molecules on APC. To achieve this pattern of responsiveness, developing T cells undergo an intricate selection process in the thymus. T cells with a low affinity/avidity for self-peptide-MHC complexes survive (positive selection), whereas T cells with a high affinity/avidity for self-peptide-MHC complexes are deleted (negative selection) (5–7). It has been shown that up to half of the T cells that are selected to mature in the thymus are then deleted because they are autoreactive (8–10). Moreover, several studies have linked autoantigen expression in the thymus to resistance to autoimmune disease (11–13). Thus, negative selection is a first line of defense against autoimmunity, and it is important to understand how this process is regulated.

In addition to a high avidity TCR stimulus, negative selection also requires costimulatory signals from APC (14, 15). The identification of specific costimulators, however, has been very controversial. Although CD28, Fas, and TNFR have been implicated in negative selection (16–18), thymocyte deletion is apparently intact in mice lacking these molecules (19–20). The receptor/ligand pair that has emerged as a master regulator of negative selection in several systems is CD40-CD40 ligand (L). Negative selection of CD4+ T cells by class II MHC molecules is profoundly defective in CD40- or CD40L-null mice (21–24). Since CD40 stimulation of APC increases the expression of many costimulatory molecules, we have hypothesized that CD40 regulates several costimuli that are required for negative selection (21, 25). These costimuli could include the CD28 ligands CD80 and CD86, adhesion molecules such as CD54 (ICAM-1) or CD58 (LFA-3), death receptor ligands such as Fas ligand (FasL), and/or cytokines such as TNF and IL-12 (26). Recently, we investigated whether several costimulators might be jointly controlling thymocyte deletion. Using a combination of blocking Abs in fetal thymus organ culture (FTOC), we found that CD5, the CD28 ligands CD80 and CD86, and TNF cooperatively regulated negative selection of CD4+ T cells by class II MHC in three different systems (27). Correspondingly, Kishimoto and Sprent (28) found that CD28, CD43 (a receptor for CD54), and Fas were involved in negative selection of medullary thymocytes induced by injection of bacterial superantigens or peptide Ag into TCR-transgenic mice. Taken together, these studies suggest that CD40 stimulation of APC induces costimuli that cooperatively regulate negative selection of CD4+ T cells, including at least CD80, CD86, CD54, TNF, and FasL. CD40 stimulation of APC may also induce CD5L; however, the expression and regulation of CD5L are presently unknown (29–31).

Although these experiments provided strong evidence for the action of several costimulators in negative selection, we wished to demonstrate a role for these molecules in an in vivo model where thymocyte deletion occurs in response to an autoantigen. Such a model is both a more stringent test of costimulator involvement in negative selection and also more applicable to studies of autoimmunity. Therefore, we examined thymocyte deletion caused by endogenous superantigens (SAg), which are produced from an open reading frame in the 3′ long terminal repeat of various mouse mammary tumor viruses (Mtv) (32, 33). SAg delete thymocytes bearing specific TCR-Vβ chains, and this deletion has been studied extensively as a model of autoantigen-induced negative selection (33, 34). We chose this model for our investigation because it is a physiologically natural model of negative selection, in that there is no manipulation of TCR or Ag expression in the thymus. Moreover, CD40- or CD40L-null mice are defective in SAg-mediated...

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deletion (21, 23) and thus we could directly compare negative selection in costimulator-null mice to that seen in CD40L-null mice. Surprisingly, we found that the costimulators CD5 and CD28 are required in only one of three models of SAg-dependent negative selection examined. Our results further indicate that the involvement of costimulatory molecules in negative selection in vivo is extraordinarily complex and will probably be different for each system that is examined.

Materials and Methods

Mice

CD5-null, CD28-null, BALB/c and D1.LP mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facilities under specific pathogen-free conditions. CD40L-null mice that had backcrossed to C57BL/6J were obtained from Randolph Noelle (Dartmouth College, Hanover, NH).

Abs and flow cytometric analysis

Mice were sacrificed by CO2 inhalation, and lymphocytes were released from thymus, spleen, and/or mesenteric lymph nodes as previously described (18). Surface expression of CD5, CD28, CD4, CD8, heat-stable Ag (HSA), CD44, CD62L, and TCRVb chains was determined by Ab staining and flow cytometry with collection of 30,000–100,000 live cells (18). Abs to CD5, CD28, Vbeta5.1/5.2 (MR9-4), Vbeta6 (RR4-7), Vbeta8.2/8.3 (MR5-2), Vbeta11 (RR3-15), HSA, CD44, and CD62L were purchased from BD PharMingen (San Diego, CA). Anti-CD4-PE and anti-CD8-Tri-Color were purchased from Caltag (Burlingame, CA).

PCR and Southern blotting

Mtv-6, -8, and -9 were detected by PCR of tail DNA (35). Mtv-7 was detected by Southern blot analysis of tail DNA, as described elsewhere (33). Mtv-6, -8, and -9 were detected by PCR of tail DNA (35). Mtv-7 was detected by Southern blot analysis of tail DNA, as described elsewhere (33).

Statistical analyses

Statistical analyses were performed by the Biostatistics Shared Resource Facility (Elizabeth Gilpin, Cancer Center, University of California, San Diego). Examination of the data for the variables of interest indicated that an ANOVA would be appropriate for establishing whether there were differences among groups of mice. Accordingly, if the overall F ratio was significant, costimulator-null mice were compared with wild-type mice using Dunnett’s test to control for multiple testing (36). A further comparison of CD5-/- CD28-/- mice to CD5-/- mice was also performed in some cases using a modified t test (Duncan’s procedure).

Results and Discussion

Experimental design

To investigate whether costimulators are required for negative selection in vivo, we examined thymocyte deletion in response to SAg in three model systems. We chose to analyze the roles of CD5 and CD28, since these molecules exhibited a strong cooperative effect on negative selection in our previous experiments in FTOC (27). We obtained CD5-/- and CD28-/- mice on the C57BL/6 background (H2b) and crossed them to BALB/c (H2d) or D1.LP mice (H2k) for all of the experiments reported here. Additionally, we analyzed very young mice (ages 2–3 wk for the BALB/c experiments and 2–5 wk for the D1.LP experiments) to avoid any cumulative effects of peripheral tolerance on the T cell repertoire. To obtain enough littermates for these experiments, the wild-type mice were heterozygous at both loci (CD5+/- CD28+/—), CD5-null mice were CD5—/- CD28+/- and CD28-null mice were CD5+/- CD28—/-.

Table I. CD4 and CD8 populations in costimulator-null mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Thymus</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% CD4</td>
<td>% CD8</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7.0 ± 1.2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>CD5-/-</td>
<td>9.0 ± 2.2*</td>
<td>2.5 ± 0.4*</td>
</tr>
<tr>
<td>CD28-/-</td>
<td>9.4 ± 1.8*</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>CD5-/- CD28-/-</td>
<td>13.2 ± 2.3*</td>
<td>2.6 ± 0.8*</td>
</tr>
</tbody>
</table>

* Statistical significance difference as compared to wild-type mice (p < 0.05, Dunnett’s test); $, statistically significant difference as compared to the CD5-/- mice (p < 0.05, Duncan’s test).

T cell repertoire in CD5- and CD28-null mice

No significant differences in total numbers of thymocytes or splenocytes were noted in these animals. However, the percentage of CD4 and CD8 T cells was increased in the thymus and spleen of CD5—/- CD28—/- mice (Table I). Both CD4+ and CD8+ thymocytes were increased in CD5-null mice, and CD4+ thymocytes were further increased in CD5—/- CD28—/- mice. Although Table I reports only the results from the H2b mice from the (CD5—/- CD28—/- × BALB/c) crosses, similar results were obtained in the H2kd mice from these crosses and also in the older H2b mice studied in the D1.LP model (data not shown).

These results are consistent with several previous observations. Teh et al. (37) noted an increase in CD4+ and CD8+ thymocytes in CD5—/- CD28—/- mice. Pena-Rossi et al. (38) showed that the block in CD4 lineage development in CD4—/- mice was partially reversed in CD5—/- CD4—/- mice. Because T cells from CD5—/- mice are hyperresponsive to TCR stimulation, CD5 has been reported to be a negative regulator of T cell activation; several groups have proposed that in the absence of CD5, positive selection is enhanced (38–40). Yet, another explanation is also possible. We found that CD4+ thymocytes were increased in FTOC treated with blocking Abs to CD5 and the CD28 ligands; this increase was observed only in thymuses from mice undergoing negative selection (27). Moreover, these CD4+ thymocytes were autoreactive (27). Thus, we proposed that the additional CD4+ thymocytes in these cultures had escaped from negative selection rather than experienced enhanced positive selection. In support of this notion, Kishimoto and Sprent (28) found that CD5, CD28, and CD43 were the only three costimulatory receptors of 10 tested that could participate in negative selection in vitro. Now we show that CD5—/- CD28—/- mice likewise have increased numbers of CD4+ and CD8+ thymocytes and T cells. It is likely that these cells have escaped from negative selection, although we have not been able to show that they are autoreactive as they respond poorly to APC stimulation due to their CD28-null phenotype (data not shown; Refs. 41 and 42).
Deletion of Vβ11⁺ thymocytes and T cells by Mtv-8,9

CD5- and CD28-null mice on the C57BL/6 background (H2b, Mtv-8, -9, -17, -30⁺) were crossed to BALB/c mice (H2d, Mtv-6, -8, -9⁺) to obtain mice that were H2b or H2d and that expressed two copies of Mtv-8,9 and one copy of Mtv-6. Mtv-6 deletes Vβ3- and Vβ5-bearing T cells, and Mtv-8,9 delete Vβ5-, Vβ11-, and Vβ12-bearing T cells (43). Since Mtv-30 is probably not expressed, and since Mtv-17 has very little effect on Vβ11 deletion in comparison to Mtv-8,9 (43), we did not screen for Mtv-17,30. An examination of SAg-mediated deletion previously showed that CD40L-null mice are profoundly deficient in deletion of Vβ5⁺ and Vβ11⁺ thymocytes, partially deficient in deletion of Vβ12⁺ thymocytes, but not at all deficient in deletion of Vβ3⁺ thymocytes (21). Correspondingly, Vβ3⁺ thymocytes were not rescued from SAg-induced deletion in CD5⁻⁻ CD28⁻⁻ mice (data not shown). Thus, we focused our examination on Mtv-6,8,9-mediated deletion of Vβ5⁺ and Vβ11⁺ T cells for these experiments (summary in Table II). Since SAg-mediated deletion is more efficient in the presence of H2-E, we first analyzed Mtv-6,8,9-induced-deletion in H2b⁻⁻ mice.

Fig. 1 shows the Vβ11 profile of thymocytes obtained from these mice. In wild-type mice, negative selection induced by Mtv-8,9/H2-E caused the percentage of Vβ11⁺ thymocytes to decrease from 6 to 4% among the CD4⁺ cells and from 5 to 2% among the CD8⁺ cells (Fig. 1, compare wild-type H2b to wild-type H2b⁻⁻). As expected, CD40L-null, H2b⁻⁻ mice exhibited a complete rescue from Mtv-8,9-induced deletion of both CD4⁺ and CD8⁺ Vβ11-bearing thymocytes (Fig. 1, compare CD40L⁻⁻ H2b⁻⁻ to wild-type H2b⁻⁻ and wild-type H2b). Interestingly, CD28⁻⁻ or CD5⁻⁻ CD28⁻⁻ H2b⁻⁻ mice exhibited a partial rescue of Vβ11⁺ CD4⁺ thymocytes from negative selection (Fig. 1, top, compare mice in H2b⁻⁻ group). This rescue appeared to be due solely to the lack of CD28, since increased rescue of Vβ11⁺ CD4⁺ cells was not consistently observed in mice lacking both CD5 and CD28. Since the total numbers of thymocytes were similar in wild-type or costimulator-null mice, the increased percentage of Vβ11⁺ CD4⁺ thymocytes represents an increase in the total number of these cells. For example, in four experiments, the average number of Vβ11⁺ CD4⁺ thymocytes obtained was 1.9 ± 0.4 × 10⁵, 2.0 ± 0.2 × 10⁵, 2.6 ± 0.7 × 10⁵, and 3.3 ± 0.2 × 10⁵ cells for the wild-type, CD5⁻⁻, CD8⁻⁻, and CD5⁻⁻ CD8⁻⁻ mice, respectively. These results suggest that CD28 is involved in Mtv-8,9-induced deletion of Vβ11⁺ CD4⁺ thymocytes.

Although Vβ11⁺ CD8⁺ thymocytes were rescued from negative selection in CD40L-null mice, they were not significantly rescued in the costimulator-null mice (Fig. 1, bottom, H2b⁻⁻ mice). Thus, CD5 and CD28 are not required for Mtv-8,9-induced deletion of Vβ11⁺ CD8⁺ thymocytes. Instead, CD40 stimulation of APC apparently induces costimuli that act through other receptors to delete these cells.

Fig. 2 shows the Vβ11 profiles of the splenocytes obtained from these mice. In previous reports, the cells that were rescued from negative selection in CD40L-null mice did not accumulate in the periphery; rather, the autoreactive cells were apparently deleted by uncharacterized mechanisms of peripheral tolerance (21, 23).

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Rescue of CD4⁺ Cells</th>
<th>Rescue of CD8⁺ Cells</th>
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<tbody>
<tr>
<td>Thymi</td>
<td>Mtv-8,9 SAg</td>
<td>Mtv-8,9 SAg</td>
</tr>
<tr>
<td>CD5⁻⁻</td>
<td>Vβ11⁺ TCR</td>
<td>Vβ5⁺ TCR</td>
</tr>
<tr>
<td>CD28⁻⁻</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CD5⁻⁻ CD28⁻⁻</td>
<td>+ +</td>
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</tr>
<tr>
<td>CD40L⁻⁻</td>
<td>+ + +</td>
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</tr>
<tr>
<td>Periphery</td>
<td>CD5⁻⁻</td>
<td>No</td>
</tr>
<tr>
<td>CD28⁻⁻</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CD5⁻⁻ CD28⁻⁻</td>
<td>+ +</td>
<td>No</td>
</tr>
<tr>
<td>CD40L⁻⁻</td>
<td>+ + +</td>
<td>+</td>
</tr>
</tbody>
</table>

*Rescue from negative selection is reported on a subjective scale, with +++ denoting complete rescue. For the Mtv-6,8,9 model, results are summarized only for coreceptor-null mice expressing H2-E. Rescue from negative selection was determined by comparing cell populations obtained in H2-E⁻⁻, coreceptor-null mice to those obtained in H2-E-negative, wild-type mice. For the Mtv-7 model, rescue from negative selection was determined by comparing cell populations obtained in Mtv-7⁻⁻, coreceptor-null mice to those obtained in Mtv-7-negative, wild-type mice.

FIGURE 1. Deletion of Vβ11⁺ thymocytes by Mtv-8,9 in costimulator-null mice. The CD4⁺/CD8⁺/Vβ11 profile was examined in the thymus of mice expressing the indicated costimulators and H2 haplotypes. Reported are the percentages of Vβ11⁺ cells in the CD4⁺ (top) or CD8⁺ (bottom) thymocytes, where each dot represents one mouse (77 mice total) and the line represents the mean value. *, Significant increase in the percentage of Vβ11⁺ cells as compared with the wild-type, H2b⁻⁻ animals (p < 0.05, Dunnett’s test).
However, CD4<sup>+</sup>Vβ11<sup>+</sup> T cells were present in the periphery of the CD40L<sup>-/-</sup> mice in these experiments (Fig. 2, top). One possibility is that CD4<sup>+</sup> cells were still present because the mice in this analysis were quite young. However, we note that even these young CD40L<sup>-/-</sup> mice still deleted the peripheral CD8<sup>-</sup>Vβ11<sup>+</sup> cells (Fig. 2, bottom). The mechanisms of peripheral tolerance underlying these differing effects have not yet been characterized.

CD5<sup>-/-</sup> CD28<sup>-/-</sup>, H<sub>2</sub>bd mice likewise showed an accumulation of CD4<sup>+</sup>Vβ11<sup>+</sup>, but not CD8<sup>-</sup>Vβ11<sup>+</sup>, T cells in their spleens (Fig. 2). Although there appeared to be an increased percentage of Vβ11<sup>+</sup> CD4<sup>+</sup> splenocytes in CD28<sup>-/-</sup> mice, the difference was not statistically significant. Instead, we noted a better accumulation in CD5<sup>-/-</sup> CD28<sup>-/-</sup> mice as compared with CD28<sup>-/-</sup> mice. It is possible that CD5 does act in conjunction with CD28 in thymic deletion, but that the effect is small and is only seen as the cells accumulate in the periphery. Alternatively, CD5 could be working through mechanisms of peripheral tolerance to induce accumulation of these cells. Taken together, the results in Figs. 1 and 2 indicate that both CD5 and CD28 can contribute to Mtv-8,9-induced deletion of CD4<sup>+</sup>Vβ11<sup>+</sup> cells. However, rescue of Vβ11<sup>+</sup> cells was always more profound in CD40L-null mice; thus, other costimulators must also be contributing to negative selection in this system (summary in Fig. 7).

Because CD4<sup>+</sup> and CD8<sup>+</sup> cells were increased in the CD5<sup>-/-</sup> CD28<sup>-/-</sup> mice in general (Table I), we considered the possibility that the rescue of Vβ11<sup>+</sup> cells in CD5<sup>-/-</sup> CD28<sup>-/-</sup> mice was not Mtv dependent. Therefore, we examined Vβ8.1/8.2 profiles, since these T cells are not deleted by Mtv-6, -8, or -9 (43), and this population is therefore commonly used as a control for Mtv-6,8,9-specific deletion (44–46). The percentage of Vβ8.1/8.2<sup>+</sup> cells did not increase in the costimulator-null mice (Fig. 3). In fact, the percentage of Vβ8.1/8.2<sup>+</sup> cells among the CD4<sup>+</sup> population decreased significantly in the CD28<sup>-/-</sup> and CD5<sup>-/-</sup> CD28<sup>-/-</sup>, but not the CD5<sup>-/-</sup>CD28<sup>-/-</sup> mice (Fig. 3, top). This result would be expected if Vβ11<sup>+</sup> T cells are being rescued from Mtv-8,9-induced deletion, because it is well documented that the Vβ8.1/8.2 population behaves in a compensatory fashion in the presence of these SAg (43–46). Similar results were observed in the Vβ8.1/8.2 profile of the splenocytes as well (data not shown). Thus, the rescue of the Vβ11 population in CD28<sup>-/-</sup> and CD5<sup>-/-</sup> CD28<sup>-/-</sup> H<sub>2</sub>bd mice appears to be Mtv-8,9 dependent.

**Phenotype of CD4<sup>+</sup> cells rescued from deletion**

It was possible that the Vβ11<sup>+</sup> CD4<sup>+</sup> cells rescued from deletion in the costimulator-null mice were not truly mature or expressed an activated phenotype. We therefore examined these cells for their HSA, CD44, and CD62L profile (Fig. 4). HSA is down-regulated as thymocytes progress from the CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) stage to the mature CD4<sup>+</sup> or CD8<sup>+</sup> stage (e.g., compare Fig. 4, G and H). In H<sub>2</sub>bd mice, 6.0% of the CD4<sup>+</sup> thymocytes were Vβ11<sup>+</sup>, and 32% of these cells were HSA<sub>low</sub> (Fig. 4, A and D). In contrast, CD5<sup>-/-</sup>, H<sub>2</sub>bd mice exhibited deletion of their CD4<sup>+</sup>Vβ11<sup>+</sup> thymocytes and only 10% of those remaining were HSA<sub>low</sub> (Fig. 4, B and E). As expected, Vβ11<sup>+</sup> CD4<sup>+</sup> thymocytes were rescued in H<sub>2</sub>bd, CD5<sup>-/-</sup> CD28<sup>-/-</sup> mice and 28% of these were HSA<sub>low</sub> (Fig. 4, C and F). Thus, the HSA profile of the thymocytes rescued from deletion matched the HSA profile of the Vβ11<sup>+</sup> CD4<sup>+</sup> cells that matured in the H<sub>2</sub>b<sup>+</sup> mice. Correspondingly, the Vβ11<sup>+</sup> CD4<sup>+</sup> splenocytes in the CD5<sup>-/-</sup> CD28<sup>-/-</sup> mice appeared to be mature naive T cells, in that they displayed a normal CD44 and CD62L profile (Fig. 4, K and N, and data not shown). In conclusion, the rescued Vβ11<sup>+</sup> CD4<sup>+</sup> cells in the costimulator-null mice are both mature and naive based on these parameters.

**Deletion of Vβ5<sup>+</sup> thymocytes and T cells by Mtv-6,8,9**

We next examined Vβ5 profiles in these same mice, expecting to see similar results as those obtained for the Vβ11 population. Surprisingly, quite different results were obtained. First, CD4<sup>+</sup>Vβ5<sup>+</sup>, but not CD8<sup>-</sup>Vβ5<sup>+</sup>, thymocytes were completely rescued from deletion in CD40L-null mice (Fig. 5, H<sub>2</sub>bd mice). Thus, CD40L is
not significantly involved in negative selection of the Vβ5+CD8+ population. Moreover, unlike the results above, CD28 and CD5 contributed either marginally or not at all to deletion of CD4+Vβ5+ thymocytes (Fig. 5, H2b/d mice). Similar results were obtained in splenocytes (data not shown, but see Table II). Thus, it appears that CD5 and CD28 are not required for deletion of CD4+Vβ5+ thymocytes by Mtv-6,8,9. Rather, CD40 stimulation of APC apparently induces costimuli that act through other receptors to delete these cells (Fig. 7).

The reasons for the discrepancy between costimulator contribution to negative selection in these two systems is not known. Mtv-6 could be responsible for the difference, in that Mtv-6-induced deletion of Vβ5+ thymocytes may not require CD5 and CD28. However, this is unlikely because in the cases where we screened for Mtv-6 expression, we did not observe a significant effect of Mtv-6 on Vβ5 deletion over and above that already due to Mtv-8,9 (data not shown). Correspondingly, single Mtv mice reveal that Mtv-9 alone can delete 80–98% of the Vβ5+CD4+ T cells (43). A more likely possibility is that deletion of Vβ5+ thymocytes occurs at different stages of thymocyte development or is regulated by different APC. Vβ11 deletion occurs somewhere during the transition between immature CD4+CD8+ precursors and mature medullary thymocytes (44), whereas Vβ5 deletion apparently occurs in the thymic medulla (47). Interestingly, bone marrow-derived APC are required for Vβ11 deletion (45), whereas Vβ5 deletion can be mediated by bone marrow-derived APC or medullary epithelium (48–50). Thus, it is possible that Vβ5 deletion by medullary epithelium is not CD5/CD28-dependent and that a requirement for participation of these costimulators is thereby overcome and the thymocytes are deleted. However, this explanation does not agree with data indicating that CD28 is involved in other models of medullary thymocyte-negative selection (28).

Deletion of Vβ6+ thymocytes and T cells by Mtv-7

Due to the differences in costimulator involvement in negative selection in the systems described above, we investigated the role of CD5/CD28 in another system of SAg-mediated deletion. Deletion of Vβ6+ T cells occurs in response to Mtv-7 in both H2-E+ and H2-E− mice (51). However, deletion in H2-E− mice (e.g., the D1.LP strain) is weaker and occurs over a period of several weeks. This type of negative selection is completely rescued in CD40-null mice, whereas the stronger H2-E+/Mtv-7-dependent deletion is only partially rescued in CD40-null mice (23). Our hypothesis was that roles for costimulators would be more apparent in a system of weak negative selection. To our surprise, this was not the case.

For these experiments, we compared deletion of Vβ6+ cells in the following D1.LP mice (H2-A+, H2-E−) from 2 to 5 wk of age: 1) CD5−/− CD8−/−, Mtv-7 (negative control for deletion); 2) CD40L−/−, Mtv-7− (positive control for deletion); 3) CD40L−/−, Mtv-7− (control for rescue from deletion); and 4) CD5−/− CD28−/−, Mtv-7−. As expected, nearly complete rescue of CD4+Vβ6+ and CD8+Vβ6+ cells from negative selection was observed in the thymi of CD40L−/− mice expressing Mtv-7 (Fig. 6). For example, the percentage of Vβ6+ cells among CD4+ thymocytes in CD40L−/− littermates expressing Mtv-7 (large dashed line with filled triangles) declined from 7.6% at age 2 wk to 5.5% at age 5 wk. In contrast, CD40L-null mice expressing Mtv-7
retained high levels of these cells (8–10%, thick line with open triangles). In fact, there was virtually no Mtv-7-induced negative selection in the CD4+ or CD8+ thymocytes from CD40L-null mice (compare thick line to Mtv-7+ control, small dashed line with filled squares). Some of the cells rescued from negative selection in CD40L-null mice also accumulated in the periphery (data not shown, but see summary in Table II).

In contrast to the rescue of V\textbeta6+ cells seen in CD40L-/- mice, no rescue whatsoever was observed in CD5-/-CD28-/- mice, even in the youngest mice examined where deletion was not yet complete. CD5-/-CD28-/- animals lacking Mtv-7 (small dashed lines with filled squares in Fig. 6, top) possessed 8–10% V\textbeta6+ cells among their CD4+ thymocytes or T cells (data not shown). In CD5-/-CD28-/- animals expressing Mtv-7, deletion of V\textbeta6+ thymocytes proceeded normally (Fig. 6, compare plain line with open squares to CD40L+/-, Mtv-7+ control). Similar results were observed in CD5-/- or CD28-/- animals as well (data not shown, but see Table II). Thus, CD5 and CD28 are not required for Mtv-7-induced thymocyte deletion; again, CD40 stimulation of APC apparently induces costimuli that act through different receptors to delete these cells (Fig. 7).

Why are CD5/CD28 involved in deletion of V\textbeta11+ cells by Mtv-8,9, but not in deletion of V\textbeta6+ cells by Mtv-7? An obvious possibility is the strength of the signal induced by the different SAg. However, Mtv-7 deletion in an H2-E-negative environment is thought to be a weaker signal than Mtv-8,9 deletion on H2-E. Thus, one would expect the Mtv-7 model to be more costimulatory dependent. For example, Dautigny et al. (22) found that CD54 (ICAM-1) and Fas were involved in the “weak” Mtv-9-induced deletion of V\textbeta8+ thymocytes in H2-E+ mice. Thus, signal strength is not a likely explanation for the different costimulatory requirements in these systems.

Another possibility is that deletion of V\textbeta6+ and V\textbeta11+ thymocytes depend on different APC. Both models of negative selection apparently require bone marrow-derived cells (45). In an FTOC model, both dendritic cells and B cells were required to induce deletion of V\textbeta6+ cells; it was thought that B cells express large quantities of SAg, which they then pass to dendritic cells (46). Similarly, Frey et al. (52) showed that B cells were likely required for deletion of V\textbeta11+ cells. However, V\textbeta6 and V\textbeta11 deletion both proceed normally in B cell-deficient mice (53). Moreover, recent studies of thymic dendritic cells show that they do express Mtv-7,8 by PCR and functional studies (54). Finally, Webb and Sprent (55) showed that CD8 T cells were responsible for inducing neonatal tolerance to Mtv-7. Thus, the APC that induce deletion in these systems are still unknown, and we currently do not understand why costimulatory requirements are different in these two systems.

**Summary**

CD40L was clearly involved in thymic deletion in all three models examined, except for deletion of V\textbeta5+CD8+ cells (Fig. 7). Thus, CD40/CD40L has again emerged as a master regulator of class II MHC-mediated negative selection. However, the precise costimulatory molecules regulated by CD40 that are required for negative selection in vivo remain elusive. Only in the case of negative selection of V\textbeta11+CD4+ cells were CD5 and CD28 shown to be significantly involved. Here, CD40 is probably regulating the CD28 ligands CD80 and CD86 (26), but the expression and regulation of CD5L are unknown (29–31). In the other models of negative selection examined here, CD40 may induce other costimuli required for thymocyte deletion, perhaps CD54 (ICAM-1), FasL, or TNF (22, 27, 28). These molecules could regulate negative selection separately or in combination with CD5 and CD28. Based on these results, we predict that a complex array of costimulatory receptors are involved in negative selection in vivo and that the cohort of receptors involved will vary depending on the timing of negative selection, the Ag, the signal strength, the APC, and whether Ag presentation occurs on class I or class II MHC molecules.