

# TNF Receptor-Deficient Mice Reveal Striking Differences Between Several Models of Thymocyte Negative Selection<sup>1</sup>

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Central tolerance depends upon Ag-mediated cell death in developing thymocytes. However, the mechanism of induced death is poorly understood. Among the known death-inducing proteins, TNF was previously found to be constitutively expressed in the thymus. The role of TNF in thymocyte negative selection was therefore investigated using TNF receptor (TNFR)-deficient mice containing a TCR transgene. TNFR-deficient mice displayed aberrant negative selection in two models: an *in vitro* system in which APC are cultured with thymocytes, and a popular *in vivo* system in which mice are treated with anti-CD3 Abs. In contrast, TNFR-deficient mice displayed normal thymocyte deletion in two Ag-induced *in vivo* models of negative selection. Current models of negative selection and the role of TNFR family members in this process are discussed in light of these results. *The Journal of Immunology*, 1998, 160: 120–133.

Recognition of Ag by the TCR determines the fate of developing thymocytes. Thymocytes that recognize self MHC molecules with high avidity could develop into self-reactive T cells and are therefore induced to undergo programmed cell death or negative selection (reviewed in Ref. 1). However, thymocytes that recognize self MHC with low avidity can mature into naive T cells that are capable of populating the peripheral lymphoid organs and responding to foreign peptides bound to self MHC (reviewed in Ref. 2; 3). The factors that determine the avidity of these interactions probably include the strength of the signal through the TCR, the nature of the APC, and the state of thymocyte development.

Thymic development can be followed both anatomically and by expression of the TCR and the CD4 and CD8 coreceptors (reviewed in Ref. 4). TCR<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells develop into a population of TCR<sup>low</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells that make up most of the thymus. These cells, which are referred to as double positive (DP) cells, are located in the thymic cortex. A small number of these cells then mature into TCR<sup>high</sup>CD4<sup>+</sup>CD8<sup>-</sup> or TCR<sup>high</sup>CD8<sup>+</sup>CD4<sup>-</sup> cells, which are located in the thymic medulla. A large body of evidence has shown that negative selection can occur either at the immature DP stage of thymic development (5–8) or early in the mature stage (9–17). Furthermore, negative selection appears to require costimulatory signals from thymic APC in addition to an antigenic stimulus (18–21).

Recent evidence indicates that members of the TNF family of ligands and receptors may be involved in negative selection. Specifically, mice deficient in CD40L (20) or CD30 (21) displayed aberrant negative selection in several model systems (see Table I). Expression of CD40 and CD40L is increased on activated APC and on activated T cells/thymocytes, respectively (reviewed in Ref. 22). Expression of CD30 and CD30L are both increased on activated T cells (reviewed in Ref. 23); however, their expression patterns on thymocytes and thymic APC are unknown. Interestingly, the involvement of CD30 or CD40L in negative selection correlated with the stage of thymic development. CD30-deficient mice showed defective negative selection in models in which deletion occurred early in the DP stage of development (21). In contrast, CD40L-deficient mice (or mice treated with anti-CD40L Ab) showed defective negative selection in models in which deletion occurred late in the DP population and early in the mature CD4<sup>+</sup> population (20). Examples of early-stage negative selection are deletion caused by anti-CD3 treatment of mice (24, 25), by exogenous Ag administration to various TCR-transgenic mice (26–28), or by the endogenous male H-Y Ag in H-Y TCR-transgenic mice (5). Examples of late-stage negative selection include superantigen-mediated deletion of cells bearing TCR with specific V $\beta$  elements (reviewed in Refs. 29 and 30) and endogenous Ag-mediated deletion in several TCR-transgenic systems (12, 13, 15, 16).

Our examination of negative selection in CD40L-deficient mice indicated that the CD40/CD40L interaction itself was probably not delivering a death signal to immature thymocytes, but rather was causing an increase in the expression of costimulatory molecules on thymic APC (20). This hypothesis was supported by the following data. First, CD40 signaling causes increased expression of costimulatory molecules on thymic epithelium and peripheral APC (reviewed in Ref. 22); correspondingly, CD40L-deficient mice had reduced levels of CD28 ligands in the thymus (20). Second, anti-CD40L Abs could not block negative selection in an *in vitro* model of negative selection, presumably because the APC already expressed the necessary costimulatory molecules (D. M. Page and S. M. Hedrick, unpublished observations). Although some evidence indicates that CD28 can be involved in thymocyte deletion, especially at a late stage of development (17, 31, 32), others have not found a role for CD28 in this process (18, 19, 33–35). However, besides CD28 ligands, CD40 signaling also induces expression of other costimulatory molecules and cytokines on

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<sup>3</sup> Abbreviations used in this paper: <sup>low</sup>, low level; DP, CD4<sup>+</sup>CD8<sup>+</sup> thymocyte; <sup>high</sup>, high level; CD40L, CD40 ligand; TNFR, tumor necrosis factor receptor; p55, tumor necrosis factor receptor-I; p75, tumor necrosis factor receptor-II; LM, littermate; MCC, moth cytochrome *c*; <sup>dull</sup>, dull staining.

Table 1. *TNFR family members and negative selection models*

| Receptor                 | Early-Stage Deletion        |                    | In Vitro Thymocyte Deletion | Late-Stage Deletion |                        |
|--------------------------|-----------------------------|--------------------|-----------------------------|---------------------|------------------------|
|                          | Class I MHC Antigen H-Y TCR | Anti-CD3 Injection |                             | Superantigens       | AND TCR/A <sup>5</sup> |
| CD40 <sup>a</sup>        | Normal                      | Normal             | Normal                      | Defective           | Defective              |
| CD30 <sup>b</sup>        | Defective                   | Defective          | ND <sup>c</sup>             | Normal              | ND                     |
| Fas <sup>d</sup>         | Partly Defective            | Defective          | Normal                      | Normal              | ND                     |
| p55 TNFR-I <sup>e</sup>  | ND                          | ND                 | Defective                   | Normal              | Normal                 |
| p75 TNFR-II <sup>e</sup> | ND                          | Normal             | Partly Defective            | Normal              | Normal                 |
| p55/75 <sup>e</sup>      | Normal                      | Defective          | Defective                   | ND                  | Normal                 |

<sup>a</sup> Compiled from references 20 and 58, and unpublished data of D.M.P. and S.M.H.

<sup>b</sup> Compiled from reference 21.

<sup>c</sup> Not done.

<sup>d</sup> Compiled from references 61, 87–91, 93, 94.

<sup>e</sup> Compiled from references 49 and 50, and this paper.

APC, including TNF (36). Interestingly, Giroir et al. (37) showed that the thymus is the only organ in which the TNF promoter is constitutively active. Others have also found constitutive TNF expression in both human (38) and murine (39) thymus. Furthermore, mice that overexpress TNF in their thymocytes and T cells have small thymuses with decreased populations of DP cells (40). Correspondingly, children with Down's syndrome (trisomy 21) overexpress TNF in their thymuses and also have small thymuses with abnormal anatomy and thymocyte subsets (38). These results are intriguing, and they suggest that TNF could play a role in thymic development.

TNF exists as either a soluble or membrane-bound protein, and it is produced by many cell types, including macrophages, dendritic cells, and T cells and thymocytes themselves (reviewed in Ref. 41). There are two known receptors for TNF, and they are coexpressed on most hemopoietic cells. These are the 55- to 60-kDa TNFR-I (p55) and the 70- to 80-kDa TNFR-II (p75). In the past, TNF-induced cytotoxicity was attributed solely to the p55 receptor, whereas TNF-induced proliferation was attributed to the p75 receptor (42, 43). However, many recent papers have shown that p75 can greatly enhance p55-induced cell death (44–48). In particular, membrane-bound TNF appears to be the primary ligand for p75 and can cause cytotoxicity in cells that are not affected by soluble TNF (46). Although p55- or p75-deficient mice were previously shown to exhibit normal superantigen-induced negative selection (49, 50), thymic deletion has never been investigated in mice deficient in both p55 and p75 or in Ag-dependent models of negative selection. Thus, in light of the recent evidence suggesting an interaction between the two TNFRs, we wished to carefully examine the effect of TNF on thymocyte development in several experimental models.

## Materials and Methods

### Mice

Mice were bred at the University of California-San Diego under specific pathogen-free conditions. The production and characterization of the AND and H-Y TCR-transgenic mice have been previously described (5, 12, 51, 52). The p55- and p75-deficient mice were constructed at Immunex Corp. (J. J. Peschon et al., manuscript in preparation) and are equivalent to those previously described by other groups (49, 50). Mice used for experiments were 3 to 12 wk of age, and all experiments used either littermate (LM) controls or, in a few cases, age-matched litters whose parents were LM.

### Cell culture

Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM 2-ME, 100 µg/ml

streptomycin, 100 U/ml penicillin, and nonessential amino acids. The murine fibroblast APC lines FT16.6C5 and DCEK.Hi7 were provided by Dr. R. Germain (National Institute of Allergy and Infectious Diseases, Bethesda, MD). These are DAP3 fibroblasts (L929 derived) that have been stably transfected with H-2E<sup>b</sup> and H-2E<sup>k</sup>, respectively (53). LB7 fibroblasts are L929 fibroblasts stably transfected with B7.1 (18).

### In vitro negative selection

Single cell suspensions of thymocytes were prepared from 3- to 12-wk-old mice that had been killed by cervical dislocation. The thymocytes were then cultured essentially as previously described (18). Briefly,  $1 \times 10^6$  thymocytes were incubated in 48-well tissue culture plates with  $1.5 \times 10^5$  fibroblast APC and other reagents. Ag consisted of the 88 to 103 COOH-terminal peptide of moth cytochrome *c* (MCC). For cultures with anti-TCR, tissue culture plates were coated with anti-Vβ3 (KJ25) (54) as previously described (18). After 18 to 24 h of culture, thymocyte viability was determined by flow cytometric counting of cells that excluded propidium iodide. The propidium iodide exclusion profile was then used to set a forward scatter/side scatter gate such that dead cells were excluded from further analyses. Surface expression of CD4 and CD8 on the live cells was then determined by Ab staining and flow cytometry using this live gate as previously described (18). Analysis was performed on a FACScan flow cytometer (Becton Dickinson) using CellQuest Software with collection of 5,000 to 20,000 live cells. The percentage of DP thymocytes recovered compared with that in the no Ag controls was calculated as:  $100\% \times (\text{absolute number of DP with Ag})/(\text{absolute number of DP without Ag})$ .

### Abs and reagents

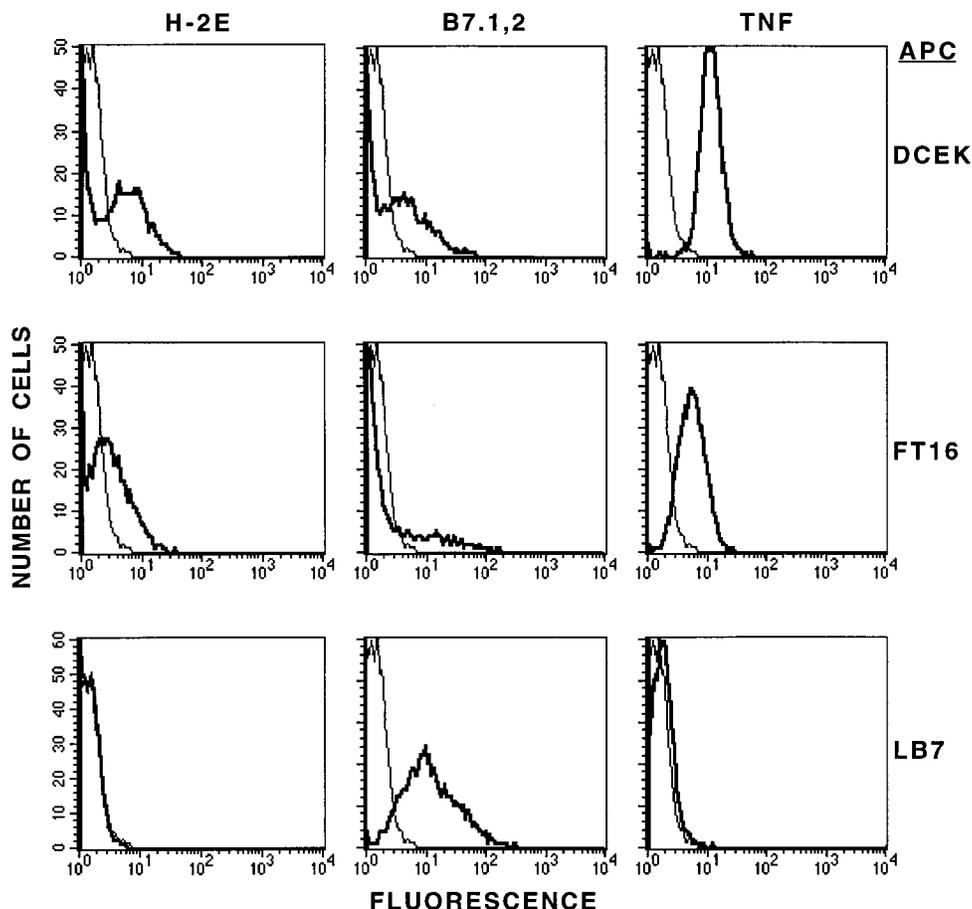
Phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-CD8 were purchased from Caltag Laboratories (Burlingame, CA). Both recombinant murine TNF and polyclonal rabbit anti-mouse TNF were purchased from Genzyme (Cambridge, MA). The Y17 Ab (55) was used to detect H-2E, and the CTLA4Ig fusion protein (provided by Dr. J. Allison, University of California-Berkeley) (56) was used to detect B7.1 and B7.2. Secondary Abs used were FITC-conjugated goat anti-rabbit, anti-rat, or anti-human (Caltag Laboratories or Fischer Biotech, Pittsburgh, PA). The hamster anti-CD3 Ab 2C11 (57) was used as a partially purified ascites, and control Syrian hamster Ig was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

### In vivo negative selection

Mice were injected i.p. with 100 to 150 µg of anti-CD3 or hamster Ig in a sterile solution of PBS. Mice were subsequently sacrificed after 1 or 2 days, and thymocytes were analyzed for live cell recovery and for CD4/CD8 expression by flow cytometry.

### Proliferation assays

Production of mature thymocytes capable of responding to Ag was monitored by assaying thymocyte proliferation. Single-cell suspensions were prepared from thymocytes and cultured for 3 or 4 days with irradiated (3500 rad) splenocytes from B10.A mice in 96-well flat-bottom tissue culture plates. Generally,  $10^5$  thymocytes were incubated with  $3 \times 10^5$  B10.A splenocytes with or without the addition of MCC peptide. The cells were



**FIGURE 1.** APC that induce negative selection *in vitro* express surface TNF. Cell suspensions were prepared from the indicated fibroblast lines and were analyzed for expression of H-2E, TNF, or B7.1 and B7.2 using monoclonal anti-H-2E, polyclonal anti-TNF, or the CTLA4Ig fusion protein. Bound Abs or fusion proteins were detected with FITC-conjugated secondary Abs, and flow cytometric data were collected from 5000 live cells. The thin lines represent staining from the secondary Abs alone, and the dark lines represent specific staining of the protein indicated.

pulsed with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]methyl-thymidine (New England Nuclear, Boston, MA) for the final 18 h of culture, and isotope incorporation was determined. Each condition was performed in triplicate.

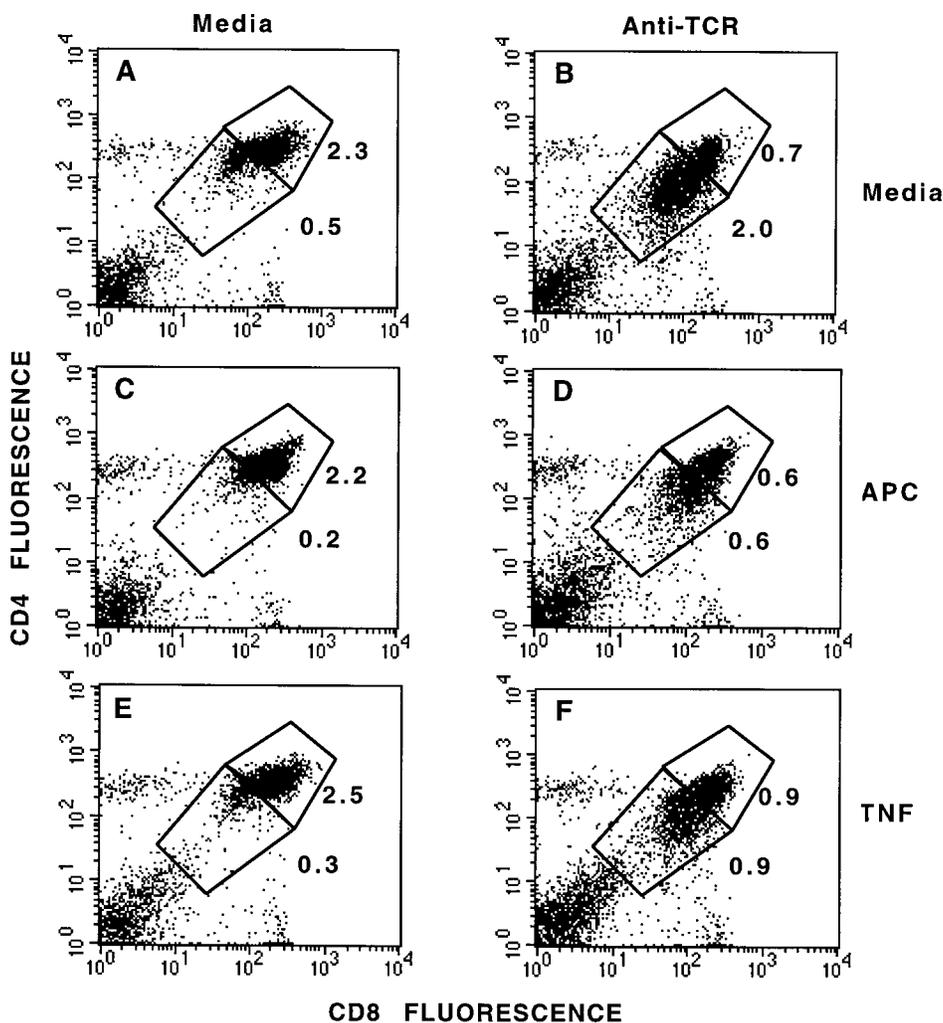
## Results and Discussion

### *TNF expression in APC and thymus*

Several laboratories have examined the mechanisms underlying thymocyte negative selection by culturing thymocytes from TCR-transgenic mice with Ag and various APC (reviewed in Ref. 58). We noticed that the ability of fibroblast APC to induce negative selection of DP thymocytes in this system correlated with their expression of TNF. Several fibroblast lines were examined for expression of TNF or the CD28 ligands B7.1 and B7.2 (CD80 and CD86). The FT16 and DCEK fibroblast APC were derived by stable transfection of H-2E<sup>b</sup> or H-2E<sup>k</sup> into an L929-derived fibroblast subline named DAP3 (53). FT16 and DCEK express H-2E<sup>b</sup> or H-2E<sup>k</sup>, B7, and TNF (Fig. 1). As previously reported (12), these APC are also able to cause deletion of DP thymocytes (see also Figs. 2-5). In contrast, the fibroblast line LB7, which was derived by stable transfection of B7.1 into L929, expresses no TNF (Fig. 1) and cannot mediate negative selection in the *in vitro* cultures when used in conjunction with a stimulus through the TCR (18). Correspondingly, we found that we could easily detect TNF expression in the thymus by immunohistochemical staining of frozen sections (data not shown), which correlates well with previous work (37-39).

### *In vitro negative selection*

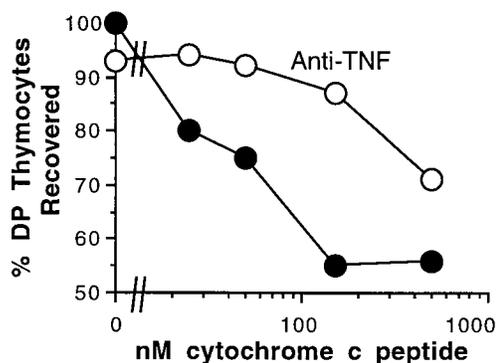
We next analyzed the role of TNF in negative selection by several methods. For these experiments, the murine AND TCR-transgenic system was chosen. These mice express a V $\beta$ 3/V $\alpha$ 11 TCR that recognizes cytochrome *c* peptides bound to the class II MHC molecule H-2E (51). This system is advantageous in that it is very well characterized both for an *in vitro* model of negative selection (12) and for the H-2 haplotypes that mediate thymocyte negative selection (A<sup>s</sup>), positive selection (A<sup>b</sup>, E<sup>k</sup>), or no selection (A<sup>d</sup>) *in vivo* (12, 52). In particular, we previously demonstrated (18) that DP thymocytes that receive only a TCR stimulus do not undergo programmed cell death, but merely down-regulate their CD4 and CD8 proteins to produce a DP<sup>du11</sup> phenotype (Fig. 2B). Apparently similar DP<sup>du11</sup> cells were recently shown to have several characteristics indicating that they are in the early stages of positive selection (59); however, addition of APC causes these cells to die and disappear from the cultures, as measured by counting the number of DP cells recovered that have not taken up propidium iodide (Fig. 2D) (18). We therefore examined whether TNF could induce negative selection in conjunction with a stimulus through the TCR. Interestingly, addition of soluble TNF to thymocytes treated with anti-TCR Abs could induce their death (Fig. 2, B vs F). In contrast, thymocytes that were not cultured with anti-TCR were relatively resistant to the effect of TNF (Fig. 2, A vs E). In control experiments, the effect of soluble TNF was blocked by either anti-TNF



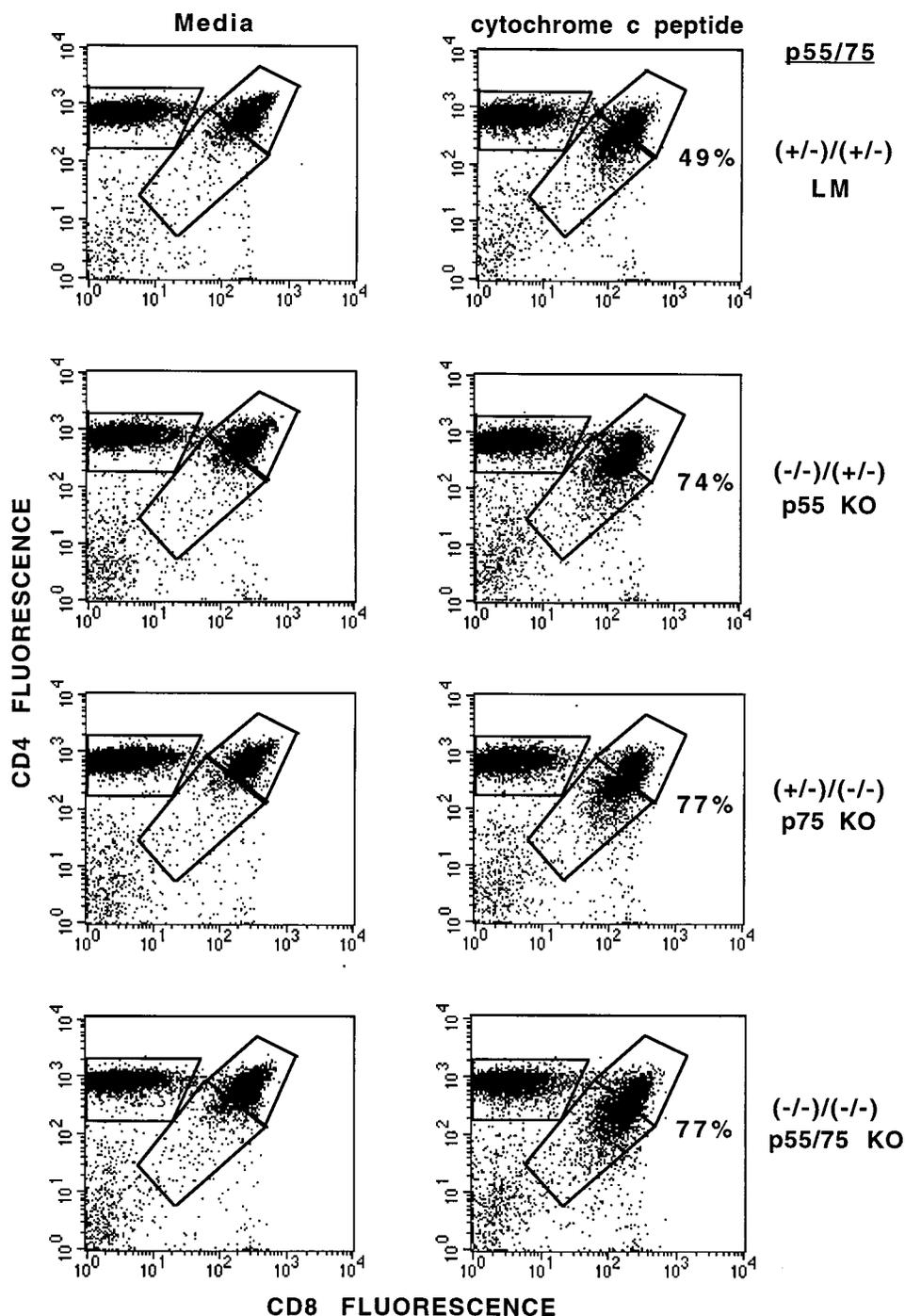
**FIGURE 2.** TNF can induce thymocyte deletion *in vitro*. Thymocytes from AND TCR-transgenic mice were cultured overnight with media or plate-bound anti-TCR Ab (KJ25) with or without the addition of murine TNF (20 ng/ml) or fibroblast APC (FT16). The cultures were subsequently analyzed by flow cytometry for CD4/8 expression and for live cell recovery by propidium iodide exclusion. The CD4/8 expression pattern and the absolute number of DP<sup>high</sup> and DP<sup>dull</sup> cells recovered ( $\times 10^{-5}$ ) are shown for each treatment. This experiment is representative of 10 experiments performed in which the percentage of DP thymocytes recovered compared with that in medium controls was  $95 \pm 8\%$  for anti-TCR,  $85 \pm 7\%$  for TNF, and  $62 \pm 17\%$  for TNF plus anti-TCR treatment (mean  $\pm$  SD).

Abs or a soluble TNFRIg fusion protein (provided by Dr. C. Ware, La Jolla Institute of Allergy and Immunology, La Jolla, CA; data not shown). We noted that APC were always better able to delete DP thymocytes than was TNF (Fig. 2, *D* vs *F*), suggesting that the APC either express other signals responsible for negative selection or that membrane-bound TNF is a more potent stimulus. Indeed, supernatants from these APC are not able to cause DP deletion (18). We tried to determine whether membrane-bound TNF could cause DP deletion without the contribution of other costimulatory molecules by transfecting TNF into several fibroblast lines that cannot mediate negative selection in the *in vitro* cultures. Despite many attempts, we were never able to obtain a stable transfectant, presumably due to TNF-induced cytotoxicity (C. Katayama and D. M. Page, unpublished data).

We next examined whether anti-TNF Abs could block Ag-induced negative selection in these cultures. Addition of cytochrome *c* and APC caused a dose-dependent deletion of DP thymocytes expressing the AND TCR (Fig. 3) (12). This deletion was blocked by anti-TNF Abs, although the blockage declined with increasing doses of Ag. It is possible that at high Ag doses, other costimulatory molecules may be able to cause negative selection. In contrast to these results, many other Abs or reagents are unable to



**FIGURE 3.** Anti-TNF Abs can block Ag-induced negative selection *in vitro*. Thymocytes from AND TCR-transgenic mice were cultured overnight with fibroblast APC (DCEK) and the indicated amounts of Ag (MCC peptide) with or without the addition of polyclonal anti-TNF. The cultures were subsequently analyzed by flow cytometry for CD4/8 expression and live cell recovery. Shown is the percentage of DP thymocytes recovered compared with that in cultures without Ag. This experiment is representative of eight experiments performed in which the percentage of DP thymocytes recovered at the highest Ag dose was  $56 \pm 13\%$  for Ag alone vs  $75 \pm 20\%$  for Ag with anti-TNF (mean  $\pm$  SD).

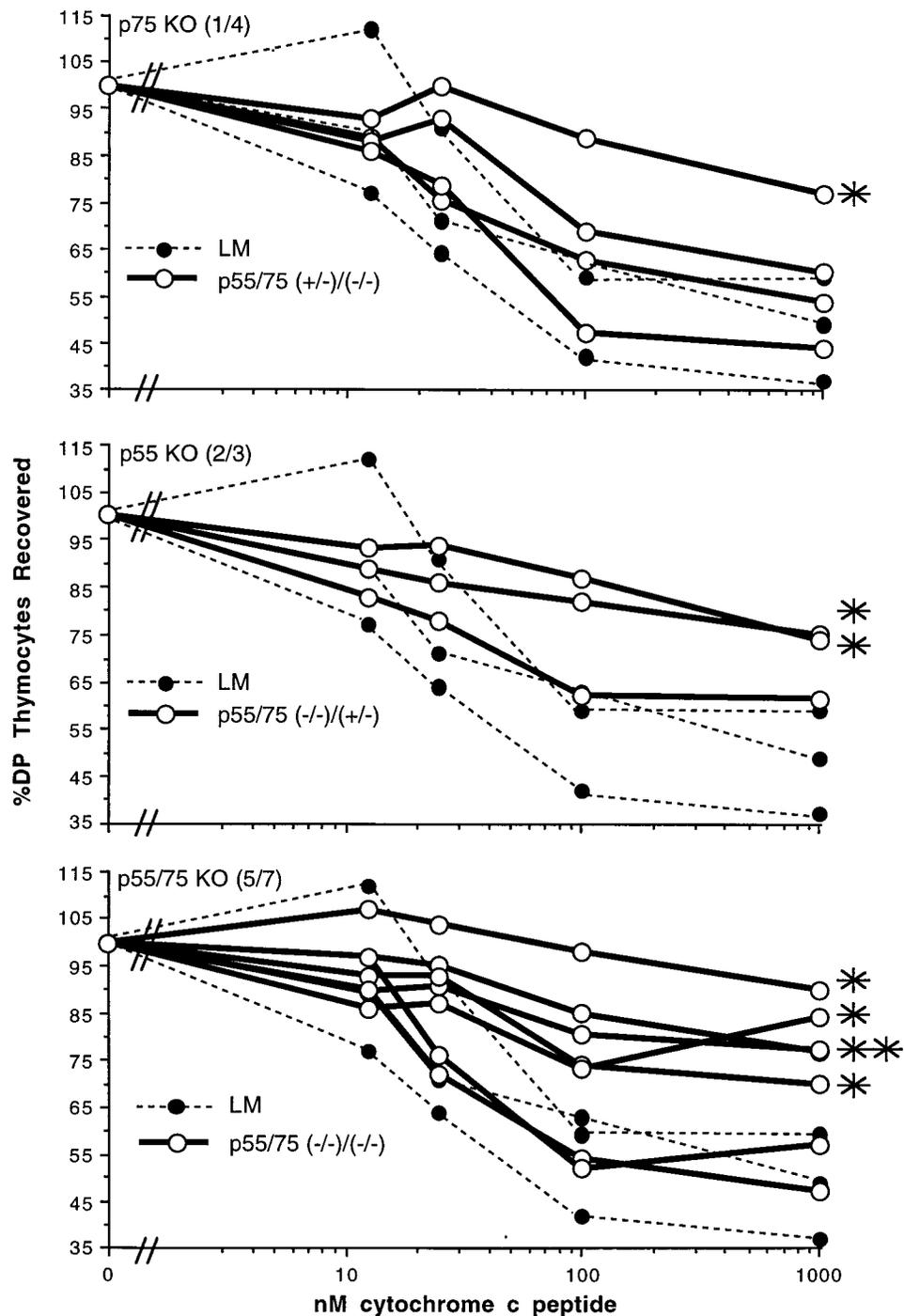


**FIGURE 4.** TNFR-deficient mice exhibit aberrant negative selection *in vitro*. Thymocytes from various p55/75-deficient mice expressing the AND TCR transgene and H-2<sup>b</sup> were cultured overnight with fibroblast APC (DCEK) with or without Ag (1  $\mu$ M MCC peptide). The cultures were subsequently analyzed by flow cytometry for CD4/8 expression and live cell recovery. The percentage of DP thymocytes recovered compared with that in the no Ag control are shown for each mouse line, and this figure is based on absolute numbers of DP recovered (see *Materials and Methods*). The percentages of mature CD4<sup>+</sup> cells in each mouse line were virtually identical: 60% (LM), 61% (p55 KO), 62% (p75 KO), and 54% (p55/75 KO).

block negative selection in these cultures, including the CTLA4Ig fusion protein, anti-CD28 Fab, anti-CD40L, the CD30Ig fusion protein (provided by Dr. R. Goodwin, Immunex Corp., Seattle, WA), anti-CD6, the CD6Ig fusion protein (provided by Drs. G. Starling and A. Aruffo, Bristol-Myers Squibb, Seattle, WA), anti-LFA-1, or anti-CD45 (18) (D. M. Page, R. Soloff, and S. M. Hedrick, unpublished observations). These results indicated that

TNF was playing a major role in negative selection in these cultures.

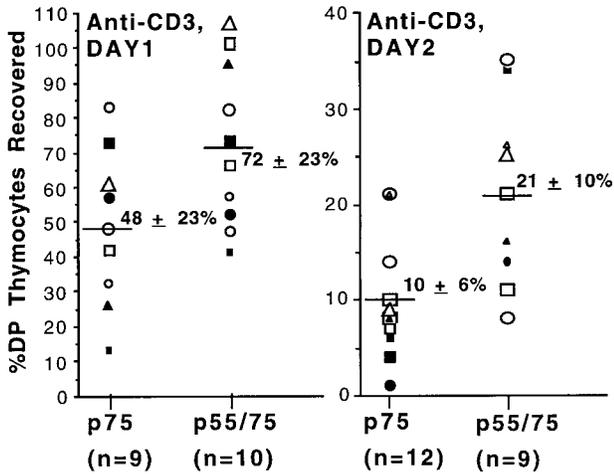
To investigate the role of TNF in thymocyte development *in vivo*, we bred the AND TCR transgene onto p55/75-deficient mice of the H-2<sup>b</sup> background. By using this animal model, we were able to analyze both positive and negative selection of thymocytes. As shown in the *left panels* of Figure 4, positive selection of the AND



**FIGURE 5.** In vitro negative selection in TNFR-deficient mice. Thymocytes from various p55/75-deficient mice were cultured and analyzed as described in Figure 4. The percentage of DP thymocytes recovered compared with that in cultures without Ag is shown for five experiments. The dashed lines represent the responses of the p55<sup>+/-</sup>/p75<sup>+/-</sup> LM controls, and the dark lines represent the responses of mice deficient in p55, p75, or p55/75, as indicated. The asterisks highlight altered negative selection responses.

TCR on H-2A<sup>b</sup> was not altered in p55<sup>-</sup>, p75<sup>-</sup>, or p55/75-deficient mice. Thymocytes from each of these mice contained a large population of mature CD4<sup>+</sup>, TCR<sup>+</sup>, Ag-responsive cells that were no different from the LM controls (Figs. 8–10 and data not shown). In contrast, if thymocytes from these mice were incubated with APC and Ag in vitro, they displayed defective deletion of their DP thymocytes compared with LM controls (Fig. 4, right panels). Significantly more DP cells were recovered in the cultures from the TNFR-deficient mice than in those from the LM controls (74–77

vs 49%). A summary of five in vitro deletion experiments is shown in Figure 5. Although the results were somewhat variable, a consistent pattern was still evident. Thymocytes from only one of four p75-deficient mice displayed aberrant negative selection, whereas thymocytes from two of three p55-deficient mice and five of seven p55/75-deficient mice displayed defective DP deletion. These data indicate that the two TNFR are playing a role in this in vitro model of negative selection. The effect seems to be mostly due to p55, although we were unable to rule out a small contribution from p75.

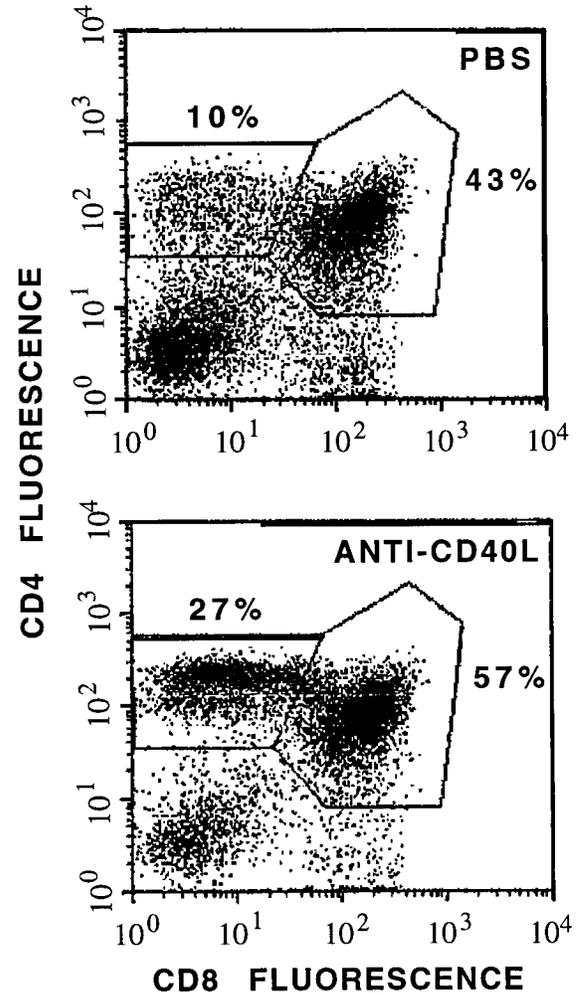


**FIGURE 6.** Negative selection induced by anti-CD3 is aberrant in TNFR-deficient mice. p75- or p55/75-deficient LM were injected i.p. with hamster Ig or anti-CD3. After 1 or 2 days, thymocytes were analyzed by flow cytometry for CD4/8 expression and live cell recovery. Shown is the percentage of DP thymocytes recovered compared with that in hamster Ig-injected controls for five experiments performed on day 1 and for six experiments performed on day 2. The total number of mice analyzed and the mean  $\pm$  SD of DP recovery are indicated.

The two TNFR are clearly not essential for deletion, however, because a high dose of Ag resulted in partial DP deletion, even in the p55/75-deficient mice. Also, in two of seven cases, the p55/75-deficient mice displayed normal negative selection. It should be noted that this type of variability in negative selection is not seen when thymocyte deletion is examined in the AND line of mice. A random sample of AND mice of the H-2<sup>b</sup> haplotype exhibited equivalent and profound negative selection in the *in vitro* culture system (data not shown). Nevertheless, since the p55/75-deficient mice derive from a mixed 129 and C57BL/6 background, we considered the possibility that the variability in negative selection was due to background genes from the 129 mouse strain. Thus, we also examined negative selection in AND mice bred to 4-1BBL-deficient mice (J. J. Peschon, unpublished observations). 4-1BBL is another member of the TNF superfamily (41), and 4-1BBL-deficient mice are likewise derived from a mixed 129 and C57BL/6 background. Thymocytes from five of five AND/4-1BBL-deficient mice, however, did not display defective *in vitro* negative selection compared with LM controls (data not shown). Taken together, these data therefore suggest that the defective thymocyte deletion observed in the majority of p55/75-deficient mice is indeed due to their lack of TNFR. However, TNF is probably not the only molecule expressed by these fibroblast APC that is able to cause DP thymocyte deletion. We believe that variable expression of other unknown costimulatory molecules on our APC lines accounts for the partial negative selection exhibited by the p55/75-deficient mice and also for the inability of anti-TNF Abs to completely block negative selection at high Ag doses (Fig. 3).

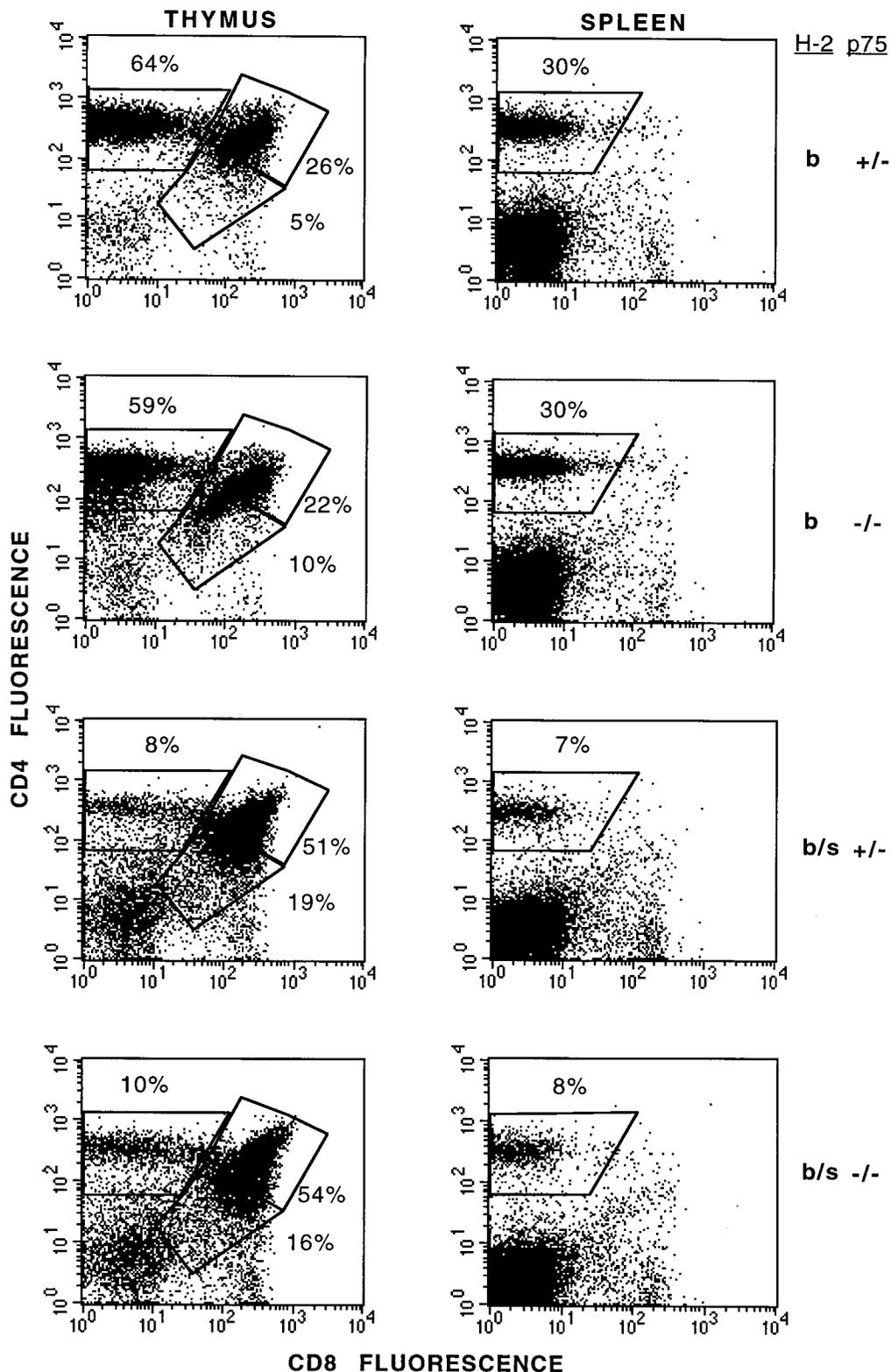
#### *Anti-CD3-induced negative selection in vivo*

Although TNF appeared to play a significant role in negative selection *in vitro*, we wished to examine its role *in vivo*. Thus, we next examined DP deletion in response to injection of anti-CD3 Abs, which has often been considered a model of negative selection. For example, CD30- or Fas (CD95)-deficient mice are both defective in this model (21, 60). Mice were therefore injected i.p. with anti-CD3 Abs and then examined after 1 or 2 days for recovery of their DP thymocytes. In early experiments, we found no



**FIGURE 7.** Anti-CD40L treatment blocks Ag-induced negative selection in AND TCR-transgenic mice. AND TCR-transgenic mice of the H-2<sup>b/s</sup> background were injected with PBS or anti-CD40L from birth as previously described (20). At 4 wk of age, the mice were killed, and their thymocytes were analyzed for viable cell recovery and CD4/8 profile. Shown are the percentages of CD4<sup>+</sup> and DP cells for each treatment. The total cell recoveries were 63 million for the PBS-injected mouse and 132 million for the anti-CD40L-injected mouse. These results are representative of three experiments.

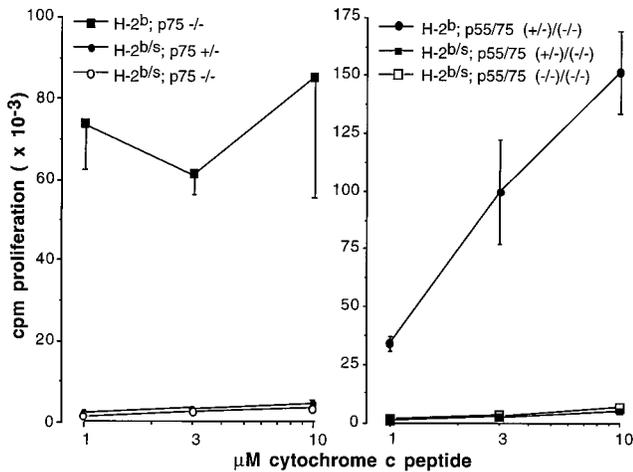
significant difference between p75-deficient mice and their LM controls ( $p = 0.1$ , by Student's *t* test; data not shown). To obtain enough mice for experiments with the p55/75-deficient line, we bred the mice such that the p75-deficient mice were the LM controls. Thus, we did not specifically examine the p55-deficient mice in this model. It was found that p55/75-deficient mice displayed aberrant anti-CD3-induced negative selection (Fig. 6). The effect was somewhat variable after only 1 day of treatment ( $p = 0.02$ , by Student's *t* test), but was more pronounced after 2 days ( $p = 0.006$ , by Student's *t* test). The effect on negative selection could be due to p55 alone or to both TNFR. In contrast with these data, Sytwu et al. (61) were not able to block negative selection with anti-TNF Abs. These investigators induced DP deletion by injecting hemagglutinin-specific TCR-transgenic mice with hemagglutinin peptide. However, it is possible that since the effects we observed were small, they could only be detected by a complete lack of TNFR stimulation, which is difficult to achieve with an Ab block. In this regard, it should be noted that the p55/75-deficient



**FIGURE 8.** In vivo Ag-induced negative selection is normal in p75-deficient mice. The p75-deficient mice expressing the AND TCR-transgene were bred onto MHC backgrounds mediating positive (H-2<sup>b</sup>) or negative (H-2<sup>b/s</sup>) selection. Thymocytes or splenocytes were isolated from LM and analyzed by flow cytometry for their CD4/8 expression pattern and live cell recovery. Shown are the percentages of CD4<sup>+</sup>, DP<sup>high</sup>, and DP<sup>dull</sup> populations for each genetic background. This experiment is representative of three performed.

mice do not express any other TNFR, as assessed by lack of binding of TNF to hemopoietic cell populations (J. J. Peschon, unpublished observations). Thus, the anti-CD3-induced DP deletion that

still occurs in these mice may be due to CD30, Fas, or other newly identified, death-inducing TNF family members such as DR-3/TRAMP/Apo-3/LARD (62–65) or TRAIL/Apo-2L (66, 67).



**FIGURE 9.** TNFR deficiency does not rescue Ag-responsive cells from negative selection. Thymocytes from LM of AND TCR-transgenic mice of the indicated genetic backgrounds were cultured with irradiated splenocytes and the indicated concentrations of Ag (MCC peptide) for 3 days. The cultures were subsequently analyzed for proliferation as described in *Materials and Methods*, and the mean counts per minute  $\pm$  SD of triplicate cultures are shown. These data are representative of three experiments performed for the p75-deficient line and four performed for the p55/75-deficient line.

#### MHC class II-dependent Ag-induced negative selection *in vivo*

Although anti-CD3 causes deletion of DP thymocytes, it is not likely to be a very physiologic model for negative selection by MHC class II-restricted Ags. Thymocyte deletion due to anti-CD3 treatment occurs throughout the cortex and affects most of the DP population (68). Similarly, injection of antigenic peptide into TCR-transgenic mice causes the death of most DP cells, and it is likely that this effect is dependent on a systemic activation of mature T cells (20, 26–28). In contrast, it is clear from the work of many laboratories that negative selection of CD4<sup>+</sup> cells is mediated in large part by dendritic cells (69–71), which are located at the corticomedullary junction and in the medulla of the thymus (72). Medullary epithelium has also been shown to cause negative selection of high avidity CD4<sup>+</sup> T cells in some cases (16, 73–75), whereas cortical epithelium (13, 76) and macrophages (70, 75, 77) are very poor mediators of this process *in vivo*. Thus, it has been deduced that negative selection due to peptides presented by class II MHC probably occurs quite late in DP development or early in the CD4<sup>+</sup> stage (11). The AND TCR-transgenic system is advantageous because negative selection can be analyzed *in vivo* in response to an Ag that causes late-stage DP deletion. When these mice express the H-2<sup>b/s</sup> haplotype, CD4<sup>+</sup> development is blocked, and many DP<sup>dull</sup> cells are observed (12). The negative selection in this system is presumably due to an unknown peptide bound to A<sup>s</sup> that causes deletion of DP thymocytes late in their development. This late deletion is evident from the fact that although the thymuses of these mice are somewhat reduced in size, most of the DP population is still present. It is also very similar to the negative selection observed when cytochrome *c* is expressed as an autoantigen along with the AND TCR (15). Accordingly, this model of negative selection appears to be a more physiologic one for class II MHC-restricted Ags.

When anti-CD40L Abs are injected into AND/H-2<sup>b/s</sup> mice, negative selection is blocked; this results in an increase in the absolute number of DP and mature CD4<sup>+</sup> cells in the thymus (Fig. 7) and

a corresponding increase in Ag-reactive cells (20). When DP deletion due to H-2A<sup>s</sup> is blocked, mature Ag-reactive CD4<sup>+</sup> cells are able to develop in AND/H-2<sup>b/s</sup> mice because the AND TCR can still be positively selected on H-2A<sup>b</sup>. Thus, we used this system to first examine negative selection in the p75-deficient line. Negative selection in these mice proceeded normally, as assessed by several criteria. First, CD4<sup>+</sup> mature thymocytes or splenocytes did not develop well in either p75-deficient mice or their LM controls when the mice expressed H-2A<sup>b/s</sup> (Fig. 8). Many of the CD4<sup>+</sup> cells that do escape have low levels of the TCR V $\alpha$ 11 chain, probably due to endogenous TCR  $\alpha$ -chain rearrangement (12) (data not shown). Second, there is no increase in Ag-reactive thymocytes in p75-deficient mice that express H-2A<sup>b/s</sup> (Fig. 9, *left*).

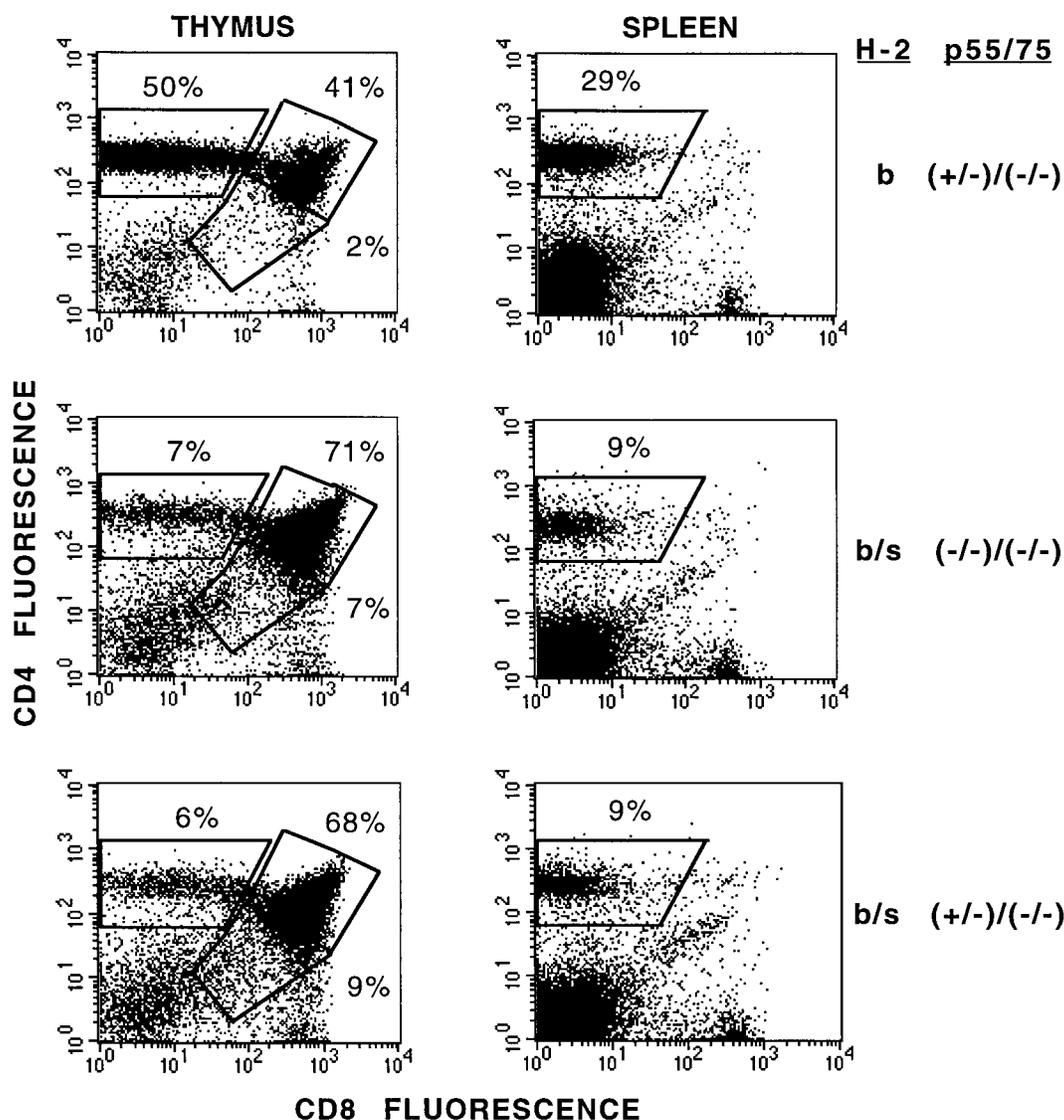
Since the p75-deficient line exhibited normal negative selection in this system, the mice were again bred such that the p75-deficient mice were the LM controls for the p55/75-deficient line. This was necessary to obtain enough mice for experiments with LM controls, as otherwise there are four independently segregating genes (p55, p75, AND TCR, and H-2A<sup>s</sup>). In striking contrast to the *in vitro* system and the anti-CD3 system, the p55/75-deficient mice displayed perfectly normal negative selection in this model. H-2A<sup>s</sup> expression blocked CD4<sup>+</sup> development and increased the DP<sup>dull</sup> population to an equal extent in these mice as in the p75-LM controls (Fig. 10). Furthermore, there was not even a slight increase in Ag-reactive thymocytes in these mice (Fig. 9, *right*). The same result was also achieved with the p55-deficient line (data not shown). Thus, in this model of late-stage negative selection, the two TNFR appear not to be required.

#### MHC class I-dependent Ag-induced negative selection *in vivo*

Although anti-CD3 treatment is unlikely to be a good model of negative selection by class II MHC-restricted Ags, it may be characteristic of negative selection that occurs early in the DP stage of development. Thus, we considered the possibility that the TNFR might play a role only in early-stage negative selection. To examine this issue, we bred the p55/75-deficient mice to H-Y TCR-transgenic mice on the H-2<sup>b</sup> background. These mice express a V $\beta$ 8.2/V $\alpha$ 3 TCR that recognizes the male H-Y peptide bound to the class I MHC molecule H-2D<sup>b</sup> (5). As previously described (5, 78), in female mice the H-2D<sup>b</sup> molecule mediates positive selection of a large population of CD8<sup>hi</sup> cells in the thymus and lymph nodes (Fig. 11A). In contrast, the H-Y Ag causes negative selection in male mice, resulting in a small thymus with many DP<sup>dull</sup> cells and a population of CD8<sup>dull</sup> cells in the lymph nodes (Fig. 11). These CD8<sup>dull</sup> cells are unresponsive to Ag (78). The lack of the two TNFR did not appear to rescue thymocytes from negative selection in the male mice as assessed by either thymus size or the appearance of CD8<sup>high</sup> cells in the lymph nodes (Fig. 11). Thus, neither p55 nor p75 appears to be required in this model of early stage *in vivo* Ag-induced negative selection.

#### Negative selection and the TNFR family

There are now numerous *in vitro* and *in vivo* models of thymocyte negative selection that include both class I and class II MHC-restricted TCR (reviewed in Ref. 4). As discussed above, however, results from many investigators indicate that negative selection due to peptides bound to class II MHC occurs quite late in DP development or early in the CD4<sup>+</sup> stage and is mediated by dendritic cells or medullary epithelium. When negative selection due to TNF was examined in this type of model, the two TNFR were not required, nor were they required in an *in vivo* model of thymocyte negative selection in which DP cells were deleted early in their development. In contrast, TNF clearly played a role in the

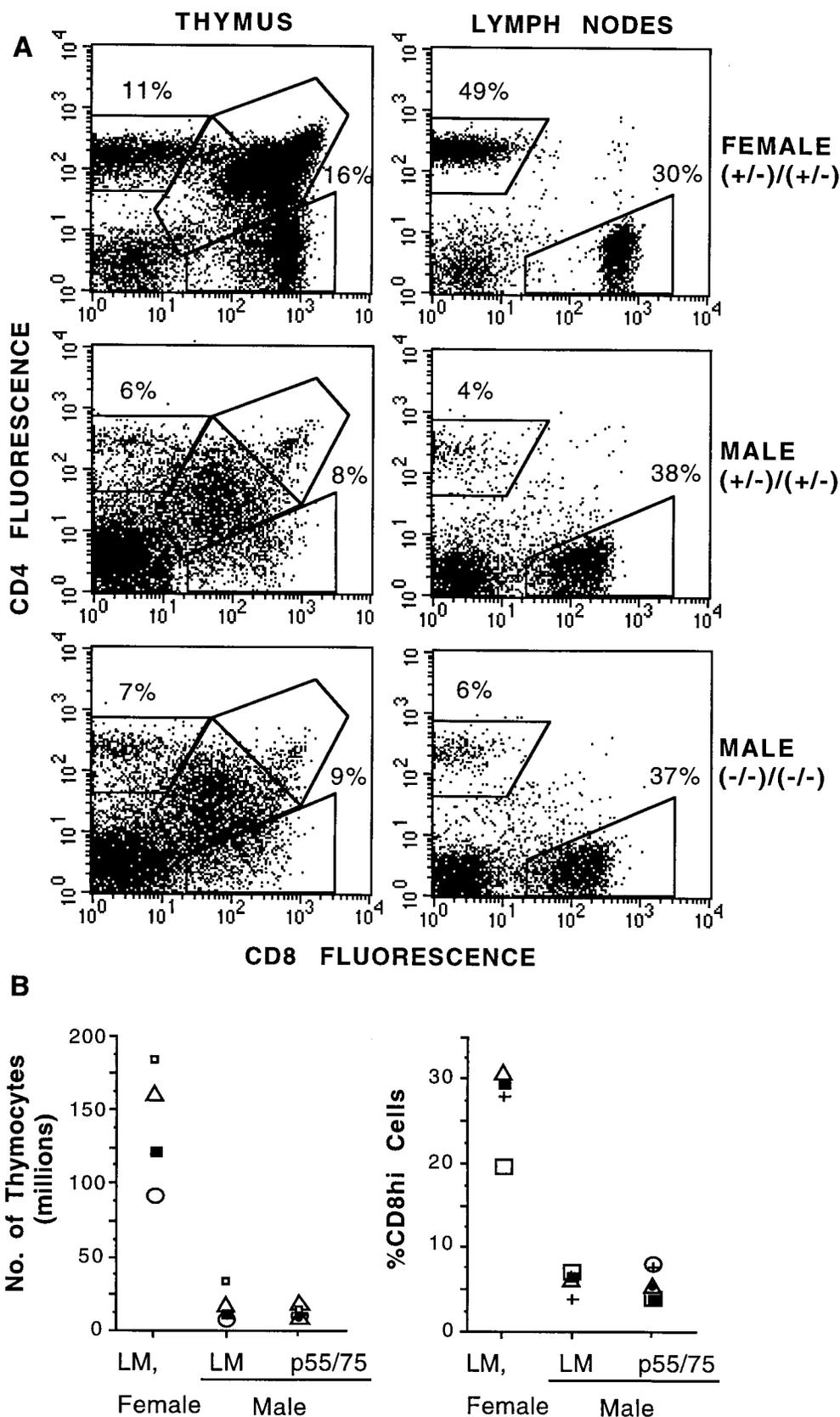


**FIGURE 10.** In vivo Ag-induced negative selection by MHC class II is normal in p55/75-deficient mice. Thymocytes and splenocytes were isolated from either p55/75-deficient mice or their p75-deficient LM expressing the AND TCR transgene and H-2<sup>b</sup> or H-2<sup>b/s</sup>. The cells were analyzed by flow cytometry for their CD4/8 expression pattern and live cell recovery. Shown are the percentages of CD4<sup>+</sup>, DP<sup>high</sup>, and DP<sup>dull</sup> populations for each genetic background, and this experiment is representative of four performed.

deletion of DP thymocytes due to APC in vitro or anti-CD3 injection in vivo (Figs. 2–6). These results have led us to question the physiologic relevance of both the anti-CD3-induced and in vitro models of negative selection, models that have been used by ourselves and others to address the fundamental issues surrounding thymic negative selection and self tolerance. The in vitro results could have been misleading for several reasons, including the use of high concentrations of TNF or the fibroblast APC. Perhaps in vitro models of negative selection that use dendritic cells would be more likely to yield results that correlate with the Ag-induced in vivo models. Unfortunately, until recently (79) pure thymic dendritic cells were difficult to obtain, which is why few investigators have used them (80–82). Another complicating factor is that dendritic cells explanted to culture acquire an activated phenotype (83).

Recent data indicate that anti-CD3 treatment of mice may cause a response that is more characteristic of inflammation than of negative selection. Specifically, Lerner et al. (84) found that anti-CD3 induces thymic stromal cell activation and production of inflam-

matory mediators such as IL-1, IFN- $\gamma$ , chemokines, and TNF. Correspondingly, Kishimoto and Sprent (17) found that anti-CD3 caused DP thymocyte deletion in adult, but not in neonatal, mice. They argue that the demise of DP cells in adult mice is probably due to cytokines or steroids that result from activated T cells present in adult, but not neonatal, mice. In agreement with this view, IL-2-deficient mice also display defective anti-CD3-induced thymocyte deletion (85). Such data may explain why TNF and Fas (60) have effects in this model system, since both these proteins can be involved in inflammatory responses (reviewed in Ref. 86). The role of Fas has also been examined in many other systems of negative selection (see Table I), including both class I and class II MHC-mediated models (61, 87–91). Although Fas is highly expressed on DP thymocytes (92), it seems to affect only anti-CD3- or injected antigenic peptide-induced negative selection (60) and, in some cases, H-Y-induced negative selection (93, 94). Even in these systems, however, the effect is only partial. Interestingly, CD30 also affects anti-CD3- and H-Y-induced, but not superantigen-induced, thymocyte deletion (21). Thus, we do not believe that



**FIGURE 11.** In vivo Ag-induced negative selection by MHC class I is normal in p55/75-deficient mice. Thymocytes and lymph nodes cells were isolated from either p55/75-deficient mice or their LM expressing the H-Y TCR-transgene on the H-2<sup>b</sup> background. The cells were analyzed by flow cytometry for their CD4/8 expression pattern and live cell recovery. Shown are the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> populations recovered in each organ (A) and a summary of thymus size and percentage of CD8<sup>hi</sup> cells recovered in the lymph nodes for each genetic background (B). These data are taken from three experiments, including analysis of four female LM, four male LM, and six male p55/75-deficient mice 4 to 12 wk of age. LM consisted of either p55<sup>+/-</sup>/p75<sup>+/-</sup> or p55<sup>+/-</sup>/p75<sup>-/-</sup> mice, as we found no differences in positive or negative selection of the H-Y TCR in p75-deficient mice (data not shown).

the current evidence supports an important role for Fas, CD30, or TNF in class II MHC-mediated negative selection. Data from the H-Y TCR transgenic model, however, indicate that Fas and CD30, but not TNF, may play partial roles in class I MHC-mediated negative selection.

In contrast, the CD40/CD40L interaction appears to be required in several models of class II MHC-mediated negative selection (20). As discussed above, we believe that this interaction is important to up-regulate costimulatory molecules on thymic APC (20, 58). In this model, negative selection would be caused by the sum total of stimulation received through the TCR and perhaps several costimulatory molecules that are up-regulated on dendritic cells. This hypothesis may explain why examination of the role of any one costimulatory molecule in negative selection, e.g., CD28, gives conflicting results. This model may also afford insight into the mechanism by which central tolerance contributes to the avoidance of autoimmunity. For example, it has been shown that dendritic cells are often the primary APC for naive CD4<sup>+</sup> T cells in the periphery (95–97). Correspondingly, an early event in Ag presentation is CD40-induced up-regulation of costimulatory molecules on APC (reviewed in Ref. 22). Thus, to avoid autoimmunity, naive T cells need to have already surveyed the baseline avidity of the costimulatory molecules on dendritic cells (98). According to our hypothesis, they would use the CD40/CD40L interaction during thymic negative selection to accomplish this survey.

One wonders, of course, why TNF is constitutively expressed in the thymus, when it does not appear to be required for thymic development (99) or for positive and negative selection. One role of TNF may be to increase the phagocytic capacity of thymic macrophages, as it does in the periphery (100, 101). Such a role for TNF might also explain its effect in anti-CD3-induced negative selection. Apoptotic cells are normally rare in the thymus, and it is only when the macrophages are overwhelmed that these cells can be seen in significant numbers (68, 102, 103). Perhaps in TNFR-deficient mice, the capacity of macrophages to ingest dying cells is partially compromised, resulting in an increased number of thymocytes in the anti-CD3-induced model of negative selection. Finally, it is formally possible that TNF could play a role in other systems of class I or II MHC-mediated thymocyte deletion.

In summary, although these data suggest that TNF does not play a role in negative selection mediated by Ags bound to class I or class II MHC, the results do provide valuable insight into several current models of negative selection and the roles of TNFR family members in this process.

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