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TGFβ1 Inhibits Ca^{2+}-Calcineurin-Mediated Activation in Thymocytes[1]

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Abstract

TGFβ1 is a polypeptide growth modulatory and differentiation factor involved in many biological processes including immune homeostasis and self-tolerance. *Tgfb1* knockout mice die around weaning age due to severe inflammation in most major organ systems, but the mechanism underlying this disease is not understood. In this study we demonstrate that *Tgfb1*$^{-/-}$ $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes are hyperresponsive to receptor-mediated and receptor-independent mitogenic stimulation. A suboptimal concentration of ionomycin in the presence of PMA fully activates *Tgfb1*$^{-/-}$ thymocytes, whereas the inhibitors of Ca^{2+} influx and calcineurin, EGTA and FK506, eliminate the hyperresponsiveness. Hence, the hypersensitivity of *Tgfb1*$^{-/-}$ thymocytes is due to a lowered threshold for Ca^{2+}-dependent activation. Further, we demonstrate that the hypersensitivity of thymocytes results from the absence of TGFβ1 and not from the inflammatory environment because the thymocytes are hyperresponsive in preinflammatory-stage *Tgfb1*$^{-/-}$ mice. Our results suggest for the first time that TGFβ1 functions to inhibit aberrant T cell expansion by maintaining intracellular calcium concentration levels low enough to prevent a mitogenic response by Ca^{2+}-independent stimulatory pathways alone. Consequently, TGFβ1 prevents autoimmune disease through a Ca^{2+} regulatory pathway that maintains the activation threshold above that inducible by self-MHC-TCR interactions.

Transforming growth factor β1 is a polypeptide growth regulatory factor that functions both during development and in the adult by affecting cell differentiation, growth, apoptosis, adhesion, and immune response (1–5). *Tgfb1* knockout mice have defects in preimplantation development (6), yolk sac development (7), tooth development (8), genetic stability (9,10), platelet activation (11), and cancer (9,12–14). In the immune system, TGFβ1 plays an important role in regulating inflammation (15,16), induction of self- and oral-tolerance (17–19), and autoimmunity (20). TGFβ1 has been shown to induce Ag-specific unresponsiveness in naive T cells (21), and TGFβ1-secreting Th3 type regulatory T cells and $CD8^+$ suppressor T cells maintain immune homeostasis and inhibition of autoimmune disorders (22–24). TGFβ1 is

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secreted by T cells that are activated by IL-2 or PHA-P/PMA (25), by cross-linking of CTLA-4, and by activation-induced cell death, suggesting an involvement in lymphocyte homeostasis (26,27). Fetal thymic organ culture studies have demonstrated that TGFβ1 and TGFβ2 inhibit growth and induce differentiation of thymocytes (28). In vitro studies also have shown that TGFβ1 can act as a positive as well as a negative regulator of lymphocyte proliferation and apoptosis (29,30).

Tgfb1 knockout mice develop severe inflammatory lesions in multiple organs and die within 3 wk after birth (15,16). Anti-LFA-1 Ab (31) and fibronectin peptides (32) rescue these mice from inflammatory disorders, suggesting that either extravasation or T cell activation is affected. T lymphocytes are the primary effectors in the pathological phenomenon in these mice as *Tgfb1*$^{-/-}$ *Scid* (31), *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ (14), *Tgfb1*$^{-/-}$ *Rag1*$^{-/-}$ (33), and *Tgfb1*$^{-/-}$ athymic nude mice (T. Doetschman, unpublished observations) survive months longer than do immunocompetent *Tgfb1*$^{-/-}$ mice. A study using mice expressing dominant-negative TGFβ receptor type II transgenes under T cell-specific promoters revealed that T cells are hyperresponsive, acquire a memory phenotype, generate autoantibodies, and produce inflammatory lesions in lung and colon tissues (34). However, another study found that only CD8$^+$ T cells are hyperresponsive in dominant-negative TGFβ receptor type II transgenic mice (35). Also, TGFβ1 can inhibit differentiation of T cells into the Th2 phenotype by inhibiting IL-4 induction (36). Breeding of *Tgfb1*$^{-/-}$ mice onto an *Ifn*$\gamma^{-/-}$ background but not onto an *Il4*$^{-/-}$ background enhances the life span by 3–4 wk and eliminates necroinflammatory hepatitis (37). Finally, *Tgfb1*$^{-/-}$ mice on a MHC class I-deficient background (β_2-microglobulin null mice) live much longer than on a class II-deficient background, suggesting that abnormal expression and presentation of self-Ags by class I molecules to CD8$^+$ T cells may also contribute to the inflammatory phenotype (38).

Complicating these studies is the possibility that the inflammatory disorder may have secondary effects that are independent of or synergistic with the absence of TGFβ1. For example, in *Tgfb1* knockout mice inflammatory cytokine levels are increased (15), the percentage of CD4$^+$ single-positive (SP)[3] thymocytes increases 7-fold, and hyperproliferation leads to enlarged spleens and lymph nodes (39). However, these are common inflammatory stress responses. Consequently, it is not clear how TGFβ1-deficient lymphocytes would behave in the absence of inflammatory stress. To avoid these complications we have investigated thymocyte function in very young *Tgfb1*$^{-/-}$ mice. We have utilized PMA and ionomycin to stimulate thymocytes in a TCR- and APC-independent manner (40,41). Our results indicate that thymocytes from mice with no inflammatory load are hyperresponsive to calcium-calcineurin-mediated mitogenic stimuli.

Materials and Methods

Mice

Tgfb1$^{-/-}$ mice (129 × CF-1) were generated as described (15). These mice were bred onto BALB/c, C3H, and 129 backgrounds. *Tgfb1* heterozygous male and female mice were housed in a specific pathogen-free mouse facility at the University of Cincinnati Medical Center.

Reagents

RPMI 1640 medium, AIM-V medium, Dulbecco's PBS, and HBSS were purchased from Life Technologies (Rockville, MD). RBC lysing buffer, trypan blue, Con A, ionomycin, PMA, rapamycin, EGTA, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). FK506

[3]Abbreviations used in this paper: SP, single-positive; BrdU, 5-bromo-2′-deoxyuridine; CLM, cell loading medium; DP, double-positive; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular calcium concentration.

was purchased from Biomol (Plymouth Meeting, PA). Indo-1 acetoxmethyl ester (Indo-1-AM) and NF-κB SN50 (cell-permeable inhibitor peptide) were purchased from Calbiochem (La Jolla, CA). Pluronic F-127 was purchased from Molecular Probes (Eugene, OR). Paraformaldehyde was purchased from Electron Microscopy Sciences (Fort Washington, PA). [*methyl*-^{3}H]Thymidine (sp. act., 6.7 Ci/mmol) was purchased from NEN (Boston, MA). FALCON flat-bottom 96-well Microtest Primaria tissue culture plates and 24-well Multiwell tissue culture plates were purchased from BD Biosciences (Franklin Lakes, NJ). FK506 (200 nM), rapamycin (100 nM), PMA (10 μM), and ionomycin (100 μM) stocks were prepared in DMSO, aliquoted, and stored at −80°C in a freezer.

Abs and staining kits

Purified anti-mouse CD3ε, anti-mouse CD28, anti-mouse CD16/CD32 (FcγRIII/II), FITC anti-mouse CD3ε, FITC anti-mouse CD4 (L3T4), Cy-Chrome anti-mouse CD4, R-PE anti-mouse CD8α (Ly-2), fluorochrome-conjugated isotype control Abs, Annexin V FITC apoptosis kit, Annexin V PE apoptosis kit, and 5-bromo-2′-deoxyuridine (BrdU) Flow kit were purchased from BD PharMingen (San Diego, CA).

PCR genotyping

Genotype of the newborn pups from heterozygous matings was determined by PCR amplification of tail DNA and size fractionation on agarose gels (14).

Inflammation index

After the thymus was removed for in vitro studies, *Tgfb1* null mice were fixed in 10% neutral-buffered formalin. Tissue sections were scored for inflammatory lesions with a severity scale from 0 to 4. Although inflammation in 25–30 organs was measured, only 8–12 organs were found to be the most severely affected (15,39). The sum of scores for all organs was divided by the number of organs analyzed to generate an inflammation index. Mice severely affected by multifocal inflammation had inflammation indices ranging from 0.5 to 1.0 (3 wk old), whereas wild-type mice had no inflammation. *Tgfb1*$^{-/-}$ mice up to 1 wk of age had inflammation indices ranging from 0 to <0.1. Inflammation indices for older mice are given in the figures.

Preparation of thymocytes

Newborn pups and young mice (<3 wk old) were sacrificed by isoflurane overdose and thymii were dissected out aseptically into a petri dish containing RPMI 1640 medium. Tissue was mechanically separated into individual cells with a syringe and 22-gauge needle. Cells were centrifuged at 1000 rpm for 5 min and RBCs were depleted with RBC lysing buffer. The cells were washed once with RPMI 1640 medium and were suspended in Aim-V serum-free medium. Viable cells were counted using trypan blue dye exclusion as an indicator. Cells were resuspended at 2×10^6/ml in Aim-V serum-free medium for all in vitro culture studies. Thymocytes were prepared in Dulbecco's PBS or HBSS for staining and flow cytometry.

Phenotype analysis of thymocytes

One million thymocytes from each mouse were stained on ice for 30 min for CD3, CD4, and CD8 expression using fluorochrome-conjugated mAbs in the presence of 10% FBS and Fc blocking Ab. After the cells were washed once they were resuspended in HBSS containing 0.09% NaN_3 and analyzed in Beckman Coulter Epics XL flow cytometer (Fullerton, CA), FACSCaliber, or BD-LSR flow cytometers (BD Biosciences). Side and forward angle light scattering was used to electronically gate the cells of choice and exclude debris. Some 10,000 events within the gate region were collected for each sample. Fluorochrome-conjugated isotype control Abs were used to control for the nonspecific binding. Cells stained with each Ab

individually were used to set compensation networks for each fluorochrome. In all experiments, control cells were taken from either *Tgfb1*$^{+/+}$ littermates or *Tgfb1*$^{+/-}$ littermates when a wild-type littermate was not available. Data were analyzed by System II or CellQuest software (BD Biosciences). Cells were stained for apoptosis using annexin V according to the manufacturer's protocol.

Assessment of thymocyte proliferation

A total of 2×10^5 thymocytes were cultured in 200 μl Aim-V serum-free medium in the presence of various concentrations of mitogens in triplicate in FALCON flat-bottom 96-well Microtest Primaria tissue culture plates (BD Biosciences). After 3 days of in vitro culture at 37°C and 5% CO_2, cultures were pulsed with 0.5 μCi of tritiated thymidine (NEN) for a period of 14–16 h, cells were harvested onto glass fiber filters, and radioactivity was counted in a Beckman Coulter scintillation counter. Data are represented as mean dpm from triplicate cultures ±SD. Background incorporation of unstimulated cultures was always <600 dpm. Optimization of cell number, mitogen concentration, and serum replacements were conducted in the preliminary studies. The optimum concentration of Con A was 0.5 μg/ml; anti-CD3 Ab, 0.5 μg/ml; PMA, 1 nM; and ionomycin, 250 nM in Aim-V serum-free medium supplemented with L-glutamine, streptomycin sulfate, gentamicin sulfate, and HSA. For phenotype analysis of hyperresponsive thymocytes, 2×10^6 cells were cultured in 2 ml Aim-V serum-free medium in the presence of mitogens in 24-well Multiwell tissue culture plates at 37°C and 5% CO_2 and were pulsed with 10 μM BrdU for the last 12–14 h. Cultures were harvested after 2 days of culture and were stained for surface Ags and incorporated BrdU according to the manufacturer's protocol.

Analysis of intracellular calcium concentration $[Ca^{2+}]_i$ levels by flow cytometry

A total of 1×10^7/ml day 3–11 thymocytes in cell-loading medium (CLM; HBSS-containing Ca^{2+} and Mg^{2+}, 1% FBS) were loaded with 10 μM Indo-1-AM (stock solution; 2 mg/ml dissolved in Pluronic F-127) for 40 min at 30°C (42). Cells were washed three times with CLM and resuspended in CLM, surface stained with azide-free fluorochrome-conjugated anti-CD4 and anti-CD8 Abs for 20 min on ice, washed once with CLM, and left on ice until analyzed. Cells were prewarmed at 37°C for 10 min before acquiring for Ca^{2+} measurements. Cells were acquired for 30 s before adding the agonist. For each analysis, cells were acquired for 512 s at a flow rate of 300–400 events per second in a BD-LSR FACS machine equipped with UV lasers. Ratio metric analyses of Ca^{2+}-bound Indo-1 (FL5)/Ca^{2+}-free Indo-1 (FL4) were done by CellQuest (BD Biosciences) and Flow Jo (Tree Star, San Carlos, CA) software programs. Basal $[Ca^{2+}]_i$ levels and changes in $[Ca^{2+}]_i$ levels in $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^+CD8^+$ subpopulations were analyzed after gating on these cells. Based on the concentration of $[Ca^{2+}]_i$ levels after acquiring each sample for 512 s, cells were further grouped into low (M1), intermediate (M2), medium high (M3), and high (M4) $[Ca^{2+}]_i$ cells (43).

Statistical analysis

Significance of the differences between wild-type and mutant mice responses was calculated using Student's *t* test.

Results

Normal thymic phenotype in young *Tgfb1*$^{-/-}$ mice

The total number and percent of SP and double-positive (DP) cell populations in the thymus of young *Tgfb1*$^{-/-}$ mice (postnatal day 3–7) is not different from those of *Tgfb1*$^{-/-}$ mice (Fig. 1, *A* and *B*). This suggests that there is no obvious defect in the selection and maturation of thymocytes in *Tgfb1*$^{-/-}$ mice. However, in older mice (2–3 wk old) we have also noticed a

decrease in the DP population and an increase in both $CD4^{-}CD8^{-}$ double-negative and $CD4^{+}$ SP cells (39). Older mice also show increased apoptosis similar to a corticosteroid-induced stress response (data not shown).

Thymocytes are hyperresponsive to Ca^{2+}-mediated mitogenic stimulus

Engagement of TCR by anti-CD3 or Con A results in activation of T cells and mitogenesis. However, immature T cells undergo apoptosis instead of mitogenesis upon TCR stimulation. Consequently, the mitogenic response of APC-dependent, receptor-mediated activation of postnatal day 7–17 *Tgfb1*$^{-/-}$ thymocytes by Con A suggests that they are immature. However, *Tgfb1*$^{-/-}$ thymocytes are relatively hyperresponsive (Fig. 2*A*). Stimulation of thymocytes from day 5 pups by optimal concentrations of PMA plus ionomycin results in activation and proliferation of both wild-type and mutant thymocytes, but at suboptimal concentrations only mutant cells are responsive (Fig. 2*B*). Either PMA or ionomycin is not mitogenic on its own but both of them act synergistically and induce mitogenic response in lymphocytes (data not shown and Fig. 2*B*).

Hyperresponsiveness of thymocytes to anti-CD3 plus anti-CD28 stimulation

To address whether dysregulation of $[Ca^{2+}]_i$ mobilization causes the hyperresponsiveness of *Tgfb1*$^{-/-}$ thymocytes, cells were treated with anti-CD3 plus anti-CD28 Abs. Treatment with anti-CD3 causes elevation of $[Ca^{2+}]_i$ and activation of protein kinase pathways resulting in activation and nuclear translocation of NF-ATc, NF-κB, AP-1, and other transcription factors involved in lymphokine gene transcription. Treatment with anti-CD28 provides a costimulatory signal resulting in activation of phosphatidylinositol 3 kinase and protein kinase pathways such as mitogen-activated protein kinase, c-Jun N-terminal kinase, and protein kinase C (PKC). Thymocytes from day 3 *Tgfb1*$^{+/+}$ mice do not respond to either anti-CD3 alone or in combination with anti-CD28 because they are immature cells. However, thymocytes from age-matched *Tgfb1*$^{-/-}$ mice respond to anti-CD3 plus anti-CD28 stimulation, suggesting that these cells are inherently hyperresponsive (Fig. 2*C*).

To address whether altered $[Ca^{2+}]_i$ levels cause the hyperresponsiveness in *Tgfb1*$^{-/-}$ thymocytes, cells were next treated with PMA and anti-CD28. Treatment with PMA results in activation of the PKC pathway, which in turn activates NF-κB but does not elevate $[Ca^{2+}]_i$. Hence, PMA plus anti-CD28 stimulate lymphocytes in a Ca^{2+}-calmodulin-independent manner. Without additional stimulation through the Ca^{2+}-calmodulin pathway, normal lymphocytes will not proliferate in response to either PMA, anti-CD28, or both (44,45). As expected, treatment of *Tgfb1*$^{+/+}$ thymocytes with PMA plus anti-CD28 does not result in a mitogenic response, whereas *Tgfb1*$^{-/-}$ thymocytes do respond to the same mitogenic stimulation (Fig. 2*D*). The data also indicate that the Ca^{2+}-calmodulin-calcineurin signaling pathway may be up-regulated and in synergy with PMA and anti-CD28 signaling. Similar responses are also observed with anti-CD3 plus PMA treatments (Fig. 2*E*), although it must be noted that the magnitude of mitogenesis in response to PMA plus ionomycin is much larger than that achievable by any other mitogenic regimen tested. Together, these results suggest that *Tgfb1*$^{-/-}$ thymocytes are inherently hyperresponsive to mitogenic stimulation.

Our results on preinflammatory-stage *Tgfb1*$^{-/-}$ thymocytes demonstrate that mitogenic responses to Con A, anti-CD3 plus anti-CD28, and PMA plus ionomycin are clearly altered. Consequently, the hypersensitivity of *Tgfb1*$^{-/-}$ thymocytes to the suboptimal concentration of ionomycin (125 nM) in the presence of the optimal concentration of PMA (1 nM) is due to the absence of TGFβ1 and not due to the inflammatory environment.

TGFβ1 inhibits Ca^{2+}-calcineurin-mediated thymocyte activation

The mitogenic response of thymocytes is dependent, in part, on the mobilization and elevation of $[Ca^{2+}]_i$, which leads to calcineurin activation, nuclear translocation of dephosphorylated NF-ATc, and transcriptional activation of IL-2. Our PMA plus ionomycin experiments suggest that the *Tgfb1*$^{-/-}$ thymocyte response could be due to elevated $[Ca^{2+}]_i$ levels. To determine whether TGFβ1 alters the $[Ca^{2+}]_i$ levels and/or calcineurin activation, we have tested the effects of EGTA and FK506, which block Ca^{2+} mobilization and NF-ATc activation, respectively. If the absence of TGFβ1 leads to high $[Ca^{2+}]_i$ levels, then *Tgfb1*$^{-/-}$ thymocytes should require more EGTA or FK506 to reach the same level of inhibition as in wild-type cells.

Thymocytes from *Tgfb1*$^{-/-}$ mice are less sensitive to FK506, indicating increased NF-ATc activation. The proliferation of wild-type cells stimulated with 125 nM or 250 nM ionomycin, plus 1 nM PMA, is completely blocked by 0.1 nM FK506. Mutant thy-mocyte proliferation, on the other hand, is not completely blocked by 0.1 nM FK506 when stimulated by 250 nM ionomycin plus 1 nM PMA (Fig. 3*A*). FK506 (1.0 nM) completely blocks 250 nM ionomycin-stimulated mitogenesis of both cell types in the presence of 1 nM PMA. Hyperresponsiveness of *Tgfb1*$^{-/-}$ thymocytes to 125 nM ionomycin plus 1 nM PMA is also inhibited by EGTA (Fig. 3*B*), indicating increased Ca^{2+} mobilization. However, 0.4 mM EGTA does not completely abolish the mitogenic response of 125 nM ionomycin plus 1 nM PMA-stimulated mutant thymocytes because it is still ~60-fold higher than a background at ~140 dpm. On the other hand, 0.4 mM EGTA completely inhibits the mitogenic response of 125 nM ionomycin plus 1 nM PMA-stimulated wild-type cells because the response is not above background levels. At 0.8 mM EGTA, the mitogenic response is not above background levels either in mutant or wild-type mice. Likewise, 1.0 nM FK506 reverses the hyperresponsiveness of *Tgfb1*$^{-/-}$ thymocytes to stimulation by PMA plus anti-CD28 (Fig. 3*C*). Together, these results suggest that the Ca^{2+}-calcineurin pathway could be one of the primary targets of TGFβ1 function in thymocytes.

To determine whether TGFβ1 specifically regulates calcineurin rather than p70 S6 kinase- or PKC-mediated pathways, we treated thymocytes with rapamycin or NF-κB SN50 and stimulated with 1 nM PMA and 250 nM ionomycin. Rapamycin and NF-κB SN50 inhibit p70S6 kinase- and PKC-mediated signaling, respectively. Thymocytes from both *Tgfb1*$^{+/-}$ and *Tgfb1*$^{-/-}$ mice are equally sensitive to rapamycin and NF-κB SN50 (Fig. 3*D*), demonstrating that TGFβ1 provides a check on thymocyte activation by inhibiting Ca^{2+}-calcineurin signaling rather than p70 S6 kinase or PKC signaling.

Thymocyte $[Ca^{2+}]_i$ levels altered in *Tgfb1*$^{-/-}$ mice

To confirm that TGFβ1 regulates calcium-calcineurin signaling, we directly measured thymocyte $[Ca^{2+}]_i$ levels and changes in $[Ca^{2+}]_i$ levels upon stimulation. Basal $[Ca^{2+}]_i$ levels are higher in mutant thymocytes, and the $[Ca^{2+}]_i$ levels increase with age of the mice (Fig. 4*A*, compare *top panels* with *bottom panels*) and as the cells mature from DP stage to SP stage (Fig. 4*A*, compare *right panels* ($CD4^+CD8^+$DP thymocytes) with *left* ($CD4^+CD8^-$SP) and *middle* ($CD4^-CD8^+$SP) *panels* in the same row). Percentages of cells in the M2 region of *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ mice also suggest that there is a 2-fold increase in the number of cells with elevated $[Ca^{2+}]_i$ in *Tgfb1*$^{-/-}$ mice. Numbers in the histograms of Fig. 4*A* represent the percentage of cells in the M2 region. Stimulation of thymocytes with anti-CD3 results in elevation of $[Ca^{2+}]_i$, which is more evident in $CD4^+CD8^-$ SP than in $CD4^+CD8^+$ DP thymocytes. There are noticeable differences in percentages of cells in gates M1 and M2 between *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ $CD4^+CD8^-$ SP thymocytes. However, there are no differences in DP thymocytes (Fig. 4*B*, compare *top left panel* with *bottom left panel*). Stimulation with a suboptimal concentration of ionomycin (100 nM) leads to elevation of $[Ca^{2+}]_i$ to a greater extent as against anti-CD3 stimulation and is more elevated in mutant than wild-type

thymocytes (note the rightward shift of the red tracing relative to the green in Fig. 4*B*, *right panels*).

Upon anti-CD3-stimulation, increase in $[Ca^{2+}]_i$ levels in DP thymocytes of both wild-type and mutant mice is not significantly different, and the magnitude of $[Ca^{2+}]_i$ levels is much lower (mean ratio of 180, Fig. 4*C*, *bottom panel*) than in mature $CD4^+CD8^-$ thymocytes (mean ratio of 260, Fig. 4*D*, *upper panel*). As expected, anti-CD3-induced elevation in $[Ca^{2+}]_i$ levels in mature $CD4^+CD8^-$ thymocytes is higher in mutant mice (mean ratio of $Tgfb1^{-/-}$ SP thymocytes is 20% more than $Tgfb1^{+/+}$ SP thymocytes upon stimulation with anti-CD3, Fig. 4*D*, *upper panel*), and anti-CD28 and PMA do not alter $[Ca^{2+}]_i$ levels in mutant or wild-type thymocytes (Fig. 4*D*, PMA data not shown). Hence, lack of sufficient elevation in $[Ca^{2+}]_i$ levels explains why the immature thymocytes do not respond to receptor-mediated mitogenic stimuli in wild-type mice.

Phenotype of hyperresponsive thymocytes in *Tgfb1*$^{-/-}$ mice

The thymus is composed of mainly DP cells (85–90%) and a small proportion of mature SP cells and double-negative T cell precursors. Based on the thymidine incorporation data previously presented, it is reasonable to conclude that DP cells are the primary effectors in the hyperresponsive cultures because there would not be enough SP cells to yield such a massive proliferation seen at suboptimal concentrations of ionomycin in the presence of PMA. Upon stimulation, thymocytes down-regulate one of their coreceptors and mature into either $CD4^+$ SP or $CD8^+$ SP cells (46). Mutant, but not wild-type cells, undergo mitogenesis in response to a suboptimal concentration of ionomycin in the presence of PMA in day 9 mice (Fig. 5*A*). This again confirms that DP thymocytes respond to suboptimal mitogenic stimulus and differentiate to $CD4^+$ SP cells in $Tgfb1^{-/-}$ mice (Fig. 5*B*). A similar response is observed in day 16 $Tgfb1^{-/-}$ mice, except that DP thymocytes differentiate to both $CD4^+$ SP and $CD8^+$ SP cells (data not shown). This suggests that $CD4^+$ SP cells are the initiators of inflammation and assist in the generation of effector $CD8^+$ SP cells in $Tgfb1^{-/-}$ mice. These data also suggest that only a portion (10–20%) of thymocytes proliferate in response to mitogenic stimulus even at optimal concentrations of PMA and ionomycin. The majority of thymocytes undergo apoptosis in both wild-type and mutant thymocyte cultures upon stimulation with PMA plus ionomycin (data not shown). Hence, the differences in mitogenic responses observed between wild-type and mutant mice is actually due to the presence of a small percentage of hyperresponsive thymocytes in mutant mice.

Discussion

$Tgfb1^{-/-}$ mice develop severe inflammatory lesions in multiple organs and die around 3 wk of age. Backcrossing these mice onto immunodeficient backgrounds has revealed that T cells are the primary effectors in the inflammatory phenotype of $Tgfb1^{-/-}$ mice. It is clear that there is a positive relationship between age and the degree of inflammation in $Tgfb1^{-/-}$ mice (39), and that many phenotypes, such as epithelial cell proliferation and ICAM and MHC class II up-regulation, are eliminated if the inflammation is removed by genetically combining $Tgfb1^{-/-}$ and *Scid* mice (31). The present study demonstrates that the distribution of thymocyte subsets is not affected up to 1 wk after birth. Our proliferation studies unequivocally demonstrate for the first time that $Tgfb1^{-/-}$ thymocytes are inherently hyperresponsive to a suboptimal concentration of ionomycin plus PMA, irrespective of the age or inflammation index of the mice. They are also hyperresponsive to other forms of mitogenic stimulation such as Con A and anti-CD3 plus anti-CD28. However at ages ≥2 wk, the inflammation-induced stress response makes it difficult to interpret whether the observed decrease in thymic cellularity results from the presence of inflammation or the absence of TGFβ1. $Tgfb1^{-/-}$ thymocytes are also hyperresponsive to Ca^{2+}-independent (PMA plus anti-CD28) mitogenic stimulus. Because

Tgfb1$^{+/+}$ thymocytes do not respond well to stimulation by PMA plus anti-CD28 or to suboptimal concentrations of ionomycin plus PMA, TGFβ1 could be acting on the $[Ca^{2+}]_i$ channels through the FK506 binding protein, FKBP12, and calcineurin.

FKBP12 is known to be released through association with the TGFβ type I receptor upon ligand binding (47) and to recruit calcineurin to the inositol 1,4,5-triphosphate (IP_3) receptor complex (48), where together they are required for fully functional IP_3 receptor activity (49,50). That FKBP12 can function in TGFβ signaling is strongly suggested by the fact that *Fkbp12* knockout mice have cardiomyocyte defects that involve abnormal Ca^{2+} channel function (51), and *Fkbp12* and *Tgfb2* knockout mice have similar morphological defects in the developing heart (51,52). However, it is still unclear which isoform of FKBP is involved in TGFβ signaling in T cells (53–56). In the absence of TGFβ1, there could be disequilibria of TGFβ type I receptor-associated FKBPs, resulting in leaky Ca^{2+} release channels. In this study we have shown that *Tgfb1*$^{-/-}$ thymocytes are less sensitive than wild-type thymocytes to Ca^{2+}ion chelation by EGTA and to calcineurin inhibition by FK506. This suggests that TGFβ1 inhibits activation of thymocytes in vivo by blocking $[Ca^{2+}]_i$ mobilization and uptake by activated cells. The observation that both *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ thymocytes are equally sensitive to rapamycin and NF-κB SN50, while *Tgfb1*$^{+/+}$ cells are more sensitive to FK506, suggests that TGFβ1 does not affect PKC and p70S6 kinase signaling. Finally, BrdU incorporation studies demonstrate that DP thymocytes are hyperresponsive and that $CD4^+$ SP thymocytes develop at a higher rate than $CD8^+$ SP thymocytes. $[Ca^{2+}]_i$ measurements of unstimulated and stimulated thymocytes confirm that the basal $[Ca^{2+}]_i$ levels in *Tgfb1*$^{-/-}$ thymocytes are elevated, and that the increase in $[Ca^{2+}]_i$ levels is greater upon stimulation with suboptimal concentration of ionomycin and anti-CD3. Also, the difference between *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ is more evident in $CD4^+$ SP thymocytes.

TGFβ1 is known to have multiple suppressive actions on immune cells. Based on the data presented in this study, we hypothesize that TGFβ1 prevents autoimmune disorders by increasing the signal threshold level of activation. It is known that alteration of signal threshold levels due to mutations affecting the TCR complex and its downstream effectors leads to altered thymic selection and hyperresponsiveness (42,57–59). It is therefore possible that in the absence of TGFβ1, more hyperresponsive, self-reactive T cells that escape thymic negative selection are exported to the periphery. Escape to the periphery of a few autoreactive thymocytes with a lowered threshold for activation could result in enough expansion to cause autoimmune disease. It is also known that a few autoreactive cells always escape to the periphery, but their activation is inhibited by TGFβ1-secreting regulatory T cells (60). Thymocytes and mature T cells must recognize self-MHC molecules in the thymus and periphery for their survival (40,61). Lowering of the activation threshold could therefore result in positive selection of T cells that would otherwise have been deleted. However, recognition of self-MHC molecules by TCR alone can lead to activation if there are disturbances in the signaling environment (57,58). Activated T cells are known to exhibit enhanced adhesion to endothelial cells (62), TGFβ1 has been shown to inhibit the adhesion of neutrophils and T cells to endothelial cells (63,64), splenocytes from *Tgfb1*$^{-/-}$ mice exhibit enhanced adhesiveness to endothelial cells (32), and TGFβ1 is known to affect integrin activity (11). Hence, it is reasonable to speculate that hyperresponsive *Tgfb1*$^{-/-}$ T cells adhere to endothelial cells and extravasate into peripheral organs more rapidly in *Tgfb1*$^{-/-}$ mice.

Thymectomy of neonatal mice in the first week of birth but not on the day of birth or 7 days after birth results in the development of autoimmune disease that can be reversed by adoptive transfer of either thymocytes from neonates or lymphocytes from spleen and lymph nodes of adult mice (60,65). Suppressor and regulatory T cells secrete TGFβ1 (22,23). Because TGFβ1 is produced in the thymus and secreted by both suppressor and regulatory T cells, depletion of TGFβ1 with Ab reverses the regulatory function of $CD4^+CD25^+$ T cells and makes

them proliferate in response to mitogenic stimuli (66). Hence, it is reasonable to speculate that absence of TGFβ1 would lead to export of autoreactive T cells into the periphery, resulting in autoimmune disease.

Autoreactive T cells are deleted in the thymus by negative selection. In this process T cells that recognize self-Ags with a low enough affinity that they are not activated will survive negative selection and be exported to the periphery. Autoimmune disease can occur if there is up-regulation of self-MHC molecules and presentation of self-Ags, up-regulation of costimulatory molecules, or a lowering of the threshold level of T cell activation due to increased or decreased production of stimulatory or inhibitory cytokines (67). Naive T cells need to interact with self-MHC molecules for their survival in the periphery. Normally, interaction of TCR with self-MHC per se is not sufficient to activate a naive T cell in the periphery (61). However, because TGFβ1 is a negative regulator of T cells, TGFβ1-deficient T cells that exhibit a lower threshold level of activation may become activated upon engagement with self-MHC molecules.

TGFβ1 mediates its inhibitory effects on many cell types including lymphocytes through SMAD2 and SMAD3. However, there is evidence suggesting that TGFβ1 can exert its effects such as inducing fibronectin expression through SMAD-independent mechanisms. Consequently, *Smad3* null thymocytes and mature T cells were found to be normal in their mitogenic response, although resistant to TGFβ1-mediated suppression of mitogenic response (68,69). Because SMAD3-deficient mice live much longer than TGFβ1-deficient mice due to less severe forms of inflammatory disorders and because SMAD3-deficient thymocytes are not hyperresponsive to mitogenic stimulation, our data demonstrate that regulation of $[Ca^{2+}]_i$ levels by TGFβ1 is SMAD3 independent and is critical for maintaining immune homeostasis and prevention of autoimmunity.

In summary, our studies demonstrate that *Tgfb1*$^{-/-}$ thymocytes taken from animals with no complicating effects of multifocal inflammation are hyperresponsive to mitogenic stimuli. Analysis of thymocytes from these animals has led to the conclusion that TGFβ1 functions to inhibit aberrant T cell expansion by maintaining low enough $[Ca^{2+}]_i$ levels to prevent a mitogenic response by Ca^{2+}-independent stimulatory pathways alone. Consequently, TGFβ1 ensures that all T cell stimulatory pathways must be extant before a mitogenic response occurs. In its absence, there is a low level of stimulation through the Ca^{2+}-calcineurin pathway, which when combined with stimulation through other pathways becomes sufficient for T cells to proliferate rather than die, thereby leading to autoimmune disease. Because mature SP thymocytes have more free $[Ca^{2+}]_i$ levels than their precursor DP cells, they may be more prone to autoreactivity when they emigrate to the periphery.

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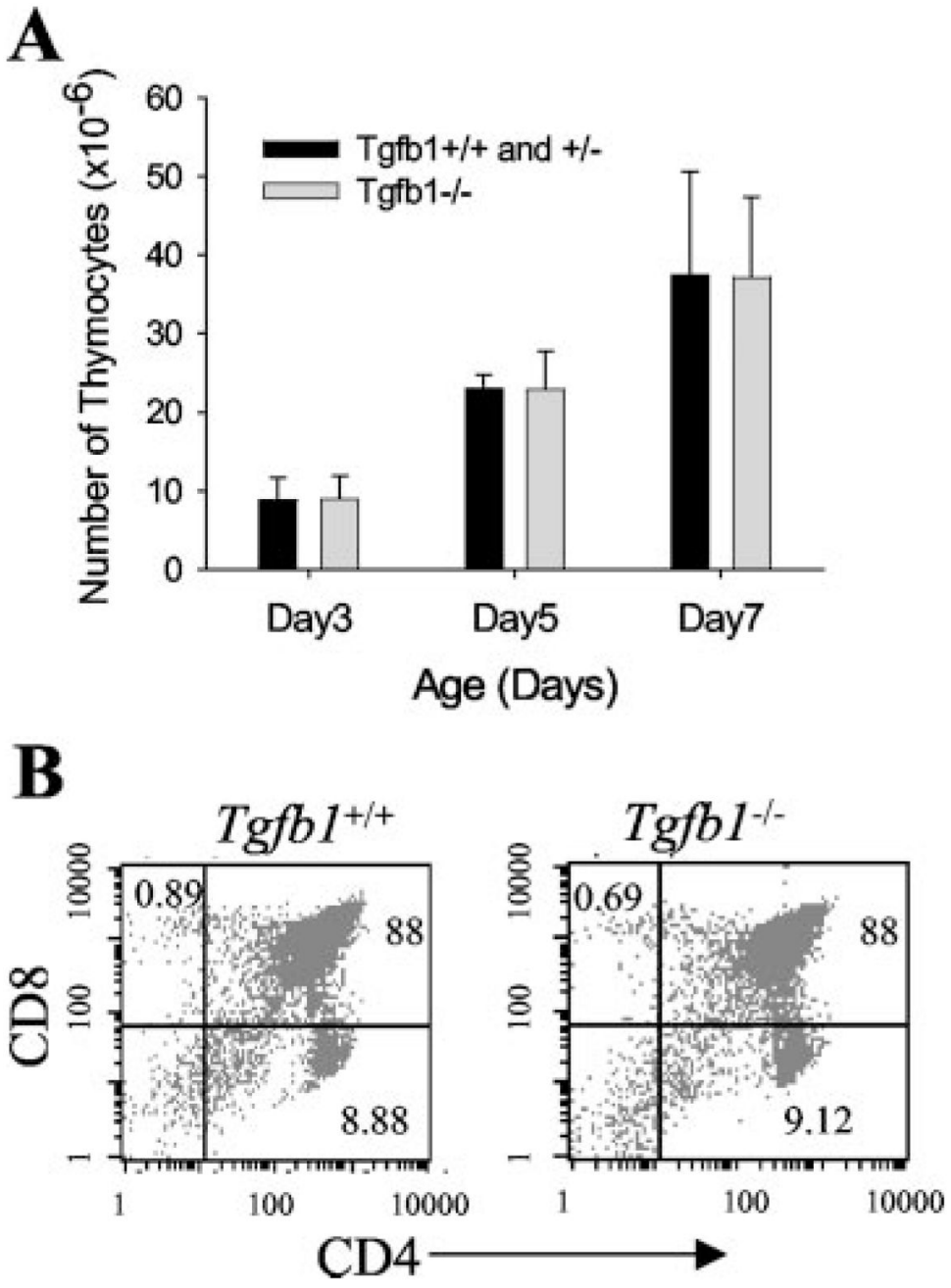

FIGURE 1.

Normal thymic phenotype in young *Tgfb1*$^{-/-}$ mice. *A*, Total number of cells from thymii of three to five mice for each age (days) represented with error bars as mean ± SD. *B*, Phenotypic analysis of thymocytes. Thymocytes from day 7 mice were stained with fluorochrome-labeled anti-CD4 and anti-CD8 Abs as described in *Materials and Methods*. Numbers in each quadrant represent the percentage of cells positive for the indicated surface Ag(s).

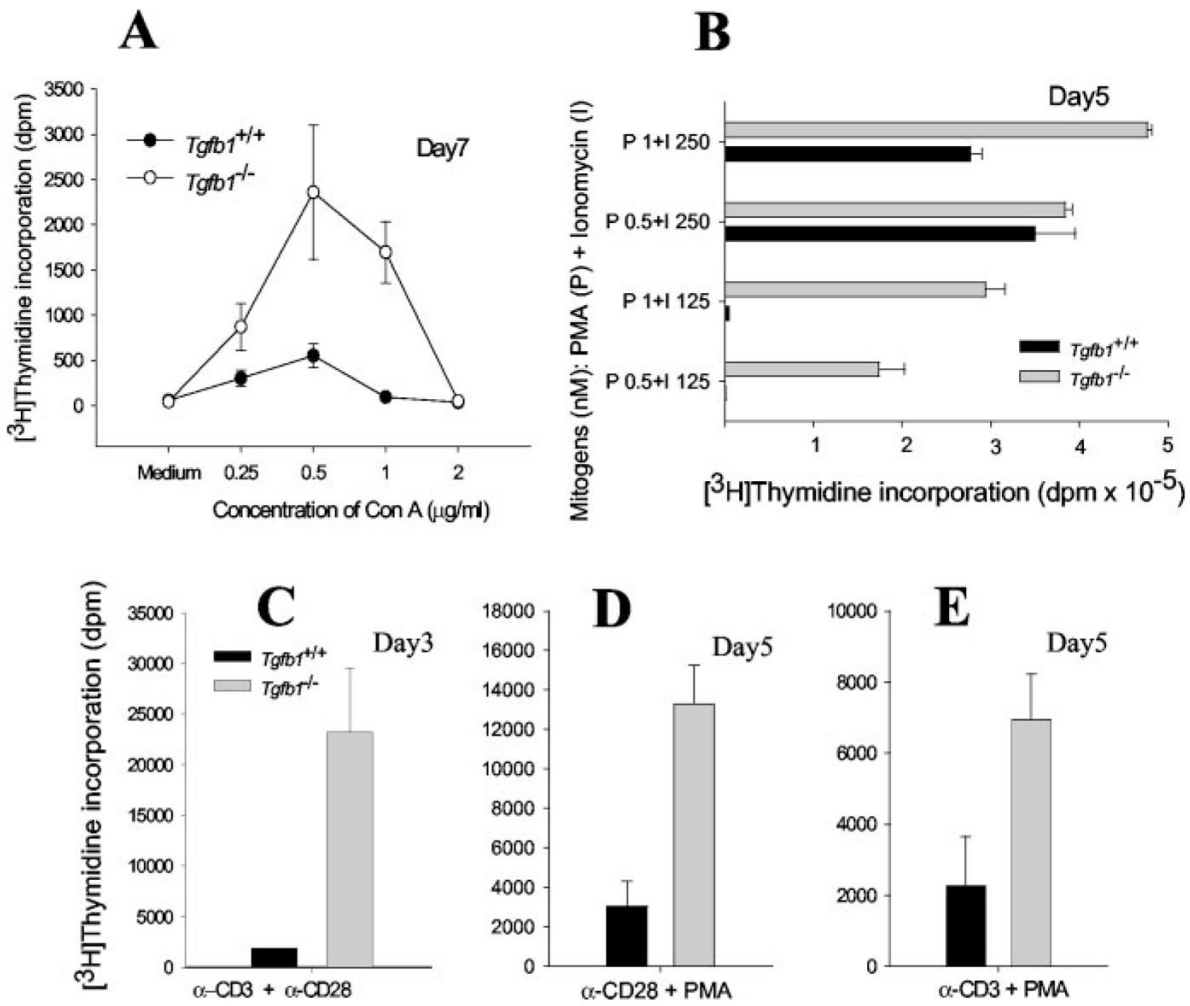

FIGURE 2.

Tgfb1$^{-/-}$ thymocytes are hyperresponsive to mitogenic stimulation. Thymocytes from day 3–7 mice were cultured either in the presence of Con A (*A*), PMA + ionomycin(*B*), anti-CD3 + anti-CD28(*C*), anti-CD28 + PMA (*D*), or anti-CD3 + PMA (*E*) and pulsed with [^{3}H]thymidine as described in *Materials and Methods*. Legend and ordinate within *D* and *E* are the same as that in *C*. Data are presented as mean value of dpm from triplicate wells and error bars represent ±SD. Similar responses are observed in all day 3–21 mice tested, independent of severity of inflammation (*p* for all mice from day 3–21 is <0.0005 for PMA + 125 nM ionomycin stimulation, <0.02 for anti-CD3 + PMA stimulation, and <0.05 for anti-CD3 + anti-CD28 and anti-CD28 + PMA stimulations).

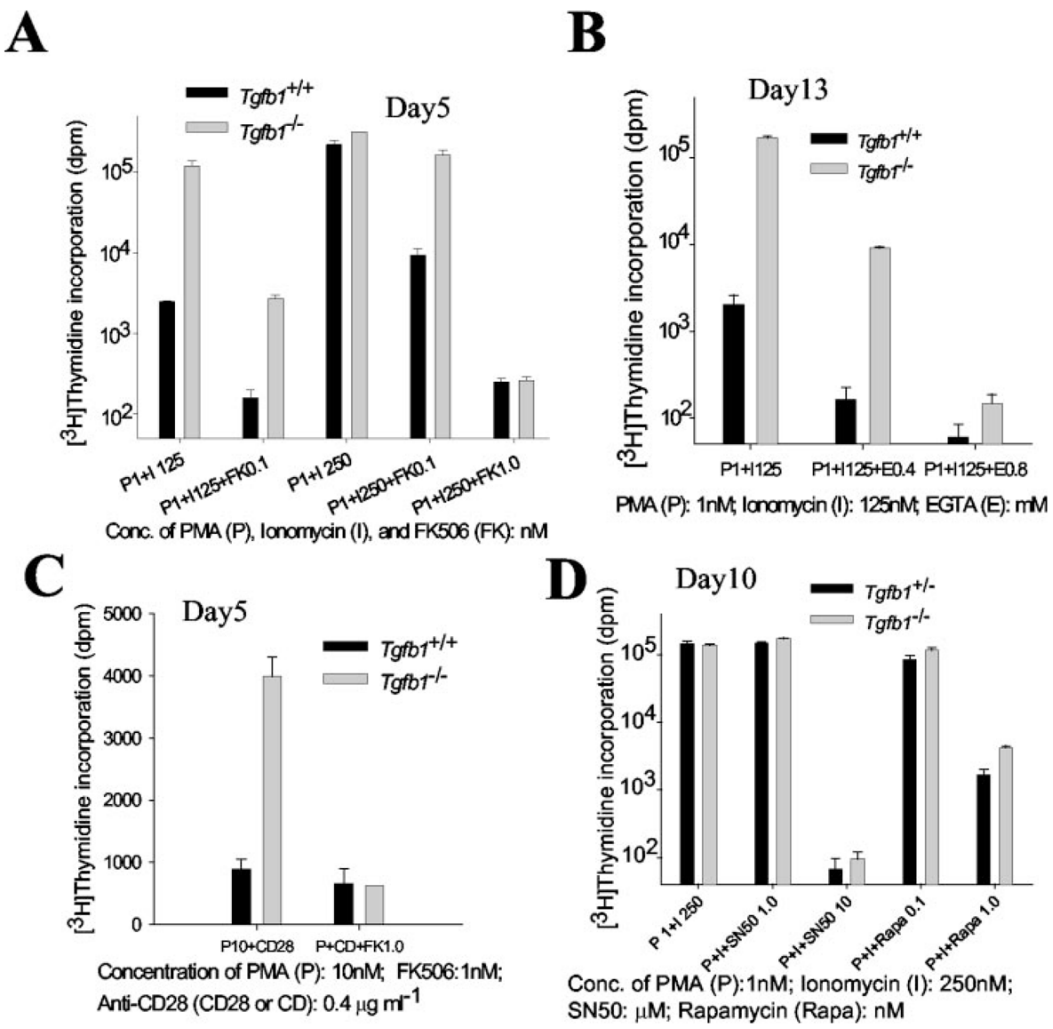

FIGURE 3.

Thymocytes from *Tgfb1*$^{-/-}$ mice are less sensitive to FK506 and EGTA, and equally sensitive to rapamycin and SN50. Thymocytes from day 5 mice (*A* and *C*), day 13 mice (*B*), or day 10 mice (*D*) were cultured in the presence of indicated concentrations of PMA + ionomycin with or without FK506 (*A*), PMA + ionomycin with or without EGTA (*B*), anti-CD28 + PMA with or without FK506 (*C*), or PMA + ionomycin with or without rapamycin or NF-κB inhibitor SN50 (*D*) and pulsed with [^{3}H]thymidine as described in *Materials and Methods*. Inflammation indices: 0 for the day 5 *Tgfb1*$^{-/-}$ mouse (*A* and *C*), and 0.18 for the day 10 *Tgfb1*$^{-/-}$ mouse (*D*). Inflammation index for the day 13 mouse used in *B* was not determined, but average index for four other day 13 mice was 0.18 ± 0.15. For comparison, inflammation index for the 50% of *Tgfb1*$^{-/-}$ mice that have not yet died by day 21 from severe inflammation ranges from 0.5 to 1.0. Data are presented as mean value of dpm from triplicate wells and error bars represent ±SD. Control cultures were included and either received one of the mitogens or medium only. A value of $p < 0.01$ for 0.1 nM FK506 mediated inhibition of 1 nM PMA + 250 nM ionomycin stimulation of thymocytes from six mutant mice compared with age-matched littermate control mice.

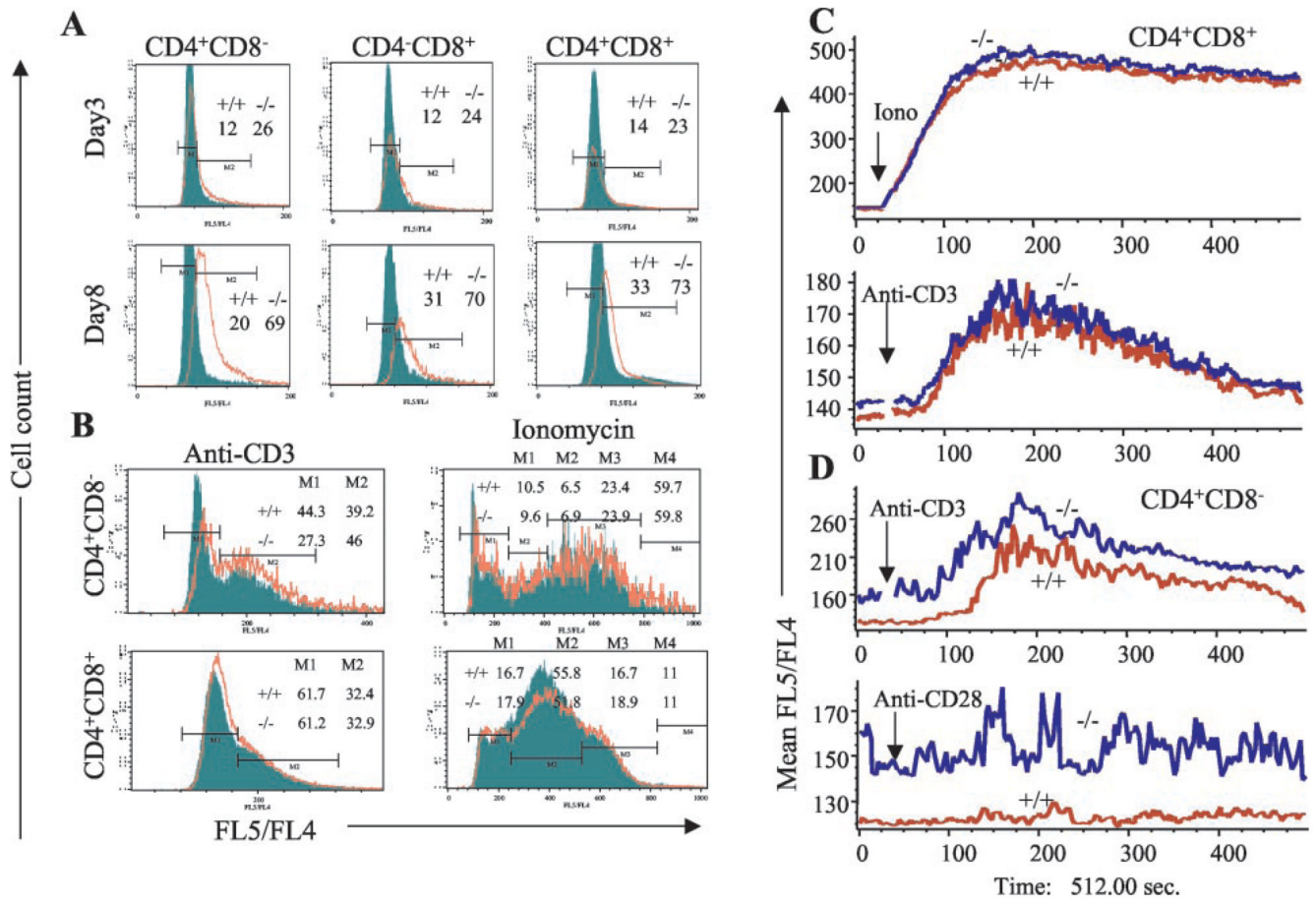

FIGURE 4.

$[Ca^{2+}]_i$ levels are higher in *Tgfb1*$^{-/-}$ thymocytes. Fluorescence ratio of Ca^{2+}-bound Indo-1 (FL5)/Ca^{2+}-free Indo-1 (FL4) was measured as described in *Materials and Methods*. *A*, Overlays of FL5/FL4 vs cell-count histograms of *Tgfb1*$^{+/+}$ (filled) and *Tgfb1*$^{-/-}$ (open) $CD4^+$ SP thymocytes (*left panels*), $CD8^+$SP thymocytes (*middle panels*), $CD4^+CD8^+$DP thymocytes (*right panels*), from day 3 (*upper panels*) and day 8 (*lower panels*) mice. Percentages of cells in M2 gate are shown. *B*, Overlays of FL5/FL4 vs cell-count histograms of day 7 *Tgfb1*$^{+/+}$ (filled) and *Tgfb1*$^{-/-}$ (open) $CD4^+$ SP thymocytes (*upper panels*) and $CD4^+CD8^+$DP thymocytes (*lower panels*) of anti-CD3-stimulated, 20 μg/ml (*left panels*) and ionomycin-stimulated, 100 nM (*right panels*). *C*, Overlays of mean FL5/FL4 ($[Ca^{2+}]_i$ levels) vs time of *Tgfb1*$^{+/+}$ (red) and *Tgfb1*$^{-/-}$ (blue) $CD4^+CD8^+$DP thymocytes stimulated with ionomycin 100 nM (*upper panel*) or anti-CD3 (*lower panel*). (D) Overlays of mean FL5/FL4 ratio ($[Ca^{2+}]_i$ levels) vs time of *Tgfb1*$^{+/+}$ (red) and *Tgfb1*$^{-/-}$ (blue) $CD4^+CD8^-$ SP thymocytes stimulated with anti-CD3 (*upper panel*) or anti-CD28, 2 μg/ml (*lower panel*). Note that upon stimulation with anti-CD3, the magnitude of increase in $[Ca^{2+}]_i$ levels in DP thymocytes (*C*, *lower panel*) is lower than the $CD4^+$ SP thymocytes (*D*, *upper panel*). Arrow indicates the time of addition of agonist in *C* and *D*. Genotype of *Tgfb1*$^{+/+}$ (+/+) and *Tgfb1*$^{-/-}$ (−/−) mice are shown.

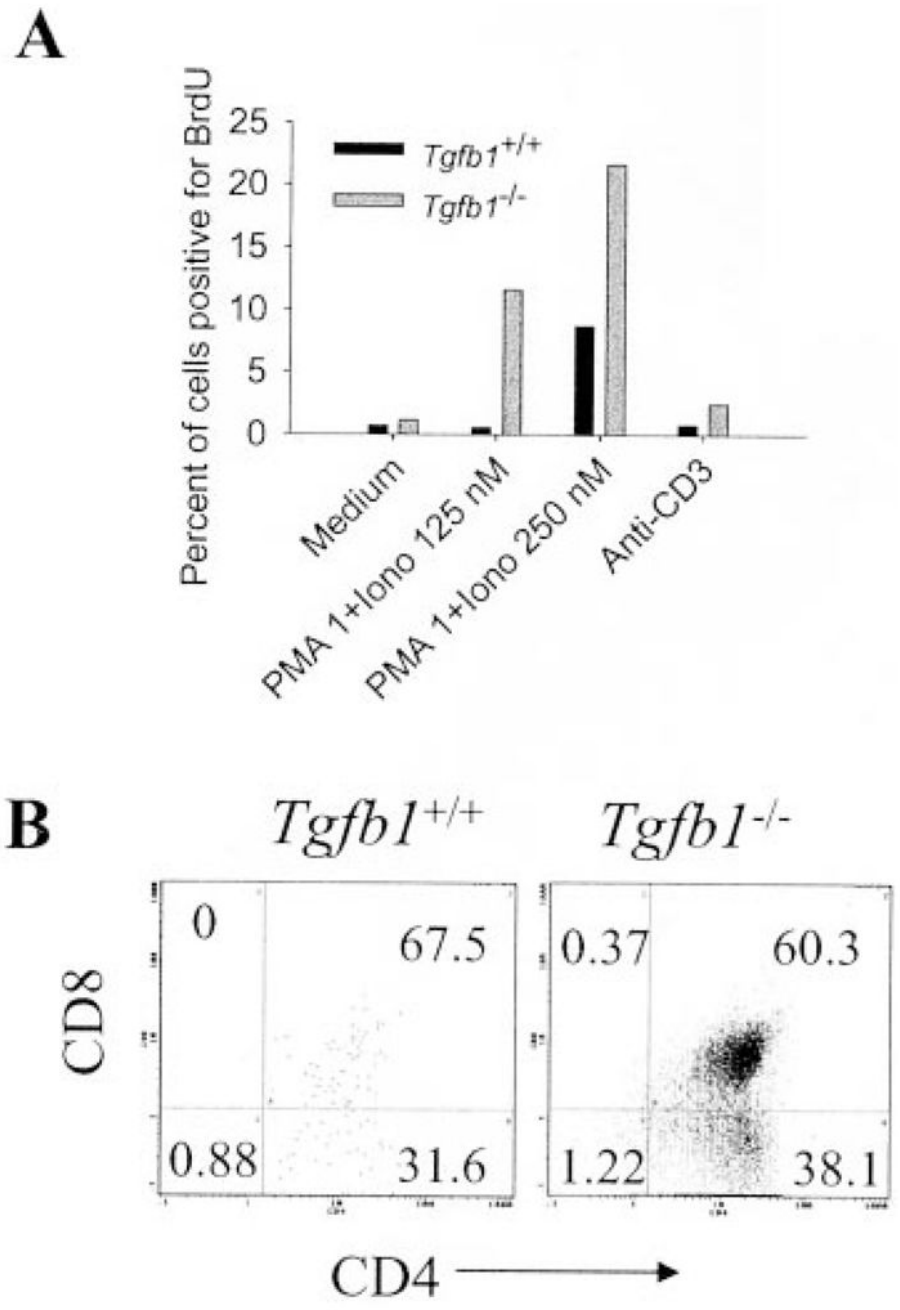

FIGURE 5.

CD4$^+$ SP *Tgfb1*$^{-/-}$ thymocytes initiate hyperresponsive phenotype. Thymocytes from day 9 mice were cultured in the presence of PMA plus ionomycin, anti-CD3 or medium, pulsed with the thymidine analog BrdU, and stained for surface Ags and BrdU. Percentage of cells positive for BrdU (*A*) and phenotype of 1 nM PMA plus 125 nM ionomycin-stimulated, BrdU-positive cells (*B*) was determined by FACS analysis as described in *Materials and Methods*. Note that both the immature DP and mature CD4$^+$ SP thymocytes are hyperresponsive.