Immune responses are shaped by several processes that promote responses to pathogens and hinder responses to self. One mechanism that contributes to this polarization in response is negative selection, in which thymocytes that can respond to self-peptide/MHC complexes are deleted from the T cell repertoire. I found here that several coreceptors known to contribute to mature T cell activation also participate in negative selection. Interestingly, these molecules appeared to act in a cooperative fashion. Blocking the contribution of these molecules in fetal thymus organ culture not only prevented negative selection in the CD4^+ lineage, but also induced the appearance of autoreactive thymocytes. This is the first demonstration that blocking coreceptor interactions during thymic development can produce autoreactive T cells. The contribution of negative selection to the mature T cell repertoire and to autoimmunity is discussed in light of these results.

**Cutting Edge: Thymic Selection and Autoreactivity Are Regulated by Multiple Coreceptors Involved in T Cell Activation**

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To produce a repertoire of T cells capable of responding to foreign peptides bound to self-MHC but not able to respond to self-peptide/MHC complexes, T cell maturation is tightly controlled by selective events in the thymus. Immature T cells with a TCR of low avidity for self-peptide/MHC complexes survive, while cells with a TCR of high avidity for self-peptide/MHC complexes are deleted (1). In addition to a high avidity TCR stimulus, negative selection also requires costimulatory signals from APC (2), but the identification of these costimulatory molecules has been extraordinarily elusive and controversial. Class II MHC-dependent negative selection is defective in CD40 ligand (CD40L)^-null mice, yet the mechanisms underlying this deficiency have not been explained (3). Stimulation of CD40 increases the costimulatory function of APC by up-regulating levels of the CD28 ligands B7-1 (CD80) and B7-2 (CD86), the adhesion molecules CD54 and CD58, and cytokines such as IL-12 and TNF (4). However, conflicting evidence has been obtained regarding the role of these costimulatory molecules in negative selection. For example, TCR stimulation in conjunction with TNF or CD28 can cause thymocyte death, but TNFR-I/II-null mice and CD28-null mice undergo normal negative selection to both class I and class II MHC-dependent Ags (5–8).

I reasoned that several costimulatory molecules might be cooperatively regulating negative selection, which would account for the conflicting data surrounding examination of any one of them. Here, the role of coreceptors in negative selection was examined by using a fetal thymocyte organ culture (FTOC) system. FTOC preserves the thymic environment via culture of a whole thymus with intact cortex, medulla, and endogenous APC, but one can still manipulate thymic selection by the addition of soluble mediators. In addition, I used models of negative selection, either with or without TCR transgenes, that do not involve an inflammatory response. This issue is absolutely crucial to the study of negative selection, as thymic deletion induced by the addition of Ag or anti-TCR Abs in the presence of mature T cells is complicated by the production of proinflammatory cytokines (9). I found that several costimulatory molecules that regulate mature T cell activation cooperatively participate in negative selection of the CD4^+ T cell lineage. Blocking the contributions of these molecules prevented negative selection and induced the appearance of autoreactive thymocytes.

**Materials and Methods**

**Mice**

AND TCR-transgenic (Tg) mice have been previously described (10). CD8^- and CD28-null mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Cell culture**

Thymuses were removed from fetuses at day 16–17 of gestation and cultured for 4–5 days as described (11). Following culture, thymocytes were released by straining through nylon mesh. Surface expression of CD4, CD8, TCR-Val11, and heat stable Ag (HSA) was determined by Ab staining and flow cytometry, as described (2, 11).

**Abs and reagents**

Anti-CD5 (53.7.3) was a generous gift from Dr. Gary Starling (Bristol-Myers Squibb, New York, NY). Abs to CD80 (16-10A1), CD86 (GL1), and H-2K^d^ were purchased from PharMingen (San Diego, CA); rabbit...
anti-mouse TNF was purchased from Genzyme (Cambridge, MA). Anti-H-2K\(^{b}\) and hamster Ig (Hlg; Jackson Immunoresearch, West Grove, PA) were used at 60 \(\mu\)g/ml. The other Abs were titrated and used at concentrations that caused optimal maturation of CD4\(^{+}\) thymocytes in FTOC: anti-TNF sera at 1.25–2.5\% and all others at 10–20 \(\mu\)g/ml.

**Proliferation assays**

Production of mature thymocytes capable of responding to Ag was monitored by assessing thymocyte proliferation. A total of 1.25 \(\times\) 10\(^{5}\) thymocytes from FTOC were cultured for 3–4 days with 3 \(\times\) 10\(^{5}\) mitomycin C-treated splenocytes from B10.A mice with or without the addition of Ag (the 88-103 COOH-terminal peptide of moth cytochrome \(c\) (MCC)). For autopostrafficitive responses, 2.5 \(\times\) 10\(^{5}\) thymocytes were cultured for 5 days with 10 U/ml IL-2 and mitomycin C-treated APC that were enriched for dendritic cells by collagenase digestion of splenocytes and subsequent centrifugation over dense BSA (12), as described (13). A dose-response of 10\(^{-10}\)–10\(^{5}\) APCs was used in these cultures, but for purposes of brevity, only the response to the highest APC dose is reported in Fig. 4. The cells were pulsed with 1 \(\mu\)Ci of [\(^{3}H\)]methyl-thymidine (New England Nuclear, Boston, MA) for the final 18 h of culture, and isotope incorporation was determined.

**Results**

**Abs to several costimulatory molecules rescue CD4 maturation in AND.b/9R mice**

To examine the effect of coreceptors on thymocyte negative selection, I first used the well-characterized AND TCR-Tg system. These mice express a V\(_{\beta}3/V\alpha11\)-TCR that recognizes cytochrome \(c\) peptides bound to H-2E class II MHC molecules (10). Thymocytes bearing this TCR are positively selected on H-2A\(^{b}\) (AND.b mice) such that a large population of CD4\(^{+}\) cells develops in the thymus. Conversely, if AND.b mice are crossed with B10.S(9R) mice (AND.b/9R), then the presence of H-2A\(^{b}\) causes a dominant loss of CD4\(^{+}\) thymocytes (14, 15). I used FTOC of AND.b/9R thymuses to investigate the molecules that contribute to negative selection. This system is advantageous because if one can block negative selection to H-2A\(^{b}\), then positive selection on H-2A\(^{b}\) would result in the appearance of mature CD4\(^{+}\) thymocytes. For example, anti-CD40L treatment of AND.b/9R neonates (3) or FTOC (data not shown) rescued the development of CD4\(^{+}\) thymocytes.

Previous reports indicated that CD5-null mice have altered thymic maturation (16). Moreover, signals from CD28 (6) or TNFR (5) can cause thymocyte death in vitro in conjunction with a TCR stimulus. Thus, I used Abs to CD5, TNF, and the CD28 ligands B7-1 and B7-2 to block the interactions of these molecules with their ligands. Although the identity of CD5L is controversial (17–19), anti-CD5 blocks the interaction of CD5L with CD5 (17). Anti-B7-1 and anti-B7-2 likewise block T cell activation (20, 21), and the anti-TNF sera blocks TNF cytolytic activity (22). Fetal thymuses from AND.b/9R mice at day 17 of gestation have very few CD4\(^{+}\) cells (data not shown). After 4 days in FTOC, some CD4\(^{+}\) cells developed (Fig. 1, top), similar to the low level of development seen in AND.b/9R adults (14). A population of CD8\(^{+}\) cells was also present, though this population was never affected by the Ab treatments. However, when these thymuses were cultured with Abs to CD5, B7-1 plus B7-2 (5 + B), and TNF (5 + B + T), then a striking rescue of CD4 maturation occurred (Fig. 1, top). Both the percentage and number of CD4\(^{+}\) cells increased in these cultures, and the CD4\(^{+}\) cells appeared to be mature as they expressed low levels of the HSA (CD24) marker (Fig. 1, bottom) and high levels of the AND TCR (data not shown). Ag reactivity should also correlate with CD4 maturation, and the thymocytes from the cultures exhibiting rescue of CD4 maturation showed increased proliferation to Ag (Fig. 1, middle). The blocking Abs did not cause an increase in the CD4\(^{+}\)CD8\(^{+}\) (DP) population in these cultures. This result was expected because negative selection in neonatal AND.b/9R mice does not affect the DP population, but rather occurs at the transition between the DP and CD4\(^{+}\) stages of development (14, 15).

Because the blocking Abs coated the surface of both thymocytes and APC, it was possible that this could nonspecifically alter thymocyte/APC interactions, and so alter thymic selection. As a control for this possibility, the thymuses were cultured with an Ab to class I MHC (anti-H-2K\(^{b}\)), which also binds to both thymocytes and APC. This Ab could not rescue CD4 maturation in FTOC (Fig. 1, bottom).

Another possibility was that the blocking Abs were acting not by rescuing thymocytes from negative selection, but rather by stimulating proliferation of the small population of CD4\(^{+}\) cells that develop in the AND.b/9R thymuses. However, as expected from previous reports of the effects of anti-B7-1 and anti-B7-2 on T cell activation (20, 21), these Abs blocked AND thymocyte proliferation to Ag (data not shown). Moreover, the rescued CD4\(^{+}\) thymocytes were not proliferating, as measured by incorporation of bromodeoxyuridine (data not shown).

Finally, I tested the effect of these Abs on thymuses from mice undergoing only positive selection (AND.b). Although AND.b mice eventually develop a large population of CD4\(^{+}\) cells, the
number of CD4$^+$ cells in AND.b or AND.b/9R thymuses is equivalent at birth (AND.b, 0.76 ± 0.39 × 10$^6$ CD4$^+$; AND.b/9R, 0.76 ± 0.15 × 10$^6$ CD4$^+$; n = 4–6 mice). The CD4$^+$ population then declines in AND.b/9R mice but increases in AND.b mice (14, 15). Because the two types of mice have the same number of CD4$^+$ cells early in their development, AND.b FTOC is ablated in the presence of Ag (Fig. 2, top). These results strongly argue that the Abs to CD5, B7-1, B7-2, and TNF cause an increase in the CD4$^+$ population in AND.b/9R FTOC by specifically rescuing thymocytes from negative selection on H-2A$^+$ and subsequently allowing them to be positively selected.

To determine the individual contributions of CD5, the B7 molecules, and TNF to negative selection, fetal thymuses from AND.b/9R mice were cultured with various combinations of the Abs to these molecules, and the amount of CD4 maturational rescue was determined (Fig. 1, bottom). In general anti-CD5 alone (5) or anti-B7-1 plus anti-B7-2 alone (B) induced only a small rescue of the CD4$^+$ population; anti-TNF alone (T) rarely induced CD4 rescue. However, the combination of 5 + B or 5 + T induced CD4 maturational rescue in >90% of the cultures. Statistical analysis (Student’s t test) of results from paired culture conditions from 32 experiments with AND.b/9R thymuses confirmed these observations: treatment of FTOC with 5 + B or 5 + T was significantly different from treatment with 5, B, or T alone (p < 0.05), and treatment with 5 + B + T was most significant (p < 0.005). Interestingly, the combination of B + T could not rescue CD4$^+$ cells from negative selection (data not shown). Thus, it appears that the block in negative selection is not due to simply an accumulation of adhesive interactions, but that CD5 plays a central role that is augmented by CD28 or TNFR.

Rescue from negative selection in other systems

I next investigated whether CD5, B7-1, B7-2, and TNF were involved in other examples of negative selection. First, the effect of the blocking Abs on negative selection induced by graded doses of a defined Ag was examined. AND.b mice were crossed to B10.A mice (AND.b/a), which express the H-2E$^b$ molecule necessary for presentation of the MCC peptide recognized by the AND TCR. Note that at day 16 of gestation, there are no CD4$^+$ thymocytes in AND.b/a thymuses that could be stimulated to induce an inflammatory response (data not shown). The development of CD4$^+$ thymocytes in FTOC is ablated in the presence of Ag (Fig. 2, top, Hlg control). However, the blocking Abs rescued the CD4$^+$ cells from Ag-induced negative selection, even at high doses of MCC. In this negative selection model, Ag also induces DP cell death, and the blocking Abs rescued a significant portion of the DP population from Ag-induced death as well. At 1000 nM MCC, only 23% of the DP population survived, whereas 60% survived in the presence of 1000 nM MCC plus 5 + B + T.

Next, I determined whether rescue from negative selection could be observed in a non-TCR-Tg system, normal C57BL/6 mice. The blocking Abs also induced significant rescue of CD4 maturation in C57BL/6 FTOC (Fig. 2, bottom). As in the AND.b/9R cultures, the rescued CD4$^+$ cells were not proliferating (data not shown). Interestingly, Abs to CD5, B7-1, and B7-2 (and TNF, data not shown) were not able to rescue CD8 maturation (Fig. 2, bottom). These results suggest that CD5, CD28, and TNF are specifically involved in MHC class II-dependent negative selection.

Finally, I determined the effect of the blocking Abs on mice deficient for either CD5 or CD28. Based on the results above, CD5-null mice should show rescue from negative selection just by blocking interaction of CD28 with B7-1 and B7-2. Correspondingly, CD28-null mice should show rescue from negative selection just by blocking interaction of CD5 with its ligand. This is exactly what was observed. Thymuses from CD5$^{-/-}$ mice showed little or no rescue of CD4 maturation when cultured with anti-B7-1 and anti-B7-2 (Fig. 3, top). In contrast, the CD4 population in thymuses from CD5$^{-/-}$ mice treated with anti-B7-1 and anti-B7-2 increased to levels obtained when wild-type thymuses were treated with 5 + B. Analysis of data from seven experiments confirmed that the difference in recovery of mature HSA$^{low}$CD4$^+$ cells between CD5$^{-/-}$ and CD5$^{-/-}$ mice treated with anti-B7-1 and anti-B7-2 was significant (p < 0.03). Likewise, thymuses from CD28$^{-/-}$ mice showed little rescue of CD4 maturation when cultured with anti-CD5. However, anti-CD5 treatment of CD28$^{-/-}$ thymuses caused CD4 maturation rescue equivalent to that obtained when wild-type thymuses were treated with 5 + B. Again, the difference in recovery of mature HSA$^{low}$CD4$^+$ cells between CD28$^{-/-}$ and CD28$^{-/-}$ mice treated with anti-CD5 was significant (p < 0.01; five experiments). These results confirm that CD5 and CD28 cooperatively contribute to negative selection: when one receptor is missing due to genetic ablation, blocking interaction of the other receptor with its ligand(s) rescues thymocytes from negative selection and allows them to mature into CD4$^+$ cells.

Rescue from negative selection correlates with autoreactivity

It has previously been shown that cells that escape negative selection are autoreactive (23). Thus, as a final test of maturational rescue, I examined whether the CD4$^+$ cells from these cultures were able to respond to syngeneic APC in an autoproliferation assay (13). Thymocytes obtained from AND.b/9R FTOC cultured with the blocking Abs showed increased proliferation in response to B10.S(9R) APC but not to C57BL/6 APC (Fig. 4, left). This
however, the CD28 experiments with each mouse line. Controls in other experiments. These data are representative of five to seven
ular experiment did not contain a CD28 from C57BL/6 FTOC cultured with the blocking Abs consistently
to APC can be observed even in normal mice under these conditions. As previously reported (13), significant proliferation to au-
tion that the blocking Abs rescue cells that were destined for
lected ligand, H-2A<sup>b</sup>. Thus, these results lend solid support to the
result is exactly what would be expected if AND thymocytes are
being rescued from negative selection on H-2A<sup>a</sup>. Even though both
H-2A<sup>b</sup> and H-2A<sup>a</sup> are expressed in AND.b/9R FTOC, the thymo-
cytes are autoreactive only to APC expressing the negatively sel-
lcting ligand, H-2A<sup>a</sup>. Thus, these results lend solid support to the

Discussion

The findings presented here show that CD5 and either CD28 or
TNFR cooperatively contribute to thymocyte negative selection.
This is the first demonstration that blocking the contribution of
coreceptors during thymic selection can produce autoreactive thy-
mocytes. Furthermore, these results clarify a large body of con-
fllicting data on the signals that control thymocyte death. For ex-
ample, TCR stimulation in combination with TNF can induce DP
thymocyte death in vitro, yet negative selection proceeds normally
in TNFR-II-null mice (5). Cross-linking the TCR with CD28 can
also induce thymocyte death in vitro (6, 7), and blockade of B7-1 and
B7-2 can partially block Ag-induced thymocyte death (24). Yet
egative selection is apparently unaffected in CD28-null mice (8).

These data are readily explained by the participation of multiple
coreceptors in thymocyte death, and they are consistent with pre-
vious results showing that CD40L is involved in negative selection
(3). Although the effect of CD40 stimulation on CD5L expression
is not known (17, 19), B7-1, B7-2, and TNF are up-regulated by
CD40-stimulation of APC (4) and B7-2 expression was reduced in
CD40L-null mice (3). CD54 (ICAM-1) is also up-regulated on
CD40-stimulated APC, and a recent report showed that CD54-null
mice have a partial defect in negative selection (25). Negative
selection due to class II MHC/peptide complexes is likely caused
by the sum total of stimulation received from the TCR plus co-
stimulatory molecules that are induced by CD40 stimulation of
thymic dendritic cells. CD40 stimulation of peripheral dendritic
cells is required to initiate an immune response (4). Thus, to avoid
autoimmunity, the population of naive T cells needs only to be
purged of cells that react with self-peptides on activated dendritic
cells. By using CD40-activated coreceptors during both negative
selection and the initiation of an immune response, the immune
system has an excellent system in place for avoiding autoimmune
responses.

There has been much debate as to what percentage of thy-
mocytes that are positively selected then undergo negative selection
because the affinity of their TCR for self-peptide/MHC complexes
is too high. Estimates of negative selection among the positively
selected CD4<sup>+</sup> population have ranged from <5% (23) to >50%
(26, 27). We were initially surprised by the large proportion of
CD4<sup>+</sup> T cells rescued when normal C57BL/6 FTOC were treated
with the blocking Abs (Fig. 2). However, the 2- to 3-fold increase
in the CD4 population in these cultures correlates exactly with the
2- to 3-fold increase in mature T cell production that is seen in
mice that lack negatively selecting APC in their thymuses (27).
Thus, the results presented here also imply that a high percentage
of thymocytes that are positively selected do not exit the thymus
due to negative selection. The observed increase in autoreactivity
in these cultures supports this notion (Fig. 4). These results are
significant to the study of autoimmunity, as the contribution of
negative selection vs peripheral tolerance to autoimmune diseases
is not known. Interestingly, medullary thymic epithelium ex-
presses proteins that were originally thought to be tissue specific or
developmentally regulated (28). Moreover, autoantigen expression
in the thymus correlates with resistance to some autoimmune dis-
eases (29–31). Taken together, these results suggest that negative
selection is a significant contributor among the mechanisms that
the immune system has developed to avoid autoimmunity. The

FIGURE 3. Rescue of CD4 maturation in CD5- or CD28-null mice.
Fetal thymuses from CD5<sup>−/−</sup> or CD5<sup>−/−</sup> littermates or from CD28<sup>−/−</sup> or
CD28<sup>−/−</sup> littermates were cultured in FTOC with Hlg or the indicated Abs.
Then the thymocytes were analyzed for CD4/CD8/HSA profile, and the
absolute numbers of CD4<sup>+</sup> HSA<sup>−/−</sup> cells recovered are shown. This partic-
erular experiment did not contain a CD28<sup>−/−</sup> thymus cultured with 5 + B; however, the CD28<sup>−/−</sup>
thymuses responded similarly to the wild-type controls in other experiments. These data are representative of five to seven
experiments with each mouse line.

FIGURE 4. Ab-induced CD4 maturation rescue correlates with auto-
reactivity. Fetal thymuses from C57BL/6 or AND.b/9R mice were cultured
in FTOC with Hlg, 5 + B, or 5 + B + T. Following culture, the percentage
CD4<sup>+</sup> obtained were 5% (Hlg) and 15% (5 + B) in the C57BL/6 FTOC
and 12% (Hlg) and 30% (5 + B + T) in the AND.b/9R FTOC. Prolifer-
ation to C57BL/6 or B10.S(9R) APC was assessed, and the average (±SD)
cpm of triplicate cultures are shown. The background proliferation with no
APC added was subtracted from each point, and these values were 14,300,
10,800, 19,500, 16,000, 11,600, and 8,400 cpm for the corresponding bars
on the graph (left to right). These data are representative of four similar
experiments.

exhibited increased proliferation in response to auto-APC (Fig. 4, right). Taken together, these results show that CD5, CD28, and
TNF contribute to class II MHC-dependent negative selection and
that blocking the contributions of these molecules can block neg-
ative selection and so induce the maturation of autoreactive cells.
identification of specific coreceptors that regulate negative selection and autoreactivity provides a new perspective for future explorations on the development of autoimmune disease.

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Note added in proof. Kishimoto and Sprent have also demonstrated that several coreceptors regulate negative selection (J. Exp. Med. 190:65, 1999).

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