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CD27 Cooperates with the Pre-T Cell Receptor in the Regulation of Murine T Cell Development

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Summary

CD27 is a lymphocyte-specific member of the TNF receptor family and has a TNF-related transmembrane ligand, CD70. The CD27/CD70 receptor-ligand pair cooperates with the TCR in the regulation of the peripheral T cell response. The study presented here reveals that CD27 may play a similar role in thymic pre-T cell development. We have previously cloned the cDNA encoding murine CD27, prepared specific mAbs and observed that murine CD27 is expressed on virtually all thymocytes, with the exception of a subpopulation of $CD4^-8^-$ precursor T cells. It is shown here that induction of murine CD27 expression occurs at the transition from the $CD4^-8^-25^+$ to the $CD4^-8^-25^-$ precursor T cell stage and is regulated by the pre-TCR. Therefore, we investigated whether CD27 contributes to pre-TCR–mediated thymocyte development. Pre-TCR function was mimicked by the induction of CD3 signaling in thymocytes of recombination activating gene (RAG)–deficient mice. This in vivo anti-CD3ϵ mAb treatment induces an about fifty fold numerical expansion of $CD4^-8^-25^+$ thymocytes and their differentiation to the $CD4^+8^+25^-$ stage. Co-injection of anti-CD27 mAb inhibited the CD3-mediated expansion and differentiation of the $CD4^-8^-25^+$ precursor population. Also, injection of anti-CD27 mAb in TCR$\alpha^{-/-}$ mutant mice led to a reduction in the absolute number of $CD4^+8^+25^-$ thymocytes. We present evidence that in these in vivo systems, anti-CD27 mAb inhibits CD27-ligand interaction. Therefore, we conclude that CD27 may contribute to normal murine T cell development by synergizing with the pre-TCR–mediated signal.

In the murine system, recent thymic immigrants are characterized by high cell surface levels of the CD44 molecule and lack of CD4 and CD8. Upon maturation, thymocytes acquire the CD25 marker and subsequently lose CD44 expression (reviewed in reference 1). The recombination activating gene (RAG)[1] products, which catalyze TCR gene rearrangement, are crucial for further thymocyte development: elimination of the RAG-1 or RAG-2 gene results in lack of TCR protein expression and a consequent differentiation arrest at the $CD44^-$, $CD4^-8^-25^+$ stage (2, 3). Introduction of a TCRβ transgene into RAG-deficient mutant mice allows the generation of a wild-type sized $CD44^-$, $CD4^+8^+25^-$ thymocyte population (4, 5). Precursor T cell proliferation and differentiation at this stage are mediated by the pre-TCR complex, which consists of the TCRβ chain, the pTα chain and CD3 components (6, 7). Pre-TCR function can be mimicked by triggering the CD3 components expressed on RAG-deficient thymocytes with specific mAb. This treatment induces the generation of the $CD4^+8^+25^-$ thymic compartment (8–10).

While the pre-TCR is required for early thymic development, other molecules present on thymocytes and stromal cells are expected to contribute to the maturation process: cytokines and cytokine receptors controlling proliferation and differentiation, as well as molecules affecting cell survival. Members of the TNF receptor family have been implicated in the regulation of proliferation and differentiation of lymphoid cells (reviewed in reference 11 and 12). These receptors have membrane-bound or soluble TNF-related ligands, whose expression is tightly regulated. Within the lymphoid system, the function of TNF receptor family members CD40 and APO-1/fas (CD95) has been most extensively studied. CD40 has been detected on some stromal cells and B cells in the human thymus (13). Although $CD40^{-/-}$ (14) or CD40 $ligand^{-/-}$ (15) mutant mice have no obvious defects in T cell maturation, treatment of mice with anti-CD40 ligand mAb influences thymic selection (16). CD95 is essential for the deletion of periph-

[1] *Abbreviation used in this paper:* RAG, recombination activating gene.

Published August 1, 1996

eral T cells after an immune response (17, 18). However, CD95 mutant *lpr* mice or CD95 ligand mutant *gld* mice have no defects in the deletion of auto-reactive thymocytes (18, 19). Nevertheless, in vitro triggering of CD95 with antibodies can induce cell death in $CD4^+8^+$ thymocytes (20, 21).

The lymphocyte-specific members of the TNF receptor family CD27 (22), CD30 (23), 4-1BB (24) and OX40 (25) have been shown to affect the outcome of TCR- and/or B cell receptor-induced signal transduction (26–28). CD27 is expressed on most peripheral T cells in human (26) and mouse (29). TCR triggering on human peripheral T cells results in an about fourfold increase in CD27 expression within a few days (26). Murine CD27 is not subject to such dramatic upregulation; it is already expressed at relatively high levels on resting T cells (29). Anti-CD27 mAb enhance TCR-induced proliferation of both human (26) and murine (29) peripheral T cells. The antibodies most likely induce CD27 function, since interaction of CD27 with its ligand (CD70) on transfected cells has comparable effects: it enhances TCR-induced proliferation of human peripheral blood T cells and their differentiation into effector cells (30–32). It remains to be established by which mechanism CD27 contributes to peripheral T cell expansion and differentiation.

We have previously isolated the cDNA encoding murine CD27 (33), generated specific antibodies to murine CD27 in Armenian hamsters and found that these mAb, upon cross-linking by second step reagent, mimic the action of the CD27 ligand (29). An inventory of CD27 expression in the mouse showed that virtually all thymocytes express CD27, with the exception of a subset of $CD4^-8^-$ cells (29). In this paper, we focus on the expression and function of CD27 in murine thymocyte development and provide evidence that CD27 cooperates with the pre-TCR in the induction of thymocyte expansion and differentiation.

Materials and Methods

Mice. Wild-type mice were from the C57BL/6, 129/Ola or BALB/C strains and used at 4–8 weeks of age. The RAG-$1^{-/-}$ (2) and the TCR$\alpha^{-/-}$ (5) mutant mice were on a mixed background of 129/Ola and SV; both types of mice were 4–6 weeks of age when used.

Antibodies. Anti-mouse mAb used in this study were: anti-CD27, LG.3A10 (29); anti-CD3ϵ, 145.2C11 (34) and 500A2 (35); anti-TCR$\gamma\delta$, GL-3 (36); anti-F_cRIII and -$F_c\gamma$RIIb, 2.4G2 (37); anti-CD4, RM4-5; anti-CD8, 53-6.7; anti-CD25, 3C7; anti-CD45, 30 F11.1 (all from PharMingen, San Diego, CA), and 5.9 (Ossendorp, F., unpublished results). For generation of the 5.9 mAb, an Armenian hamster was immunized with murine C57BL/6 thymocytes. Hybridomas were generated as described (29) and selected for reactivity with murine thymocytes by immunofluorescence analysis. The 5.9 mAb reacts with $CD4^-8^-$ thymocytes, and to a lesser degree with $CD4^+8^+$ thymocytes. It does not show detectable staining of $CD4^+$ or $CD8^+$ single positive thymocyte populations or peripheral T lymphocytes. The antibody does react with splenic B lymphocytes and subpopulations of bone marrow cells. Immunoprecipitation of the 5.9 antigen revealed a 20–23-kD protein, that migrates at 16–18-kD under non-reducing conditions and is sensitive to digestion with PI-PLC, indicating that it is anchored in the plasma membrane by a phospholipid anchor (Ossendorp, F., unpublished results). The collected results suggest that the 5.9 mAb recognizes a novel protein related to the products of the Ly-6 gene family (38). Normal hamster IgG was from Jackson Immuno–Research Laboratories Inc. (West Grove, PA). Antibodies used for in vivo experiments were purified from tissue culture supernatant by HPLC using an ABX column (Baker, Philipsburg, NJ). Purified antibodies were biotinylated or FITC-labeled according to standard procedures and dialyzed against PBS.

In Vivo Antibody Treatment. Mice were injected intravenously with 50 μg of appropriate purified antibody or a combination of antibodies at 50 μg each in a final volume of 300 μl of PBS. Mice were killed 1–5 d after antibody injection as indicated per experiment, thymus and spleen were removed and disrupted into single cells, which were counted and analyzed by immunofluorescence. In the in vivo antibody cross-linking studies, animals were first injected with 50 μg biotinylated anti-CD27 mAb or hamster IgG. One d later, 50 μg unconjugated anti-CD3 mAb (145.2C11) as well as 50 μg biotinylated anti-CD27 mAb or hamster IgG were injected, followed 17–19 h later by 50 μg streptavidin. Mice were killed 3 d after injection with anti-CD3 mAb and cells were analyzed as described above.

Immunofluorescence Analysis. Cell suspensions were washed in culture medium and seeded in 96-well round-bottom plates at 3×10^5 cells per well. Fc receptors were blocked by incubation with 2.4G2 mAb, cells were washed and incubated with the relevant antibodies, followed by second step reagents. In between incubations, cells were washed in PBS with 0.5% BSA and 0.02% sodium azide. For two parameter analysis, cells were incubated with FITC-conjugated anti-CD8 or anti-CD27 mAb and PE-conjugated anti-CD4 or anti-CD25 mAb. For three parameter analysis, cells were incubated with biotinylated anti-CD4 and anti-CD8 mAb, PE-conjugated anti-CD25 mAb and FITC-conjugated anti-CD27 mAb, followed by incubation with streptavidin-conjugated Tricolor (Caltag, San Francisco, CA). Cells were analyzed on a FACScan® using Lysys software (Becton Dickinson, Mountain View, CA). Live cells were gated on the basis of forward and side scatter. For analysis of the $CD4^-8^-$ thymocyte population, cells were gated on the basis of negative staining with Tricolor.

Immunohistology. Thymuses were derived from 6–10-wk-old BALB/C mice and frozen tissue sections were prepared and stained as described (39) with FITC-conjugated anti-CD3 mAb (500A2) or anti-CD27 mAb. Micrographs were recorded with a Leitz Orthoplan, equipped with a Leitz Ploemopak illumination system, a 100 W HBO mercury lamp and a ProbeMaster (Perceptive Scientific International, Chester, UK), consisting of an Apple Quadra computer, color monitor, cooled CCD 24-bit camera, GPIB interface board, optical disk, and a microcope-adjusted fluorescent filter cube.

Results

CD27 Expression Is Upregulated at the Transition from the $CD44^-$, $CD25^+$ to the $CD44^-$, $CD25^-$ Pre-T Cell Stage. When anti-CD27 mAb LG.3A10 was used for immunofluorescence analysis of frozen thymus sections, virtually all thymocytes in both cortex and medulla stained with equal

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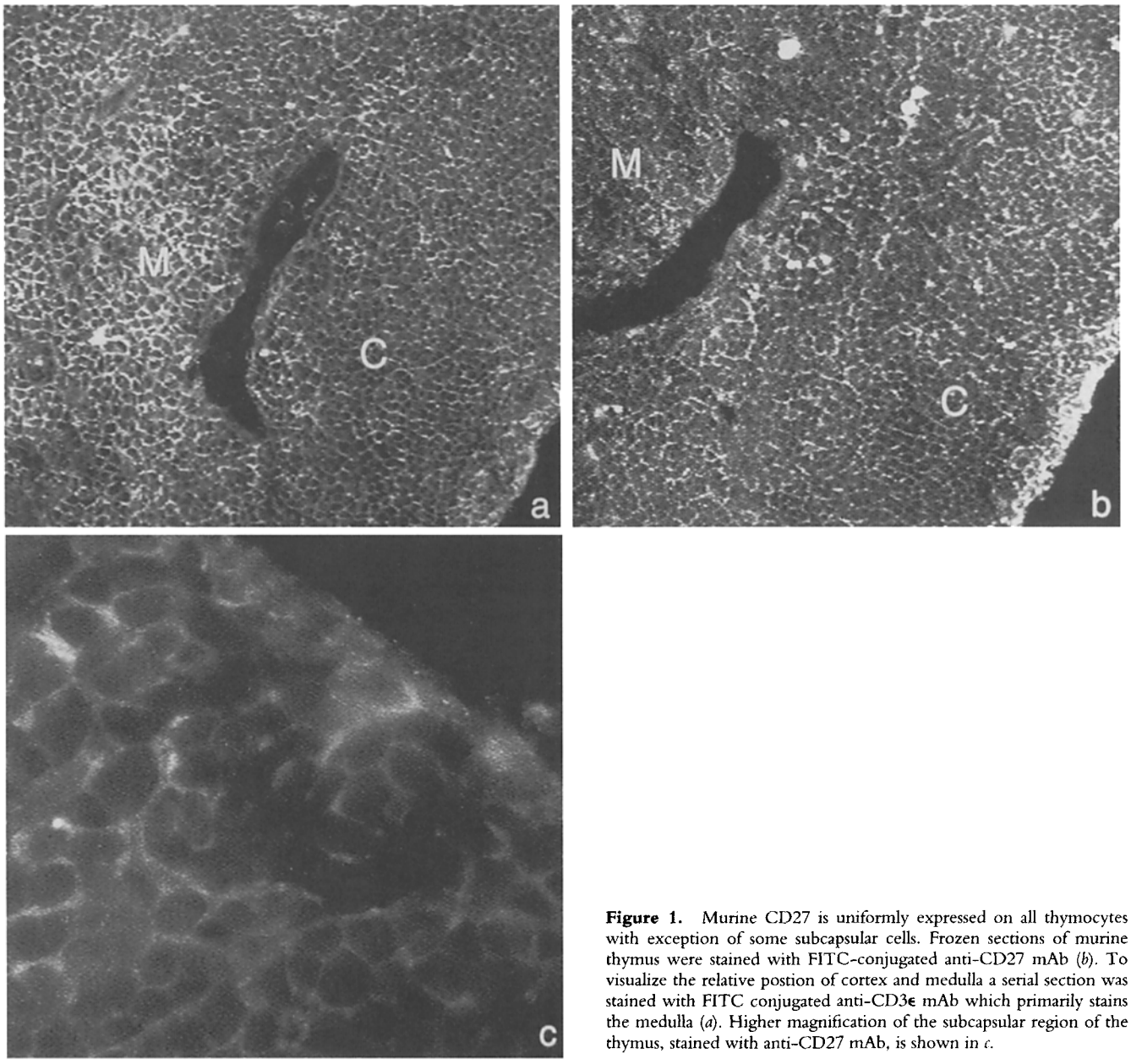

Figure 1. Murine CD27 is uniformly expressed on all thymocytes with exception of some subcapsular cells. Frozen sections of murine thymus were stained with FITC-conjugated anti-CD27 mAb (*b*). To visualize the relative postion of cortex and medulla a serial section was stained with FITC conjugated anti-CD3ε mAb which primarily stains the medulla (*a*). Higher magnification of the subcapsular region of the thymus, stained with anti-CD27 mAb, is shown in *c*.

intensity (Fig. 1 *B*). The location of cortex and medulla was determined by staining of a serial section with anti-CD3 mAb as shown in Fig. 1 *A*. However, a higher magnification revealed heterogeneity in CD27 expression among subcapsular lymphoblasts in the thymic cortex. While the majority of cells at this location is clearly CD27 positive, a minority expresses CD27 at low density or appears negative. Such cells occur in small foci under the capsule (Fig. 1 *C*).

The subcapsular localization of $CD27^{low}$ thymocytes suggested that these cells are recent thymic immigrants. To investigate whether $CD27^{low}$ and $CD27^{high}$ thymocytes could be discriminated in terms of maturity, we compared CD27 expression levels on $CD4^-8^-$ thymocytes of wild-type and RAG-$1^{-/-}$ mutant mice. In RAG-$1^{-/-}$ mice, differentiation of $CD4^-8^-$ thymocytes is arrested at the $CD44^-$, $CD25^+$ stage. In wild-type mice, the $CD4^-8^-$ population contains a significant amount of $CD44^-$, $CD25^-$ cells, which result from pre-TCR–mediated differentiation (2). Immunofluorescence analysis indicated that all $CD4^-8^-$ thymocytes of RAG-$1^{-/-}$ mice are $CD27^{low}$ (Fig. 2). In wild-type mice, the $CD4^-8^-$ population contains not only these $CD27^{low}$ cells, but also a subset of $CD27^{high}$ cells (Fig. 2). Concomitant analysis of the expression of CD44 (not shown) and CD25 (Fig. 3), allowed us to define the $CD27^{high}$ population as $CD44^-$, $CD25^-$. These results indicate that CD27 expression is upregulated upon differentiation of the $CD4^-8^-$ population from the $CD44^-$, $CD25^+$ to the $CD44^-$, $CD25^-$ stage. Throughout subsequent thymocyte development, CD27 remains uniformly expressed at this relatively high density (Fig. 2).

Published August 1, 1996

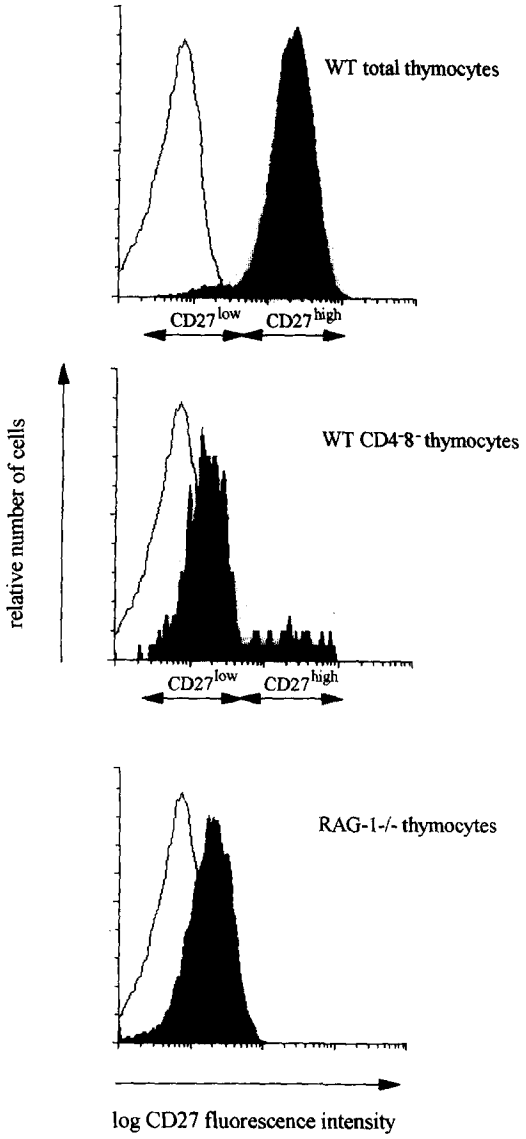

Figure 2. Cell surface expression of CD27 is upregulated on $CD4^-8^-$ thymocytes in wild type, but not in RAG-$1^{-/-}$ mice. Thymocyte suspensions of wild-type (*WT*) and RAG-$1^{-/-}$ mice were stained with anti-CD4-PE, anti-CD8-biotin plus streptavidin-conjugated Tricolor and anti-CD27-FITC mAb. CD27 staining (*black*) is compared to staining with irrelevant FITC-conjugated antibody (*white*). For analysis of wild-type total thymocytes and RAG-$1^{-/-}$ thymocytes, all viable cells were gated. For analysis of CD27 expression on $CD4^-8^-$ wild-type thymocytes, cells were gated for lack of CD4 and CD8 expression.

CD3 Signaling Results in Increased CD27 Expression on $CD4^-8^-$ Pre-T Cells. The developmental transition of $CD44^-$, $CD4^-8^-25^+$ thymocytes to the $CD44^-$, $CD4^-8^-25^-$ stage is controlled by the pre-TCR (5, 40). To investigate whether pre-TCR signaling may induce the upregulation of CD27 expression, we used an in vivo thymocyte maturation system. It has been shown previously that injection of anti-CD3ε mAb into RAG-$1^{-/-}$ mice induces the differentiation of $CD4^-8^-25^+$ thymocytes to the $CD4^+8^+25^-$ stage and an ~50-fold increase in thymocyte numbers (9, 10). RAG-$1^{-/-}$ thymocytes express very low levels of CD3 molecules at the plasma membrane. Anti-CD3ε mAb binds to these molecules and presumably induces their signaling activity, which mimicks the normal function of the pre-TCR. As described above, $CD4^-8^-$ thymocytes of untreated RAG-$1^{-/-}$ mice are uniformly $CD27^{low}$, while the great majority expresses CD25 at high levels. Anti-CD3 mAb treatment gave rise to a subset of $CD4^-8^-$ cells which were $CD27^{high}$ and lacked CD25. In fact, the staining profile of the RAG-$1^{-/-}$ $CD4^-8^-$ population after anti-CD3ε mAb treatment was comparable to that of wild-type $CD4^-8^-$ thymocytes (Fig. 3). This result is consistent with the notion that CD27 upregulation is part of the differentiation program inititated by pre-TCR signaling.

Anti-CD27 mAb Inhibits CD3-mediated Pre-T Cell Expansion and Differentiation. Since expression of CD27 increases at the transition of $CD4^-8^-25^+$ cells to the $CD4^+8^+25^-$ stage, we were interested to test whether CD27 exerts a function at this point in thymocyte development. To assess whether CD27 plays a role in the expansion and/or differentiation of $CD4^-8^-25^+$ thymocytes induced by pre-TCR signaling, we again made use of RAG-$1^{-/-}$ mice. The mice were injected intravenously with anti-CD27 mAb alone, or combined with anti-CD3ε mAb. After 5 d, when precursor cells appeared to be maximally expanded to form the $CD4^+8^+$ compartment, the animals were sacrificed and the total number of thymocytes, as well as their differentiation state was determined. As published previously (8–10), treatment of RAG-1-deficient thymocytes with anti-CD3ε mAb reproducibly induced their expansion from a few million to about 100×10^6 cells (Fig. 4 *A*). Strikingly, this effect of anti-CD3ε mAb was profoundly impeded by co-injection with anti-CD27, but not control hamster IgG antibody (Fig. 4 *A*). Injection of anti-CD27 mAb alone, or control hamster IgG did not affect thymocyte numbers (Fig. 4 *A*).

The upper panel of Fig. 4 *B* shows that anti-CD3ε mAb induced the generation of the $CD4^+8^+$ thymocyte compartment. Co-injection of anti-CD27 mAb resulted in a specific decrease in the absolute number of $CD4^+8^+$ cells, whereas the size of the $CD4^-8^-$ compartment was not affected. Apparently, anti-CD27 mAb impedes both CD3-mediated thymocyte expansion and differentiation to the $CD4^+8^+$ stage. The effect on thymocyte differentiation is also illustrated by the incomplete downregulation of CD25 expression in mice treated with a combination of anti-CD3ε and anti-CD27 mAbs, as compared to mice treated with anti-CD3ε mAb alone (Fig. 4 *B*, *lower panel*). Anti-CD27 mAb interfered with the CD3-mediated downregulation of CD25 already in the first two days after antibody injection, before the generation of $CD4^+8^+$ cells (data not shown). In conclusion, injection of anti-CD27 mAb into RAG-$1^{-/-}$ mice severely impedes the CD3-mediated expansion and differentiation of $CD4^-8^-25^+$ thymocytes to the $CD4^+8^+25^-$ stage.

Anti-CD27 mAb Inhibits pre-T Cell Expansion in TCR$\alpha^{-/-}$ Mice. As shown above, pre-TCR signaling does not take place in RAG-deficient mice, but can be mimicked by anti-CD3 mAb. To test the effect of anti-CD27 mAb on

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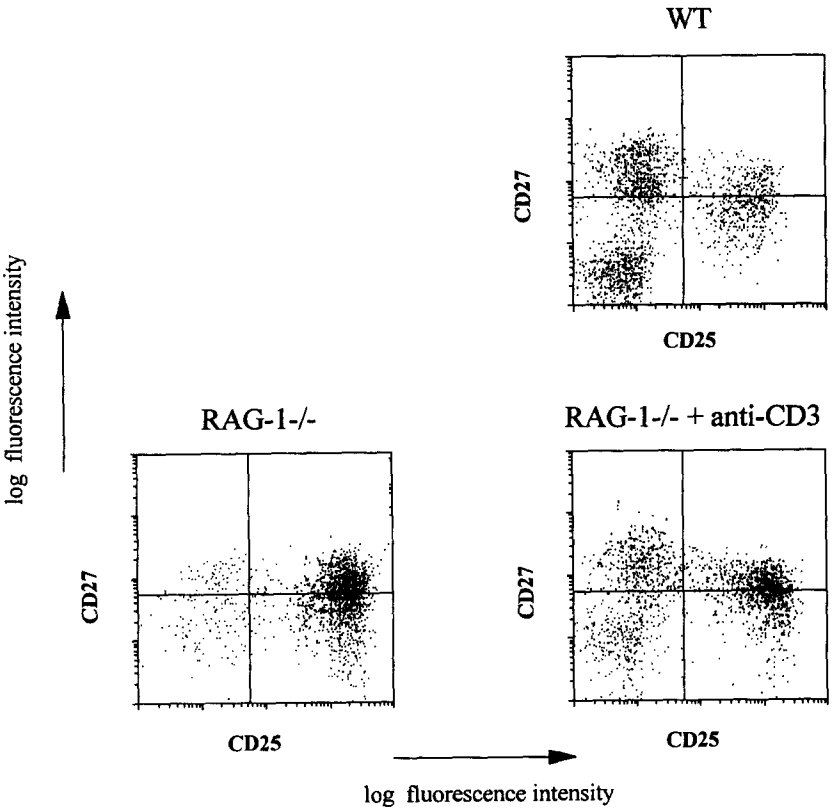

Figure 3. $CD27^{low}$ $CD4^-8^-$ thymocytes are $CD25^+$; mimicking the pre-TCR signal in RAG-$1^{-/-}$ mice induces differentiation of $CD4^-8^-$ thymocytes to the $CD27^{high}$, $CD25^-$ stage. Thymocyte suspensions of wild-type (*WT*) mice, RAG-$1^{-/-}$ mice and RAG-$1^{-/-}$ mice treated in vivo with 50 μg of anti-CD3 mAb for 5 d, were stained with biotinylated anti-CD4 and anti-CD8 mAbs plus streptavidin-conjugated Tricolor, anti-CD25-PE and anti-CD27-FITC. The $CD4^-8^-$ population was gated on the basis of lack of Tricolor staining and analyzed for CD25 and CD27 expression.

normal pre-TCR–regulated thymic development, we made use of TCR$\alpha^{-/-}$ mice, which can form a pre-TCR, but not TCR$\alpha\beta$. As a consequence, $CD4^+8^+$ thymocytes develop normally, but maturation to the $CD4^+$ or $CD8^+$ single positive stage cannot take place (5, 41). This experimental system ensures that injection of anti-CD27 mAb does not influence the process of thymic selection, which would complicate interpretation of the results.

TCR$\alpha^{-/-}$ mice were injected with anti-CD27 mAb and analyzed at 1, 2, and 3 d after injection. Thymocyte numbers were reduced about three to fourfold after 3 d of treatment with anti-CD27 mAb, whereas control hamster IgG had no effect (Fig. 5). We did not observe an increase in the percentage of $CD25^+$ thymocytes upon anti-CD27 mAb treatment, presumably because the background of $CD4^+8^+25^-$ cells in the TCR$\alpha^{-/-}$ thymus is too large. Therefore, we could not assess whether antibody treatment affected thymocyte differentiation. However, it is clear that anti-CD27 mAb treatment impeded normal pre-TCR–driven thymocyte expansion.

The Inhibition of CD3-mediated pre-T Cell Development by Anti-CD27 mAb Is Specific. To address the question whether binding of any antibody, irrespective of its specificity, to the developing thymocytes would exert an inhibitory effect, the experiments outlined above were repeated using the 5.9 mAb, which recognizes a Ly-6 like molecule. Like anti-CD27 mAb, 5.9 mAb is of Armenian hamster origin and binds to both $CD4^-8^-$ and $CD4^+8^+$ thymocytes of wild-type (Ossendorp, F., unpublished observation), RAG-$1^{-/-}$ and TCR$\alpha^{-/-}$ mice (Fig. 6 *A*).

The 5.9 mAb was injected into RAG-$1^{-/-}$ mice, either alone, or in combination with anti-CD3 mAb and its effects were compared with those of anti-CD27 mAb in the same experiment. Three days after antibody injection, mice were sacrificed and thymocyte number and differentiation stage were determined. At this timepoint, the effects of anti-CD3 mAb on thymocyte development are not yet maximal, but this shorter time period was chosen to exclude differential effects of 5.9 and anti-CD27 mAbs due to a possible difference in antibody half life. In contrast to anti-CD27 mAb, 5.9 mAb had no significant effect on CD3-mediated thymocyte expansion (Fig. 6 *B*), or differentiation (data not shown), while the antibody was present on the cells according to FACS® analysis. In addition, injection of 5.9 mAb in TCR$\alpha^{-/-}$ mice did not affect thymocyte numbers (data not shown). We conclude, therefore, that the effects of anti-CD27 mAb on thymocyte development are due to binding of the antibody to the CD27 molecule and cannot be attributed to non-specific (e.g., phagocytic) mechanisms activated by antibody coating of the thymocytes.

Cross-linking of Anti-CD27 mAb In Vivo Restores CD3-mediated Pre-T Cell Expansion and Differentiation to Normal Levels. The specific inhibition of CD3-mediated thymocyte development by anti-CD27 mAb can be explained in two ways: (*a*) the antibody induces CD27 receptor function or (*b*) the antibody inhibits the naturally occurring interaction between CD27 and its ligand and therewith inhibits receptor function. In mature T cells, the LG.3A10 mAb employed here can induce CD27 function, but only

Published August 1, 1996

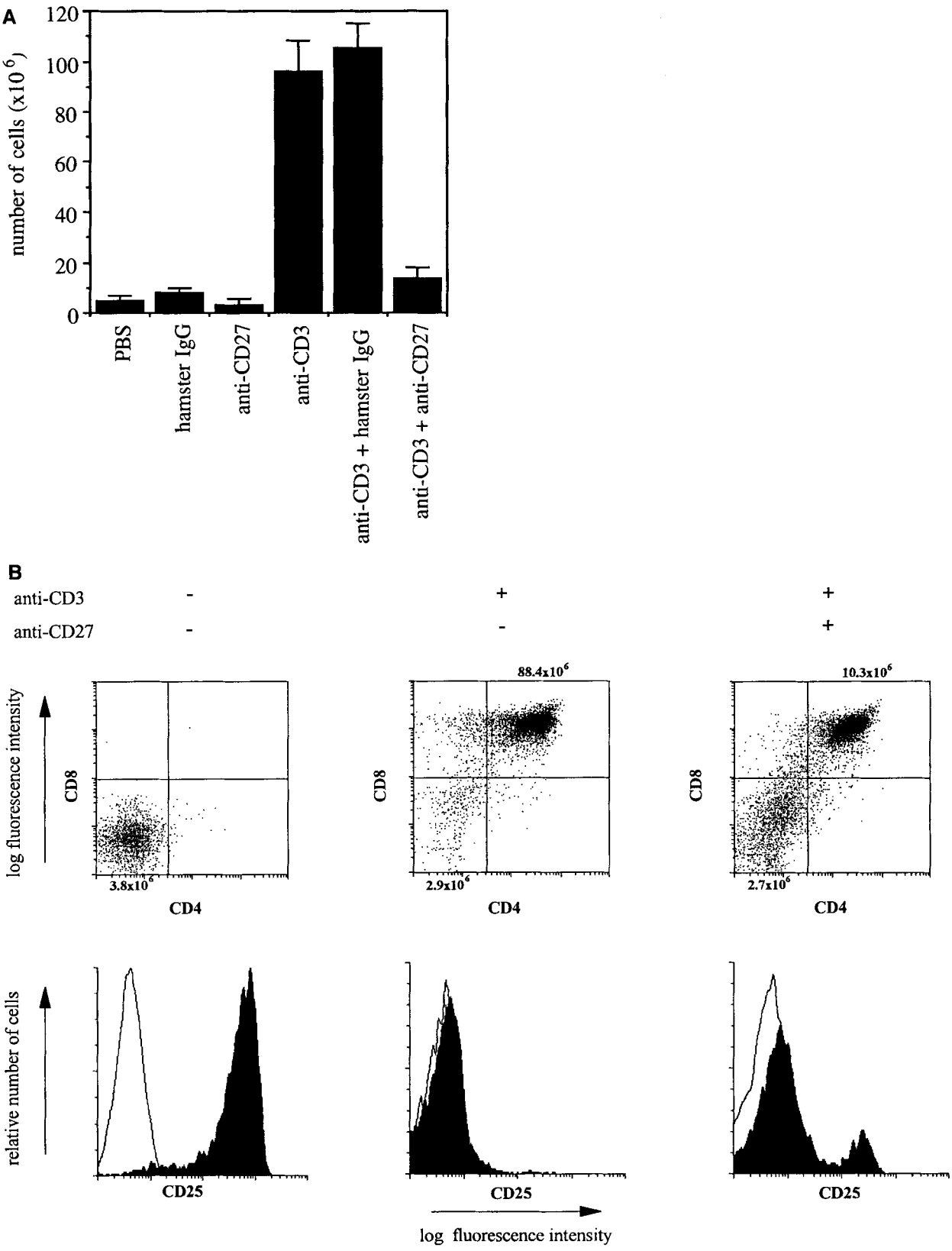

Figure 4. (*A*) Anti-CD27 mAb inhibits CD3-mediated thymocyte expansion in RAG-$1^{-/-}$ mice. RAG-$1^{-/-}$ mice were injected with PBS or with 50 μg of antibody or combination of antibodies as indicated. 5 d later, the total number of cells per thymus was determined. Shown are the means and standard deviations of four separate experiments. (*B*) Anti-CD27 mAb inhibits CD3-mediated thymocyte differentiation in RAG-$1^{-/-}$ mice. RAG-$1^{-/-}$ mice were treated with PBS, 50 μg anti-CD3 mAb alone, or with a combination of anti-CD3- and anti-CD27 mAbs as outlined in *A*. 5 d after injection, thymocytes were isolated and stained either with anti-CD4-PE and anti-CD8-FITC as shown in the upper panels, or with anti-CD25-PE (*black*) or negative control antibody (*white*) as shown in the lower panels. The absolute numbers of cells in the $CD4^-8^-$ and $CD4^+8^+$ populations are indicated in the upper panels. These numbers were calculated from the absolute number of total thymocytes.

Published August 1, 1996

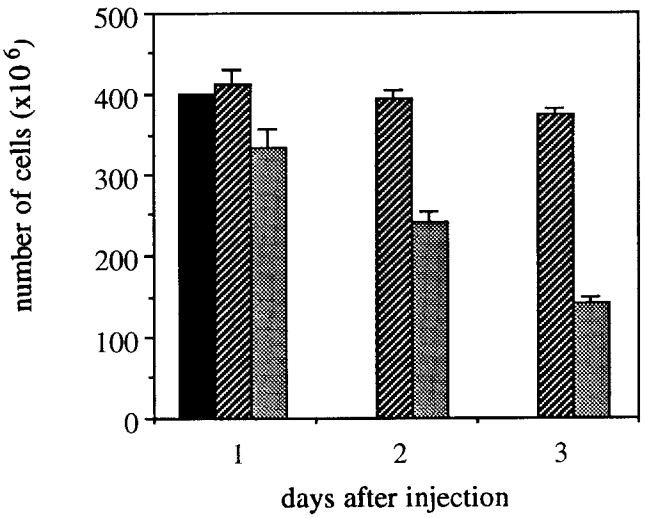

Figure 5. Anti-CD27 mAb inhibits pre-TCR-induced thymocyte expansion in TCR$\alpha^{-/-}$ mice. TCR$\alpha^{-/-}$ mice were injected with PBS, 50 μg hamster IgG, or 50 μg anti-CD27 mAb. Total number of cells per thymus was determined at 1, 2, or 3 d after antibody injection. Shown are the means and standard deviations of two independent experiments. ■, PBS; ▨, hamster IgG; ▩, anti-CD27.

when cross-linked by second step reagent (29). In our in vivo system, no second step reagent was added, but we cannot exclude that the antibody was cross-linked by Fc receptors present on thymic cells. Use of Fab fragments is not an option in this system, since their half life in vivo is very short. It was decided to resolve this issue by comparing the effect of anti-CD27 mAb alone to the effect of anti-CD27 mAb that was deliberately cross-linked by the addition of a second step reagent.

RAG-1$^{-/-}$ mice were injected with biotinylated anti-CD27 or control antibody, followed by unconjugated anti-CD3 mAb. Subsequently, streptavidin was injected to cross-link the anti-CD27 mAb. 3 d after injection of anti-CD3 mAb, the thymocyte compartment was analyzed. This proved optimal for reading out the effects of biotinylated anti-CD27 mAb, which has a shorter half life in vivo than the unconjugated antibody. Like the unconjugated antibody, biotinylated anti-CD27 mAb inhibited the CD3-mediated increase in thymocyte numbers (Fig. 7 *A*). In contrast, upon cross-linking of anti-CD27 mAb with streptavidin, no inhibition of the CD3-induced thymocyte expansion was observed. Immunofluorescence analysis indicated that in both cases similar amounts of CD27 molecules were occupied by specific antibody, indicating that streptavidin-binding did not affect the degree of receptor internalization (results not shown). Similarly, cross-linked anti-CD27 mAb did not affect CD3-induced thymocyte differentiation, as read out by downregulation of the CD25 molecule (Fig. 7 *B*). Apparently, induction of CD27 function by cross-linking of the receptor allows pre-T cell development to occur in response to anti-CD3ε mAb. Therefore, we suggest that non-cross-linked anti-CD27 mAb inhibits CD3-induced pre-T cell development by interfering with a naturally occurring receptor-ligand interaction. When physiological CD27 receptor function is allowed to take place, or when it is induced artificially with cross-linked antibody, CD3-mediated thymocyte proliferation and differentiation proceed normally.

Discussion

The most immature thymocytes in the mouse are contained within the CD4$^-$8$^-$ population, which can be subdivided into CD44$^+$25$^-$, CD44$^+$25$^+$, CD44$^-$25$^+$, and CD44$^-$25$^-$ subsets of increasing maturity (1). Functional TCRβ gene rearrangement and pTα expression (7, 42) first occur in the CD44$^-$25$^+$ population, allowing pre-TCR expression. The transition from the CD44$^-$25$^+$ to the CD44$^-$25$^-$ stage is dependent on the pre-TCR, as evidenced by the phenotype of RAG- (2, 3), TCRβ- (5) and pTα-(40) deficient mice. From our studies, CD27 appears to be a very useful marker for thymocyte maturation in the mouse, since it is not expressed, or only at very low levels, on the CD44$^+$25$^-$ and CD44$^+$25$^+$ populations, but it is found at high levels on the CD44$^-$25$^-$ and more mature thymocyte populations. In other words, the upregulation of CD27 cell surface levels coincides with the induction of pre-TCR expression (7, 42) and function. In fact, we have shown here that mimicking the pre-TCR signal in RAG-1$^{-/-}$ mice induces the upregulation of CD27. CD27 can be considered a differentiation marker in the murine system, since its expression remains high upon further thymic maturation to the CD4$^+$8$^+$ and the mature, single positive stages.

In this paper, we have demonstrated that in the mouse, CD27 can exert a function at the transition from the CD44$^-$25$^+$ stage to the CD44$^-$25$^-$ stage, when the pre-TCR mediates further thymocyte development. Injection of LG.3A10 anti-CD27 mAb in vivo counteracted pre-TCR-induced thymocyte differentiation and expansion. We have obtained similar results using fetal thymic organ cultures from RAG-1$^{-/-}$ mice: thymocyte expansion and differentiation to the CD4$^+$8$^+$ stage, as induced by anti-CD3 mAb, was counteracted by anti-CD27 mAb. For the interpretation of its effect on thymocyte development, it is essential to know whether anti-CD27 mAb inhibits or induces CD27 function in these systems. We know that the LG.3A10 mAb needs to be cross-linked to exert its costimulatory activity on TCR-activated peripheral T cells (29), in line with the multimeric nature of the ligands of the TNF receptor family (11, 12). In absence of cross-linking, the antibody inhibits interaction between CD27 and human CD70, thus abrogating costimulatory activity (J. Nieland, personal communication). Although we cannot assess whether the LG.3A10 mAb was cross-linked by Fc receptors in vivo, deliberate cross-linking of the biotinylated version of this antibody with streptavidin abrogated its inhibitory effect on CD3-mediated thymocyte differentiation and expansion. This implies that induction of CD27 function, which occurs upon cross-linking, cannot counteract the CD3 signal in RAG-1$^{-/-}$ mice. Therefore, it is

Published August 1, 1996

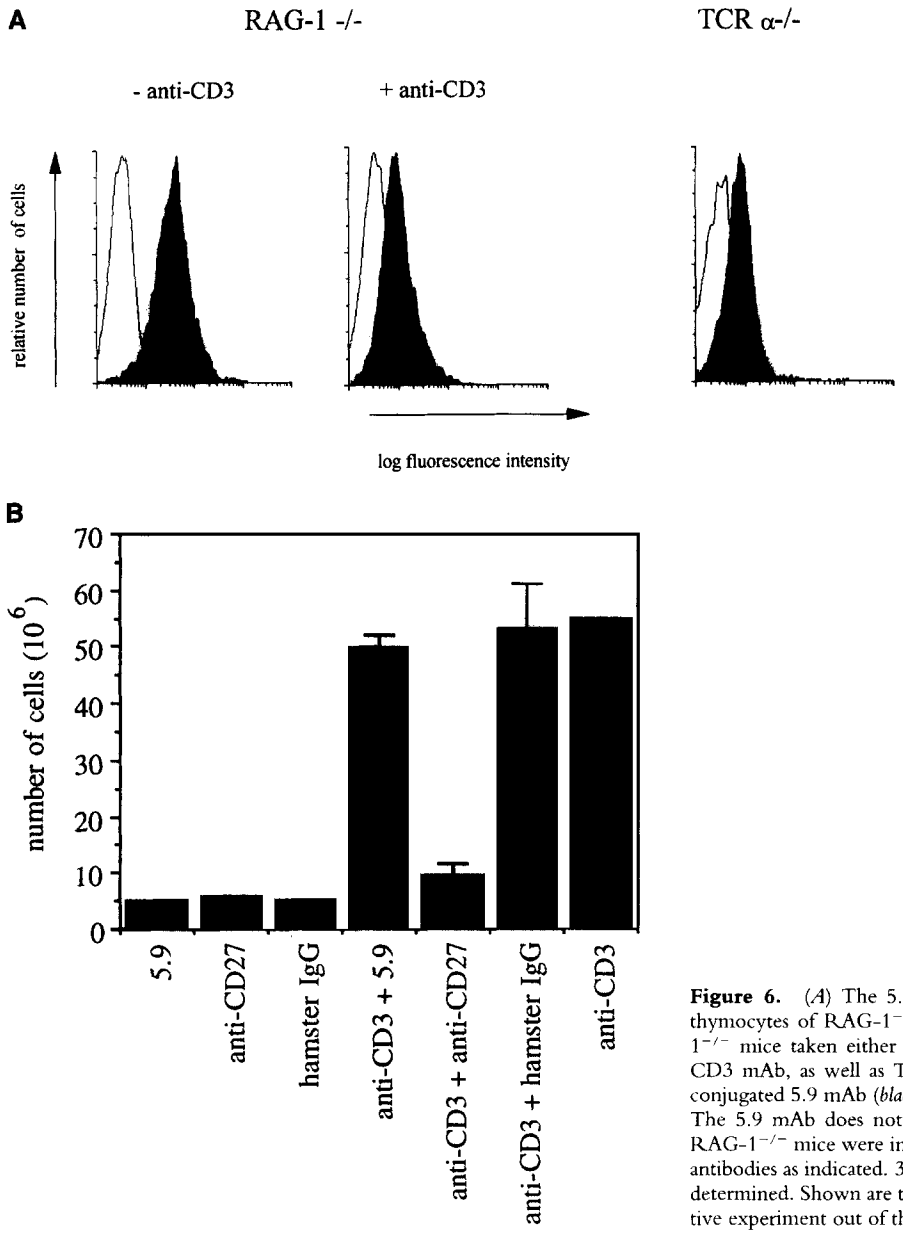

Figure 6. (*A*) The 5.9 mAb recognizes both $CD4^-8^-$ and $CD4^+8^+$ thymocytes of RAG-$1^{-/-}$ and TCR$\alpha^{-/-}$ mice. Thymocytes of RAG-$1^{-/-}$ mice taken either before or after 5 d in vivo treament with anti-CD3 mAb, as well as TCR$\alpha^{-/-}$ thymocytes were stained with FITC-conjugated 5.9 mAb (*black*), or with negative control antibody (*white*). (*B*) The 5.9 mAb does not affect CD3-mediated thymocyte development. RAG-$1^{-/-}$ mice were injected with 50 μg of antibody or combination of antibodies as indicated. 3 d later, the total number of cells per thymus was determined. Shown are the means and standard deviations of a representative experiment out of three experiments performed in duplo.

most likely that non-cross-linked anti-CD27 mAb blocks a physiological CD27-ligand interaction. Our experiments indicate that such an interaction contributes to CD3-mediated thymic development in the RAG-$1^{-/-}$ system and pre-TCR-mediated thymic expansion in TCR$\alpha^{-/-}$ mice. It remains to be established whether CD27 signaling enhances thymocyte expansion by increasing CD3-induced cell proliferation per se or by increasing cell survival. Since thymocytes appear to become very sensitive to apoptotic stimuli at the transition from the $CD44^-25^+$ to the $CD44^+25^+$ differentiation stage (43), enhanced survival of these precursor cells is expected to contribute significantly to the size of the $CD4^+8^+$ population.

Due to the lack of specific reagents, the expression of the CD27 ligand, CD70, has not yet been analyzed in the murine system. CD70 has been detected on occasional stromal cells in the human thymus (44). Therefore, it is conceivable that physiological CD27-CD70 interactions take place during thymic T cell development. However, findings in the human system cannot easily be extrapolated to the murine system, since CD27 protein expression is regulated differently in both species. In human, CD27 is found primarily on mature, medullary thymocytes (26, 45). CD27 is not detectable on $CD4^+8^+$ cortical thymocytes in situ, but inducible in vitro by TCR-mediated activation (45). Also, CD27 expression on peripheral T cells is regulated differently in both species: in the mouse, CD27 cell surface levels are relatively high on resting T cells and increase only moderately upon TCR-mediated activation (29 and data not shown), whereas in human, CD27 levels are low on

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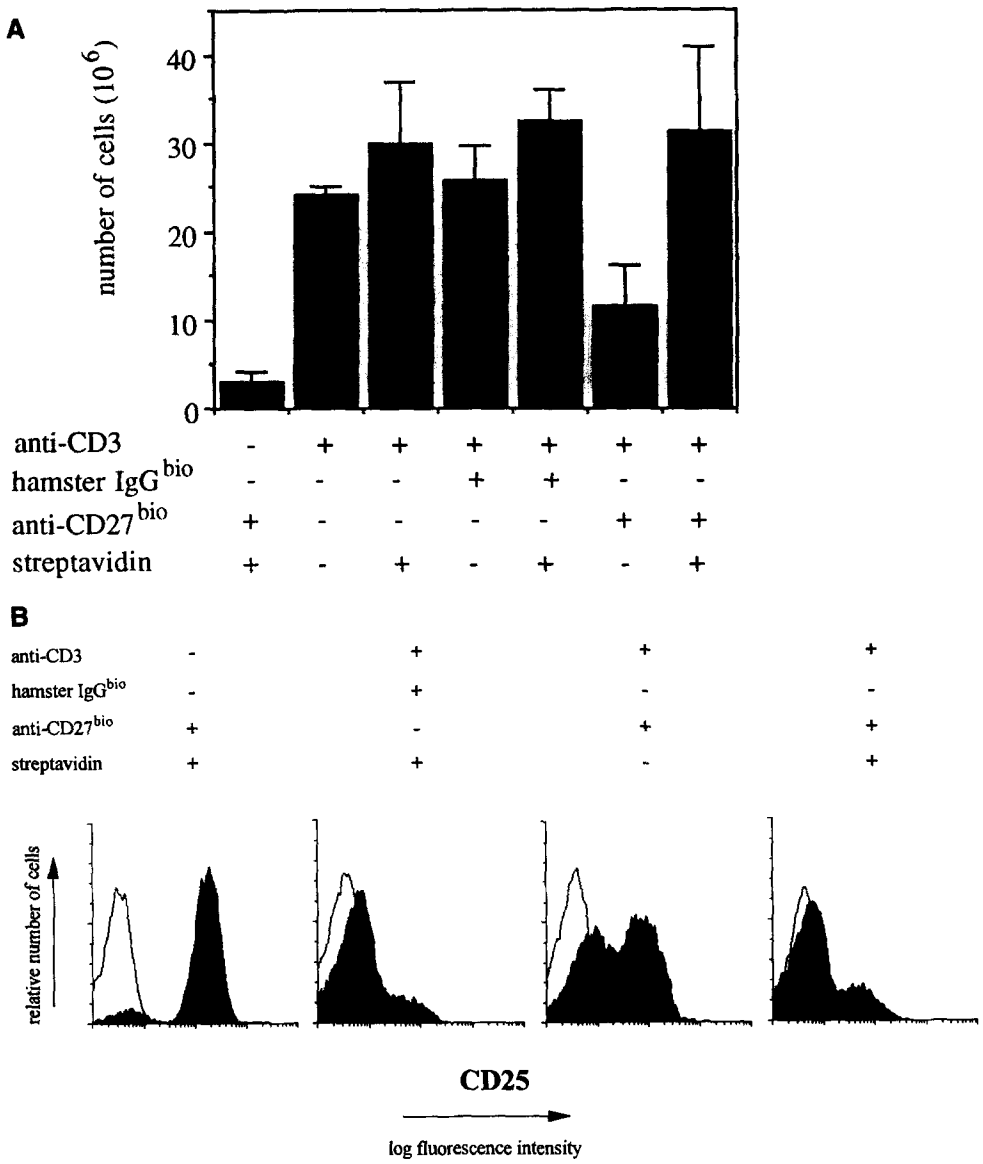

Figure 7. (*A*) Cross-linking of anti-CD27 mAb in vivo restores CD3-mediated pre-T cell expansion. RAG-$1^{-/-}$ mice were pretreated with 50 μg of biotinylated anti-CD27 mAb or control hamster IgG. 1 d later, 50 μg anti-CD3ε mAb was injected with an additional dose of biotinylated anti-CD27 mAb or control hamster IgG, followed 17–19 h later by injection of 50 μg of streptavidin. 3 d after injection of anti-CD3ε mAb, total number of cells per thymus was determined. Shown are the means and standard deviations of two independent experiments. (*B*) Cross-linking of anti-CD27 mAb in vivo restores CD3-mediated pre-T cell differentiation. RAG-$1^{-/-}$ mice were treated with biotinylated anti-CD27 mAb or control hamster IgG and cross-linked with streptavidin as outlined in *A*. 3 d after anti-CD3ε mAb injection, thymocytes were stained with anti-CD25-PE (*black*) or PE-conjugated negative control antibody (*white*).

resting T cells and transiently increase about fivefold upon TCR-mediated cellular activation (26). These data can be reconciled by the statement that once induced, CD27 expression in the mouse is of a rather constitutive nature, while its expression in the human system is more pronouncedly subject to TCR-mediated regulation. Therefore, it cannot be excluded that in the human thymus, the pre-TCR induces CD27 expression on the small transitional population that generates the $CD4^+8^+$ compartment.

According to the data presented in this paper, CD27 would cooperate with the pre-TCR in mediating thymocyte differentiation and expansion, as it cooperates with the TCR in mediating peripheral T cell differentiation and expansion (26, 29, 32). We are currently testing this hypothesis by the generation of $CD27^{-/-}$ mutant mice.

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