CD7 and CD28 Are Required for Murine $CD4^+CD25^+$ Regulatory T Cell Homeostasis and Prevention of Thyroiditis[1]

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CD7 and CD28 are T cell Ig superfamily molecules that share common signaling mechanisms. To determine roles CD7 and CD28 might play in peripheral lymphocyte development and function, we have generated CD7/CD28-double-deficient mice. CD7- and CD28-single-deficient and CD7/CD28-double-deficient mice had normal levels of CD4 and CD8-single-positive T cells in thymus and spleen. However, CD28-deficient mice had decreased $CD4^+CD25^+$ T cells in spleen compared with wild-type mice, and CD7/CD28-double-deficient mice had decreased numbers of $CD4^+CD25^+$ T cells in both thymus and spleen compared with both wild-type and CD28-deficient mice. Functional studies demonstrated that $CD4^+CD25^+$ T cells from CD28-deficient and CD7/CD28-double-deficient mice could mediate suppression of CD3 mAb activation of $CD4^+CD25^-$ wild-type T cells, but were less potent than wild-type $CD4^+CD25^+$ T regulatory cells. Thyroiditis developed in aged CD7/CD28-double-deficient mice (>1 year) that was not seen in age-matched control mice or single CD7- or CD28-deficient mice, thus suggesting in vivo loss of T regulatory cells allowed for the development of spontaneous thyroiditis. Taken together, these data demonstrated collaborative roles for both CD7 and CD28 in determination of number and function of $CD4^+CD25^+$ T regulatory cells in the thymus and peripheral immune sites and in the development of spontaneous thyroiditis. ***The Journal of Immunology*, 2004, 172: 787–794.**

The CD28 molecule is a 44-kDa homodimeric member of the Ig gene superfamily (1–3) that is expressed on thymocytes, ~95% of peripheral $CD4^+$ T cells, and ~50% of peripheral $CD8^+$ T cells (3, 4). Triggering of CD28 by B7.1 and/or B7.2 on APCs enhances TCR T cell triggering, cytokine production, and effector functions (3, 5–8). Analysis of CD28-deficient animals has demonstrated that CD28 confers an enhanced ability to expand the peripheral T cell pool (9) and is important in augmenting peripheral production of IL-2, IFN-γ, and TNF-α (10–12).

CD7 is a 40-kDa member of the Ig gene superfamily that is expressed in T and NK cells (13). CD7 signals through phosphatidylinositol 3-kinase, induces TCR$\gamma\delta$ and NK cell IL-2, TNF-α, and IFN-γ production, and up-regulates cell adhesion molecule expression (13). Recent work has shown that the natural ligand for CD7 is a secreted molecule of epithelial, polymorphonuclear, and macrophage lineage cells called K-12 or SECTM-1 (14). In mice, the role of CD7 in activating T and NK cells is not well described due to lack of availability of anti-murine CD7 mAbs. However, CD7 deficiency in mice results in Ag-specific T cell triggering defects and defective generation of Ag-specific CTL (15) and protection from LPS-induced shock syndrome (16).

We have recently reported the generation of CD7/CD28-double-deficient mice and described the combined impact of CD7 and CD28 deficiency on murine thymocyte development and function (17). CD7/CD28-double-deficient animals were healthy, reproduced normally, had normal numbers of thymocyte subsets, and had normal thymus histology. However, CD7 and CD28 deficiencies combined to produce decreased numbers of B7.1/B7.2-expressing cells in the thymus, decreased thymocyte IL-2R expression (CD25), and decreased thymocyte CD3-triggered cytokine production (17).

$CD4^+CD25^+$ T regulatory cells are a unique lineage of thymus-derived cells that have immune suppressive effects on effector T cell function in vitro and in vivo. These cells represent ~5–10% of peripheral murine $CD4^+$ T cells, constitutively express the α-chain of the IL-2R (CD25), and mediate suppression of T cell activation by CD3 mAbs or allogeneic cells (18–20). Mice depleted of T regulatory cells by a variety of mechanisms develop organ-specific autoimmune diseases such as thyroiditis, gastritis, and inflammatory bowel disease (21).

Expression of CD28 has been shown to be important for maintaining the numbers of peripheral $CD4^+CD25^+$ T regulatory cells and for suppressing the development of diabetes in nonobese diabetic mice (22). However, the role of CD7 and its interaction with CD28 in immune regulation is unknown. In this study, we demonstrate that addition of CD7 deficiency to CD28 deficiency significantly decreased the number of $CD4^+CD25^+$ T cells in the thymus and spleen. Although the $CD4^+CD25^+$ T cells that are present in CD7/CD28-deficient mice have suppressive activity, the potency of $CD4^+CD25^+$ T regulatory cells in CD7/CD28-deficient mice was less than that of T regulatory cells in wild-type mice. Moreover, the combined deficiency of both CD7 and CD28 resulted in spontaneous thyroiditis in 1- to 2-year-old animals that was not observed in age-matched control mice.

Materials and Methods

Animals

C57BL/6 mice were obtained from either The Jackson Laboratory (Bar Harbor, ME) or Charles River-National Cancer Institute (Durham, NC). Homozygous CD7-deficient ($CD7^{-/-}$) mice were generated as described previously (15). Homozygous CD28-deficient ($CD28^{-/-}$) mice were obtained from Dr. C. Thompson (University of Pennsylvania, Philadelphia,

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PA) (23). Both CD7-deficient and CD28-deficient mouse lines have been backcrossed five generations onto C57BL/6. Homozygous CD7/CD28-double-deficient ($CD7^{-/-}$, $CD28^{-/-}$) mice were generated as described elsewhere (17). Confirmation of disrupted genes was performed using specific primers to amplify genomic tail DNA by PCR before use (17). Mouse handling and experimental procedures were conducted in accordance with Duke University Institutional Animal Care and Use Committee and American Association of Laboratory Animal Care Guidelines.

Antibodies

Hybridoma 145-2C11 (anti-murine CD3ϵ) was provided by Dr. J. Bluestone (University of Chicago, Chicago, IL) (24). mAb 37.51 (anti-murine CD28) was a gift from Dr. J. P. Allison (University of California, Berkeley, CA) (25). Hybridomas were cultured in serum-free medium (Life Technologies, Grand Island, NY). Anti-CD3 (2C11) was purified from hybridoma supernatant by protein G affinity chromatography (Pierce, Rockford, IL) and used at 0.5–1.0 μg/ml. Supernatant or purified Ab was used as indicated. Anti-mouse mAbs used for immunofluorescent staining were diluted in PBS wash (1× PBS, 1% BSA, and 0.1% NaN_3) and included CD3-FITC, CD4-PE, CD4-PE-CyChrome, CD8α-PE-CyChrome, CD8α-allophycocyanin, CD25-PE, CD25-allophycocyanin, CD80-PE (B7.1), and CD86-FITC (B7.2 BD Biosciences, Franklin Lakes, NJ).

Lymph node cell and splenocyte isolation

Lymph nodes (superficial cervical, mandibular, axillary, inguinal, and mesenteric) and spleens were harvested from 8- to 12-wk-old male mice. Lymph nodes or spleens were teased with a 1-ml tuberculin syringe plunger (BD Biosciences) through a sterile 70-μm nylon cell strainer (BD Biosciences) to achieve single-cell suspensions. Each cell suspension was diluted to a final volume of 10 ml with sterile RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 1× gentamicin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (complete medium). The respective cell suspensions were centrifuged for 5 min at 1500 rpm. After removal of the supernatant, cells were resuspended in 5 ml of sterile ACK RBC lysing buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.4, filtered through a 0.2-μm membrane) and thoroughly mixed. The lysing buffer was quenched with 10 ml of complete medium and centrifuged for 5 min at 1500 rpm. Each cell type was resuspended in 10 ml of complete medium and counted with a Coulter Z1 cell counter (Beckman Coulter, Miami, FL).

Isolation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells

Two protocols were used to isolate $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from wild-type and deficient animals for in vitro functional assays. The first protocol enriched effector and regulatory T cells from lymph node cells using T Cell Enrichment Columns (R&D Systems, Minneapolis, MN) and Miltenyi beads (Miltenyi Biotec, Auburn, CA). The second protocol obtained high-purity populations of T cells by FACS.

For the enrichment protocol, T cells were isolated from lymph node cell suspensions using commercial T Cell Enrichment Columns (R&D Systems). $CD4^+$ T cells were then isolated with the Miltenyi $CD4^+$ T cell isolation system and separated using LS columns according to the manufacturer's specifications (Miltenyi Biotec). The $CD4^+$ T cells were counted, washed, centrifuged (1500 rpm for 5 min), and incubated (30 min, 4°C) in Miltenyi MACS buffer with saturating amounts of rat anti-mouse CD25-PE Ab (clone PC61; BD PharMingen, San Diego, CA). Cells were washed with MACS buffer before centrifugation (1500 rpm for 5 min). The cells were incubated (20 min, 4°C) with anti-PE microbeads (Miltenyi Biotec), washed, and centrifuged in MACS buffer before placing on MS columns (Miltenyi Biotech). $CD4^+CD25^-$ T cells were collected, centrifuged (1500 rpm for 5 min), and resuspended in complete medium. $CD4^+CD25^+$ T cells were eluted from the MS columns with MACS buffer, centrifuged, and counted. The purity of each enriched cell population was determined by immunophenotyping/flow cytometry and for $CD4^+CD25^-$ cells was: C57BL/6 (93 ± 6%, $n = 7$), CD7 deficient (92 ± 9%, $n = 7$), CD28 deficient (92 ± 9%, $n = 5$), and CD7/CD28 deficient (97 ± 1%, $n = 4$) and for $CD4^+CD25^+$ cells was: C57BL/6 (47 ± 16%, $n = 7$), CD7 deficient (47 ± 7%, $n = 7$), CD28 deficient (33 ± 6%, $n = 5$), and CD7/CD28 deficient (24 ± 9%, $n = 4$).

For the FACS protocol, lymph node cell suspensions were enriched for $CD4^+$ T cells as described above. The $CD4^+$ T cells from each mouse strain were counted, washed, centrifuged (1500 rpm for 5 min), and incubated (30 min, 4°C) in azide-free PBS plus 1% BSA with saturating amounts of rat anti-mouse CD25-PE and CD4-PE-CyChrome Abs (BD PharMingen). Cells were washed with azide-free PBS plus 1% BSA buffer before FACS separation at 4°C into $CD4^+CD25^-$ and $CD4^+CD25^+$ fractions on a FACSVantage SE-Diva flow cytometer (BD Biosciences). Cells were counted, spun down, and resuspended in 4°C complete medium at 10^6 cells/ml for functional assays. The purity of each enriched cell population was determined by immunophenotyping/flow cytometry and for $CD4^+CD25^-$ cells was: C57BL/6 (95 ± 3%, $n = 4$), CD28 deficient (98 ± 2%, $n = 3$), and CD7/CD28 deficient (97 ± 3%, $n = 4$) and for $CD4^+CD25^+$ cells was: C57BL/6 (95 ± 4%, $n = 4$), CD28 deficient (96 ± 3%, $n = 3$), and CD7/CD28 deficient (91 ± 1%, $n = 4$). Consistent with the surface phenotype and proliferative potential of regulatory T cells, the isolated $CD4^+CD25^+$ cells used in in vitro assays were $CD69^-$ $CD44^{int}$, displayed a memory phenotype of $CD62L^-CD45RB^-$, and were anergic when stimulated with Con A or T cell-depleted accessory cells plus anti-CD3 in vitro (data not shown).

Isolation of T cell-depleted accessory cells

Single-cell suspensions of RBC-depleted splenocytes from each mouse strain (e.g., wild-type, CD7 deficient, CD28 deficient, CD7/CD28 double deficient) were isolated as indicated above. Splenocytes were incubated with a mixture of biotin-conjugated Abs (CD45R, DX5, CD11b, and Ter-119) and anti-biotin microbeads (Pan T Cell Isolation kit; Miltenyi Biotec). Cells were washed, resuspended in 500 μl of MACS buffer, and placed over an MS column. T cell-depleted splenocytes were eluted from the MS column with MACS buffer, counted, and resuspended in complete medium to a final concentration of 1×10^6 cells/ml before irradiation (3000 rad).

Regulatory T cell functional assays

The suppressive properties of the $CD4^+CD25^+$ phenotype T cells were demonstrated for each mouse strain according to previously published methods utilizing an accessory cell-dependent proliferation assay that was augmented by CD3 triggering (26, 27). Briefly, $CD4^+CD25^-$ (responders) cells were cultured in 96-well round-bottom plates (final volume, 200 μl) with accessory cells (stimulators), 0.5 μg/ml purified anti-CD3 (2C11), and the indicated number of either $CD4^+CD25^+$ cells or $CD4^+CD25^-$ cells (regulators and cell-density control cells, respectively). The plates were incubated at 37°C in 7% CO_2 for 3 days. Cultured cells were pulsed with [^{3}H]thymidine (1 μCi/well; NEN, Boston, MA) for the final 6 h of culture and tritium incorporation was measured as described previously (15, 17). Percent suppression was calculated by the formula: percent suppression = 1 − (cpm with $CD4^+CD25^+$ T cells added)/(cpm with $CD4^+CD25^-$ T cells added) × 100.

Phenotypic analysis of lymphocytes by flow cytometry

Single-cell suspensions of lymph node cells and splenocytes were analyzed by flow cytometry as described elsewhere (15, 17).

Mitogen proliferation assays

Mitogen proliferation assays were performed as described previously (15, 17).

LPS-induced shock

Endotoxic shock was induced in 8-to 10-wk-old male mice by i.p. injection of low-dose LPS plus D-galactosamine (D-gal;[3] Sigma-Aldrich, St. Louis, MO) as previously described (16). Animals received 1 μg LPS and 8 mg D-gal in 0.5 ml of saline. For time course experiments, blood was taken from the retro-orbital plexus under anesthesia. Sera were stored at −20°C for cytokine determinations.

Cytokine ELISA

Quantification of murine IFN-γ and TNF-α present in mouse serum was determined using commercial cytokine-specific ELISA kits (Genzyme, Cambridge, MA).

Histological examination of thyroids

Thyroids were harvested at sacrifice from aged cohorts of wild-type, CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient mice. Tissue was fixed in Formalin, embedded in paraffin, and sectioned. Tissue sections were stained with H&E or immunohistochemically stained with anti-CD3 or anti-mouse Ig (DakoCytomation, Carpinteria, CA) as previously described (28). Slides were subjected to microscopic examination and photographed. The presence of thyroiditis was determined based on the degree of leukocyte infiltration and thyroid follicle destruction.

Statistics

Student's *t* test was used to determine *p* values at a two-tailed level.

[3] Abbreviations used in this paper: D-gal, D-galactosamine; SP, single positive.

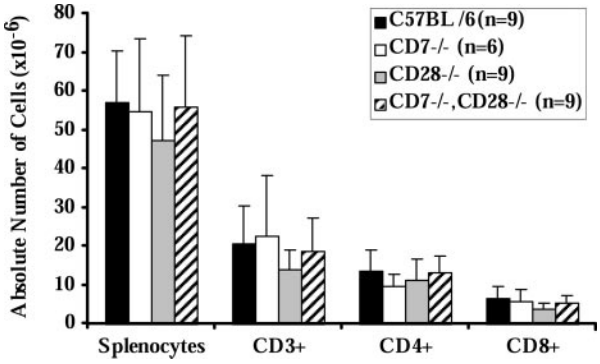

FIGURE 1. Quantitation of peripheral T cell subsets in CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient mice. Splenocytes were isolated from all four strains, counted, and surface marker expression analyzed by flow cytometry. Absolute number of T cells or T cell subsets was determined by multiplying the total number of splenocytes by the frequency of $CD3^+$, $CD3^+CD4^+$, or $CD3^+CD8^+$ T cells ($CD3^+$ gate). Data are mean ± SEM.

Results

CD7/CD28-double-deficient mice have normal levels of $CD4^+$ and $CD8^+$ peripheral T cells

To evaluate CD7/CD28-double-deficient peripheral lymphoid cells, we determined the percentage and absolute number of $CD4^+$ and $CD8^+$ T cells in splenocytes from C57BL/6 wild-type, CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient mice. No significant differences were observed in spleen histology nor frequency of splenocyte populations (B cells or myeloid cells) among the four groups of animals studied (data not shown). Analysis of absolute numbers of splenocytes, T cells, and T cell subsets indicated normal levels of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in CD7/CD28-double-deficient mice compared with wild-type, CD7-, or CD28-single-deficient animals (Fig. 1).

CD7/CD28-double-deficient mice have decreased B7.1- and B7.2-expressing APCs in spleen

We next determined whether CD7/CD28-double-deficient mice had normal levels of B7.1 and B7.2-expressing cells in spleen. We found that CD28-deficient and CD7/CD28-double-deficient mice had decreased $B7.2^+$ spleen cells compared with wild-type mice, and CD7/CD28-double-deficient mice had decreased numbers of $B7.1^+$ as well as B7.1/B7.2-double-positive spleen cells compared with wild-type mice (Table I).

Addition of CD7 deficiency to CD28 deficiency had no effect on in vitro splenocyte proliferative responses to Con A, LPS, or anti-CD3 plus anti-CD28

Mitogenic responses of freshly isolated splenocytes from wild-type, CD7-, CD28-, and CD7/CD28-deficient mice were next examined to determine the impact of CD7 deficiency on CD28 deficiency regarding T cell activation assays. Similar to data reported for CD28-deficient mice (23), we found both CD28-deficient and CD7/CD28-deficient splenocytes had significantly reduced proliferative responses to Con A (Fig. 2*A*), anti-CD3 alone, or anti-CD3 plus anti-CD28 triggering (Fig. 2*C*) compared with wild-type mice ($p \leq 0.05$) and compared with CD7-deficient mice ($p \leq 0.05$). In contrast, no significant differences in splenocyte proliferation among the four mouse strains were observed with LPS stimulation (Fig. 2*B*). These observations suggest that neither CD7 nor CD28 play a role in modulating LPS-induced signaling resulting in splenocyte proliferation. It is important to note that unlike the T cell mitogens Con A and anti-CD3, it is possible that the LPS-responsive cells in our in vitro proliferation assays are B cells and are therefore unaffected by the absence of CD7 or CD28. Taken together, these data demonstrated that combined disruption of CD7 and CD28 did not alter the splenocyte proliferative response to LPS, nor did the addition of CD7 deficiency to CD28 deficiency modulate the reduced proliferative response of CD28-deficient splenocytes to Con A or CD3 triggering.

CD7/CD28 double deficiency partially rescued impaired in vivo cytokine responses to LPS seen in single gene-deficient mice

Since both CD7-deficient and CD28-deficient mice are resistant to endotoxin shock (16, 29), we studied CD7/CD28-double-deficient mice in a lethal low-dose LPS endotoxic shock model (Fig. 3). As previously reported for this model, 72 h after i.p. injection with 8 mg D-gal and 1 μg LPS, 20% of C57BL/6 wild-type animals ($n = 10$) remained alive (16). CD7-deficient, CD28-deficient and CD7/CD28-double-deficient mice were fully resistant to the LPS/D-gal challenge, with 100% of the animals in each group surviving after 72 h ($n = 10$/group; $p < 0.005$ vs wild type). LPS/D-gal induced a peak serum TNF-α response at 2 h (Fig. 3*A*) and a peak serum IFN-γ response at 6 h (Fig. 3*B*) in wild-type control animals (16). Peak serum TNF-α responses in CD7-and CD28-single-deficient mice were significantly reduced vs wild-type mice ($p < 0.05$). There was a complete abrogation of serum IFN-γ response in CD7- and CD28-single-deficient mice ($p < 0.05$ vs wild type).

CD7/CD28-double-deficient mice also had significantly blunted peak serum TNF-α and IFN-γ responses vs wild-type control ($p < 0.05$); however, double-deficient animals had significantly greater peak serum TNF-α and IFN-γ responses when compared with single-deficient control animals ($p < 0.05$; Fig. 3). Although no difference was seen in in vitro responsiveness of splenocytes from the single- and double-deficient mice to LPS (Fig. 2), the in vivo response to LPS was significantly enhanced in double-deficient mice compared with CD28-single-deficient mice. These data support the hypothesis that signals from CD7 and CD28 are required for LPS-induced cytokine production but not proliferation. The cells involved in LPS-induced proliferation are distinct from the cells producing in vivo cytokines in response to LPS. In this regard, in the

Table I. *Frequency of B7.1 and B7.2-positive cells in fresh wild-type, CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient splenocytes*[a]

	$B7.1^+$		$B7.2^+$		$B7.1^+/B7.2^+$	
Splenocyte Source	%	Absolute no. ($\times 10^6$)	%	Absolute no. ($\times 10^6$)	%	Absolute no. ($\times 10^6$)
C57BL/6 (wild-type)	10 ± 1	8.0 ± 1.1	7 ± 1	5.7 ± 1.0	15 ± 2	11.9 ± 0.8
$CD7^{-/-}$	10 ± 1	6.9 ± 1.2	6 ± 1	4.3 ± 0.6	15 ± 2	10.4 ± 1.4
$CD28^{-/-}$	8 ± 1	6.7 ± 1.7	4 ± 0[b]	3.0 ± 0.6[b]	11 ± 2	8.8 ± 2.6
$CD7^{-/-}/CD28^{-/-}$	8 ± 1	4.5 ± 0.9[b]	3 ± 0[b,c]	1.9 ± 0.2[b,c]	12 ± 1	6.7 ± 1.6[b]

[a] Data are mean ± SEM, $n = 3$.
[b] $p \leq 0.05$ compared to C57BL/6 mice.
[c] $p \leq 0.05$ compared to $CD7^{-/-}$ mice.

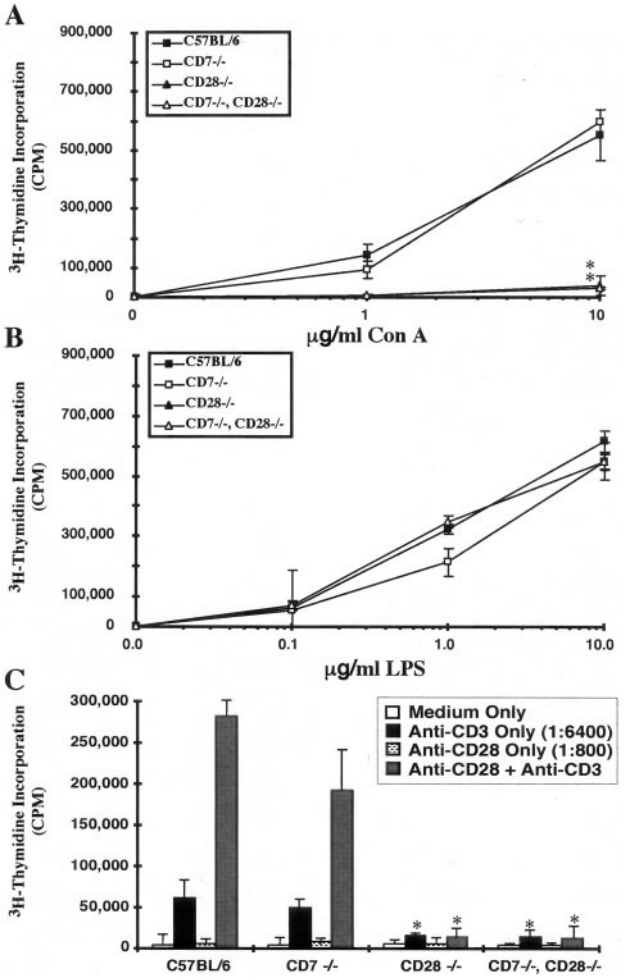

FIGURE 2. Proliferative response of CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient splenocytes to mitogenic stimulation. Splenocytes were isolated and cultured with either Con A (*A*) or LPS (*B*) for 3 days at 10^6 cells/ml as described in *Materials and Methods*. *C*, Splenocytes were cultured with medium alone, anti-CD3 only, anti-CD28 only, or anti-CD3 plus anti-CD28 for 3 days at 10^6 cells/ml as described in *Materials and Methods*. Data are expressed in cpm/10^6 splenocytes. Data are mean cpm/10^6 splenocytes ± SEM of three age-matched male mice and are representative of two to three separate experiments. ∗, $p \leq 0.05$ vs C57BL/6 and CD7-deficient mice.

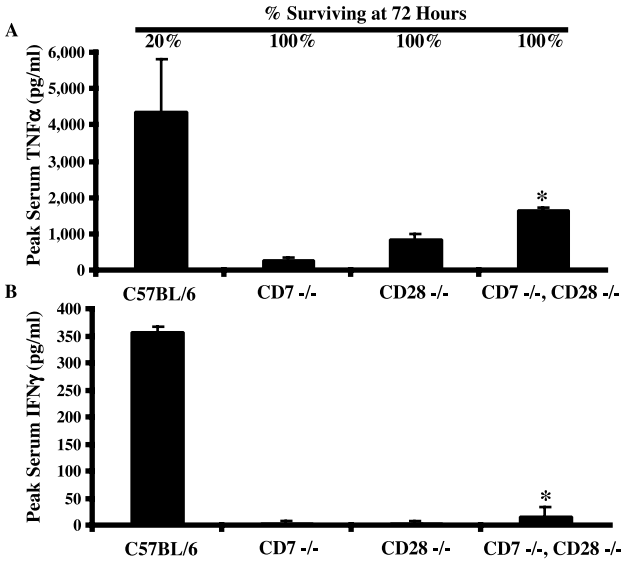

FIGURE 3. CD7/CD28-deficient mice have decreased LPS-induced peak serum cytokine responses vs wild-type mice. Animals (n = 3/group) were treated with 1 μg LPS and 8 mg D-gal to induce septic shock. *A*, Peak serum TNF-α (2 h) and *B*, peak serum IFN-γ (6 h) was determined for each group. Data are mean ± SEM of three age-matched male mice. The percentage of surviving animals after 72 h is shown in *A* (n = 10/group). ∗, $p \leq 0.05$ vs C57BL/6, CD7-deficient, and CD28-deficient mice.

low-dose LPS shock model, IFN-γ and TNF-α production by liver NK-T cells has been shown to be critical for the induction of the shock syndrome (30). In addition, in CD7-deficient mice, this population is markedly decreased (16). Therefore, in vitro LPS proliferation does not reflect the abnormalities that lead to defective cytokine production in vivo in CD7- or CD7/CD28-deficient mice.

A possible mechanism for the partial restoration of cytokine production in response to LPS (Fig. 3) is a reduction of circulating T regulatory cells in the double-deficient animals. Loss of T regulatory cell suppression may result in increased in vivo cytokine responses to LPS. Therefore, analysis of T regulatory cell number and function in the four strains of animals was next examined.

CD7/CD28-double-deficient mice have reduced numbers of $CD4^+CD25^+$ regulatory phenotype T cells in thymus and spleen

We have previously reported that CD7/CD28-double-deficient mice have a significantly reduced density of CD25 on thymocyte subsets (17). To determine whether peripheral T cells also express diminished CD25, the median fluorescence intensity of CD25 expression on freshly isolated splenic $CD4^+$ and $CD8^+$ T cells ($CD3^+$) was quantitated (Fig. 4*A*). There were no significant differences in CD25 expression on $CD8^+$ peripheral T cells from CD7- or CD28-single-deficient mice nor CD7/CD28-double-deficient mice. However, analysis of CD25 expression on $CD4^+$ peripheral T cells revealed significantly lower CD25 expression in the $CD4^+$ T cell compartment from CD28-deficient vs wild-type mice ($p \leq 0.05$) and on $CD4^+$ splenic T cells from CD7/CD28-double-deficient mice vs either wild-type, CD7-single-deficient or CD28-single-deficient mice ($p \leq 0.05$). Representative two-parameter histograms of $CD3^+$ gated splenocytes show that this loss of CD25 staining may be due to a loss in the frequency of $CD25^+CD4^+$ T cells in these animals (Fig. 4*B*).

The thymus is the source of $CD4^+CD25^+$ T regulatory cells in early life. These cells reside in the $CD3^+$, CD4-single-positive (CD4SP) thymocyte compartment (21). First, we determined the absolute number of $CD3^+CD4SPCD25^+$ thymocytes in wild-type, CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient mouse thymuses (Fig. 4*A*). We found significantly decreased absolute numbers of $CD3^+CD4SPCD25^+$ thymocytes in CD7/CD28-double-deficient mice compared with wild-type and compared with CD7- or CD28-single-deficient mice ($p \leq 0.004$; Fig. 4*C*).

Next, the absolute number of $CD4^+CD25^+$ T cells was evaluated in the periphery of the four mouse strains (Fig. 4*D*). The absolute number of $CD4^+CD25^+$ T regulatory phenotype cells in spleen was significantly decreased in both CD28-deficient and CD7/CD28-double-deficient mice compared with wild-type mice ($p \leq 0.05$). Furthermore, CD7/CD28-double-deficient mice had significantly lower numbers of $CD4^+CD25^+$ T cells per spleen when compared with either CD7- or CD28-single-deficient mice ($p \leq 0.004$ Fig. 4*D*). Thus, the combined deficiency of both CD7 and CD28 resulted in decreased numbers of $CD4^+CD25^+$ regulatory phenotype T cells in thymus and spleen.

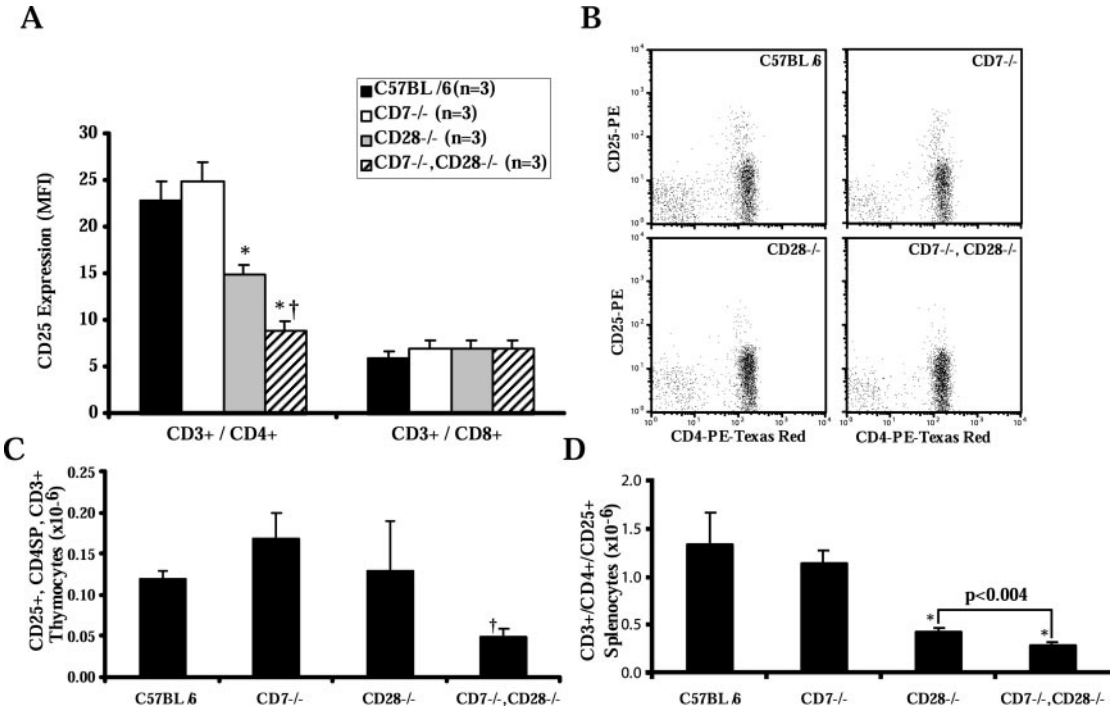

FIGURE 4. CD7/CD28-double-deficient mice have reduced absolute numbers of $CD4^+CD25^+$ T regulatory phenotype cells. *A*, Median fluorescence intensity (MFI) of CD25 expression on $CD4^+$ or $CD8^+$ T cells. *B*, Representative CD4/CD25 histogram plots of $CD3^+$ gated splenocytes from the four strains of mice. *C*, Freshly isolated thymocytes from C57BL/6 wild-type and CD7-, CD28-, and CD7/CD28-deficient mice were phenotyped by flow cytometry (CD3/CD4/CD8/CD25). Ten thousand events were analyzed per thymocyte preparation to determine the frequency of $CD3^+$, CD4SP, $CD25^+$ cells. The number of $CD3^+CD4SP/CD25^+$ T regulatory phenotype cells was calculated using the absolute number of thymocytes isolated from each animal. *D*, Freshly isolated splenocytes from wild-type and CD7-, CD28-, and CD7/CD28-deficient mice were phenotyped as above. Data are mean ± SEM (n = 3/group). ∗, $p \leq 0.05$ vs C57BL/6; †, $p \leq 0.05$ vs C57BL/6, CD7-deficient and CD28-deficient mice.

$CD4^+CD25^+$ T cells from CD7/CD28-deficient mice have present but decreased in vitro suppressive function

To evaluate the functional properties of $CD4^+CD25^+$ T cells in CD7-deficient, CD28-deficient, and CD7/CD28-double-deficient mice, we used a CD3 triggering assay previously described by Thornton and Shevach (Refs. 26 and 27 and Fig. 5). Lymph node $CD4^+CD25^-$ T cells were stimulated to proliferate in the presence of irradiated accessory cells (T cell-depleted splenocytes) plus anti-CD3 mAb (2C11). Lymph node T regulatory phenotype cells ($CD4^+CD25^+$) were then added in different ratios vs $CD4^+CD25^-$ control cells to determine percent suppression.

We found that the dose-dependent suppressive capability of $CD4^+CD25^+$ T cells from CD7-deficient mice was comparable to that of wild-type mice in assays with autologous responders, stimulators, and regulators (Fig. 5*A*). However, responder $CD4^+CD25^-$ T cells isolated from either CD28-single-deficient or CD7/CD28-double-deficient animals gave weak proliferative responses to CD3 mAb that prevented evaluation of suppressive effects of isolated $CD4^+CD25^+$ T cells from CD28- and CD7/CD28-deficient mice (Fig. 2*C* and Ref. 23).

To circumvent this complication, we tested the ability of $CD4^+CD25^+$ T regulatory cells from CD28-deficient and CD7/CD28-double-deficient mice for their ability to suppress $CD4^+CD25^-$ T cells from C57BL/6 wild-type mice triggered with accessory cells plus CD3 mAbs (Fig. 4*B*). $CD4^+CD25^+$ cells from either CD28-single-deficient or CD7/CD28-double-deficient mice were added to wild-type $CD4^+CD25^-$ responder T cells mixed with wild-type irradiated accessory cells plus anti-CD3 mAb. Percent suppression for lymph node T regulatory cells in this assay are shown in Fig. 5*B*. Now with a robust $CD4^+CD25^-$ wild-type T cell proliferative response, we were able to evaluate the suppressive capabilities of CD28-single-deficient and CD7/CD28-double-deficient $CD4^+CD25^+$ T cells vs wild-type $CD4^+CD25^+$ T cells. At the highest cell concentrations of $CD4^+CD25^+$ T cells from CD28-single-deficient and CD7/CD28-double-deficient mice (1:1 or 1:2 cell ratios), the suppressive effects were not statistically different from those of $CD4^+CD25^+$ T cells from wild-type mice, showing that the $CD4^+CD25^+$ T cells present in CD28-deficient and CD7/CD28-double-deficient mice had measurable suppressive activity. However, at the lowest ratio of $CD4^+CD25^+$ to $CD4^+CD25^-$ cells (1:10), CD7/CD28-deficient $CD4^+CD25^+$ T cell suppressive activity was significantly less than wild-type and CD28-single-deficient $CD4^+CD25^+$ T cells (Fig. 5*B*), suggesting decreased potency of CD7/CD28-double-deficient lymph node T regulatory cells.

Because the $CD4^+CD25^+$ T regulatory cell populations used in the experiments in Fig. 4*B* from CD28-single-deficient and CD7/CD28-double-deficient mice were magnetic bead enriched and were <90% pure, the data in Fig. 4*B* may not adequately analyze the potency of CD7/CD28-deficient T regulatory cells compared with wild-type cells. Thus, we determined in vitro suppressive activity with FACS-purified wild-type, CD28-deficient, and CD7/CD28-double-deficient lymph node $CD4^+CD25^+$ T cells (Fig. 5*C*). With highly purified populations of both $CD4^+CD25^-$ responders and $CD4^+CD25^+$ T regulatory cells in the assay (C57BL/6 $CD4^+CD25^+$ cells = 95 ± 4%, CD28-deficient $CD4^+CD25^+$ cells = 98 ± 2%, and CD7/CD28-deficient $CD4^+CD25^+$ cells = 91 ± 1%), we observed statistically significant less suppression of CD3-triggered $CD4^+CD25^-$ T cell activation by CD28-deficient and CD7/CD28-double-deficient $CD4^+CD25^+$ cells compared with wild-type control assays. This was apparent at $CD4^+CD25^+$ to $CD4^+CD25^-$ cell ratios of 1:10,

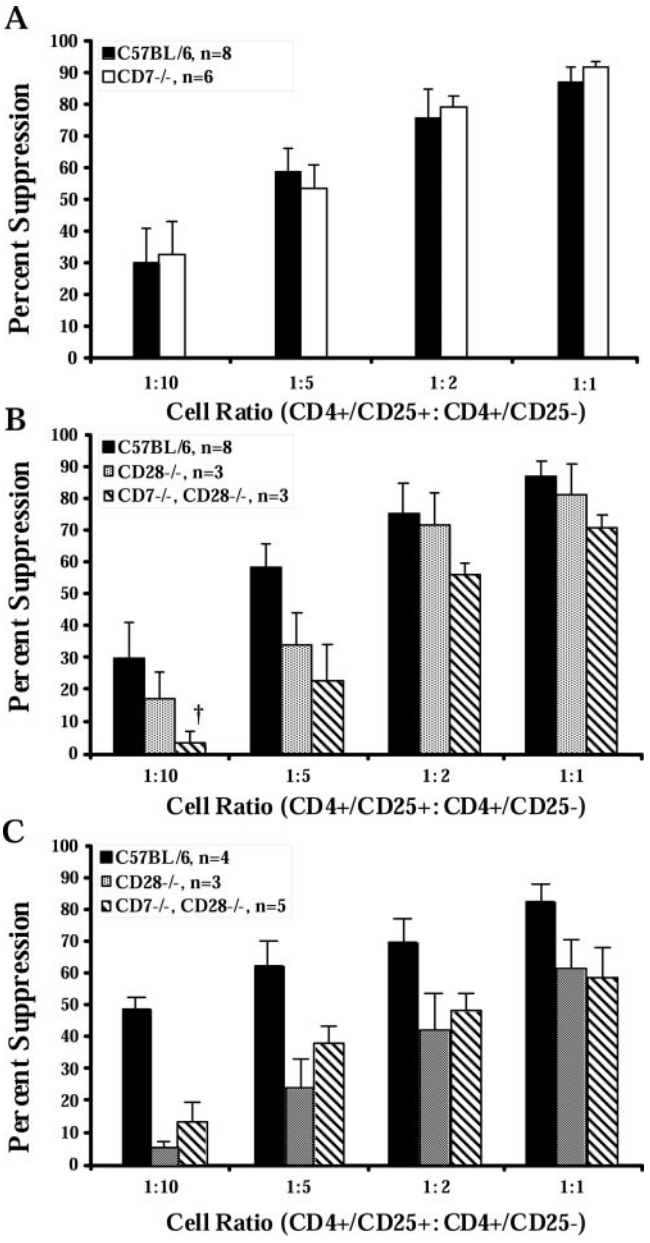

FIGURE 5. T regulatory cells from CD7/CD28-double-deficient mice have reduced suppressive function. *A,* Ability of enriched $CD4^+CD25^+$ lymph node T cells from either C57BL/6 or CD7-deficient mice to suppress autologous $CD4^+CD25^-$ T cell proliferative responses to irradiated accessory cells plus CD3 mAb as described in *Materials and Methods*. Plotted are the mean percent suppression ± SEM. Mean peak [^{3}H]thymidine responses of $CD4^+CD25^-$ T cells to CD3 mAb was 499,244 cpm/10^6 cells for C57BL/6 and 497,947 cpm/10^6 cells for CD7-deficient mice. *B,* C57BL/6 CD3 triggered base MLR to assess the in vitro potency of T regulatory cells from CD28-deficient and CD7/CD28-double-deficient mice (see *Materials and Methods*). Peak [^{3}H]thymidine response of $CD4^+CD25^-$ cells to CD3 from C57BL/6 cells was 499,244 cpm/10^6 cells, from CD28-deficient mice was 454,331 cpm/10^6 cells, and from CD7/CD28 deficient mice was 242,865 cpm/10^6 cells. Plotted are the mean percent suppression ± SEM. ∗, $p \leq 0.05$ vs C57BL/6; †, $p \leq 0.05$ vs CD28-deficient mice. *C,* FACS-sorted T regulatory phenotype cells from CD7/CD28-double-deficient mice have decreased suppressive function compared with C57BL/6 mouse-derived T regulatory phenotype cells (see *Materials and Methods*). Plotted are the mean percent suppression ± SEM. The peak [^{3}H]thymidine response to CD3 mAb of $CD4^+CD25^-$ cells from C57BL/6 was 133,062 cpm/10^6 cells, from CD28-deficient mice was 291,972 cpm/10^6, and from CD7/CD28-deficient mice was 394,872 cpm/10^6 cells. ∗, $p \leq 0.05$ vs C57BL/6.

1:5, and 1:2 (Fig. 5*C*). There were no significant differences in percent suppression between the CD28- and CD7/CD28-deficient groups. These data indicated that significantly more T regulatory cells were required from CD28- or CD7/CD28-deficient mice to achieve a comparable level of in vitro suppression to that from wild-type mice, thus confirming decreased potency of CD28-deficient and CD7/CD28-double-deficient lymph node T regulatory cells compared with wild-type T regulatory cells. These data suggest no additive impact of CD7 deficiency to the potency of CD28-deficient $CD4^+CD25^+$ T regulatory cells.

Aged CD7/CD28-double-deficient mice develop spontaneous thyroiditis

$CD4^+$ T regulatory cells are reported to regulate peripheral levels of $CD4^+$ T cells and to suppress organ-specific autoimmune disease (21). Neither CD7-deficient, CD28-deficient, nor CD7/CD28-double-deficient mice (6–119 wk) presented with elevated levels of peripheral $CD4^+$ T cells, shortened life span, decreased reproduction, or weight loss (data not shown). However, histologic analysis of the thyroid gland from aged (55–119 wk) C57BL/6 wild-type, CD7-deficient, CD28 deficient-, and CD7/CD28-double-deficient animals revealed spontaneous thyroiditis in 50% of the CD7/CD28-deficient mice compared with none in age-matched wild-type or control animals ($p \leq 0.003$; Table II and Fig. 6). Representative H&E-stained sections from the four strains of mice are shown in Fig. 6, *A–D*. Thyroid tissue sections from affected CD7/CD28-double-deficient mice were immunohistochemically stained with anti-CD3 and anti-mouse Ig to characterize lymphocytes in the leukocyte infiltrate. Both $CD3^+$ T cells and intracytoplasmic Ig^+ B cells were detected in the inflamed thyroid tissues of aged CD7/CD28-double-deficient mice (Fig. 6, *D* and *E*, respectively). These data suggested that in vivo loss of peripheral T regulatory cells allowed for the development of spontaneous thyroiditis in the CD7/CD28-double-deficient mouse model.

Discussion

In this study, we have shown that CD7/CD28-double-deficient mice have decreased numbers of thymus $CD4^+CD25^+$ T regulatory cells compared with CD7-single-deficient, CD28-single-deficient, and wild-type mice. Similarly, we found that CD7/CD28-double-deficient mice had decreased numbers of peripheral splenic $CD4^+CD25^+$ T cells and significantly reduced numbers of $B7.1^+$ as well as B7.1/B7.2-double-positive splenocytes compared with control animals. We demonstrated that CD28-deficient and CD7/CD28-double-deficient $CD4^+CD25^+$ T cells were less potent suppressors of CD3-triggered $CD4^+CD25^-$ T cells compared with those from wild-type mice. Lastly, we reported for the first time that aged CD7/CD28-double-deficient mice develop spontaneous

Table II. *Incidence of thyroiditis in aged gene-deficient mice*

Mouse Line (mean age ± SEM in weeks)	No. of Animals with Leukocyte Infiltration and Follicle Damage/Total No. Analyzed	p^a
C57B1/6 (58 ± 0)	0/9	
$CD7^{-/-}$ (73 ± 3)	0/6	
$CD28^{-/-}$ (82 ± 11)	0/7[b]	
$CD7^{-/-}/CD28^{-/-}$ (66 ± 20)	4/8	0.003

[a] Significance of thyroid disease severity vs wild-type or single gene-deficient controls.

[b] One CD28-deficeint animal had a single isolated foci of leukocyte infiltration with other areas on multiple sections showing normal histology.

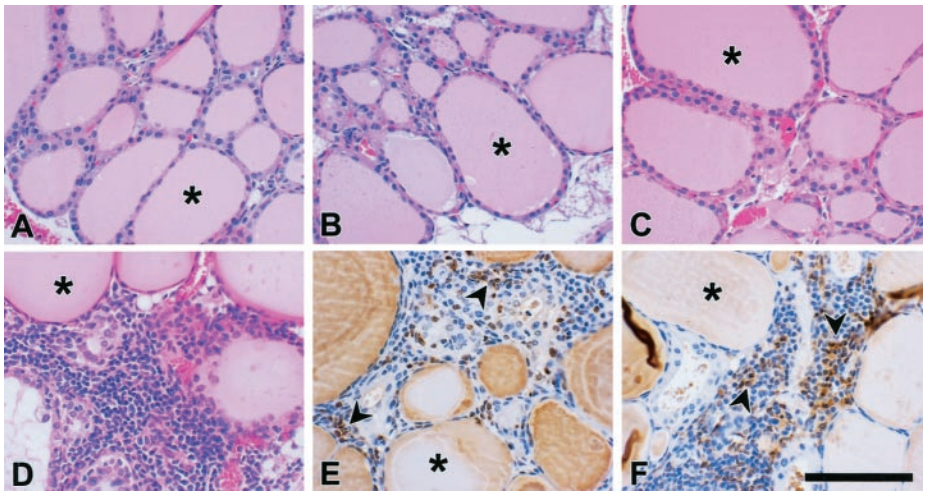

FIGURE 6. Thyroiditis with T and B cell infiltrate is present in aged CD7/CD28-double-deficient mice, but not age-matched control animals. *A,* H&E-stained C57BL/6 wild-type thyroid tissue. *B,* H&E-stained CD7-deficient thyroid tissue. *C,* H&E-stained CD28-deficient thyroid tissue. *D,* H&E-stained CD7/CD28-double-deficient thyroid tissue. *E,* CD7/CD28-double-deficient thyroid tissue stained with anti-CD3. *F,* CD7/CD28-double-deficient thyroid tissue stained with anti-mouse Ig. Brown stain within thyroid follicles (*E* and *F,* labeled with an ∗) is nonspecific and was in control sections. Arrows point out $CD3^+$ T cells (*E*) and intracytoplasmic Ig^+ B cells (*F*). All sections were photographed at the same magnification. A 100-μm bar is placed in *F* for reference.

thyroiditis, with T and B cell infiltrates, where as age-matched CD7-single-deficient, CD28-single-deficient, and wild-type mice did not.

The observation that CD7- and CD28-single-deficient mice had normal numbers of thymic $CD4^+CD25^+$ T cells in our study suggested that thymic production of T regulatory cells is not exclusively dependent on either CD7 or CD28. However, the combined deletion of both CD7 and CD28 significantly decreased the absolute number of thymic T regulatory phenotype cells. We recently reported that CD7 and CD28 deficiencies combined to produce decreased thymocyte IL-2R expression and decreased numbers of B7.1/B7.2-expressing cells in the thymus (17). Together, these findings suggest a cooperative role for CD7 and CD28 in thymic production and/or maintenance of $CD4^+CD25^+$ T regulatory cells. It has been shown that CTLA-4-Ig administration to nonobese diabetic mice depletes the thymus of $CD4^+CD25^+$ T cells, suggesting that CD28 engagement by B7.1/B7.2 is critical for thymic production of $CD4^+CD25^+$ T cells (31). One hypothesis is that loss of CD7-CD7 ligand interactions combined with loss of CD28-B7.1/B7.2 interactions leads to lack of generation and/or survival of $CD4^+CD25^+$ T cells in the thymus.

Although it has been previously reported that CD28-deficient mice have decreased numbers of peripheral $CD4^+CD25^+$ T cells (22), our study showed that addition of CD7 deficiency to CD28 deficiency added to this phenotype and further decreased the peripheral number of $CD4^+CD25^+$ T cells in the double-deficient animals. In addition, we demonstrated that deletion of both CD7 and CD28 results in significantly reduced numbers of $B7.1^+$ and B7.1/B7.2-double-positive splenocytes.

Three mechanisms have been postulated to reduce peripheral $CD4^+CD25^+$ T cell levels: decreased T regulatory cell production in the thymus, increased peripheral $CD4^+CD25^+$ T cell apoptosis rates, and increased conversion of $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cells in the periphery (18, 31). Specifically, it has been shown that CD80/CD86, CD28, and IL-2 deficiency all result in marked decreases in survival of peripheral $CD4^+CD25^+$ T regulatory cells (18). In our study, CD28-deficient $CD4^+CD25^+$ T cells are produced at normal levels in the thymus but then fail to thrive in the periphery. The ability of CD7/CD28-double-deficient $CD4^+CD25^+$ T cells to survive in the periphery is further hampered by the absence of CD7. Since IL-2 is required for $CD4^+CD25^+$ T cell generation and survival (32), it is reasonable to postulate that decreased $CD4^+CD25^+$ cell survival in CD7/CD28-double-deficient mice may be attributed to a loss of IL-2 production due to lack of CD7 and CD28 engagement. It is also possible that $CD4^+CD25^+$ T cells, which constitutively express CTLA-4 (22, 33), may be delivered an apoptotic signal by B7.1/B7.2 in the absence of CD28, thereby depleting the periphery of T regulatory phenotype cells. The additional loss of CD7-derived survival signals may further exacerbate the neglect of $CD4^+CD25^+$ T cells in the periphery of CD7/CD28-deficient animals.

Analysis of lymph node $CD4^+CD25^+$ T cells in CD7/CD28-deficient mice revealed intact suppressor cell function for CD3 mAb-triggered C57BL/6 $CD4^+CD25^-$ T cells. However, $CD4^+CD25^+$ T cells from CD28- and CD7/CD28-deficient mice were less potent than wild-type $CD4^+CD25^+$ T cells. It has been reported that activated $CD4^+CD25^+$ T cells are more potent than resting $CD4^+CD25^+$ T cells (34). Therefore, reduced function of CD28- and CD7/CD28-deficient T regulatory cells may be attributed to a lack of activation due to decreased IL-2 responsiveness and absence of CD7 ligand and B7.1/B7.2 triggering in vivo. It will be important to determine whether CD7/CD28-deficient T regulatory cells have an activation defect to explain their diminished potency as suppressor T cells.

$CD4^+$ T regulatory phenotype cells modulate peripheral levels of $CD4^+$ T cells and are known to suppress organ-specific autoimmune disease (21). Therefore, we analyzed 1- to 2-year-old mice to determine whether the reduced numbers and potency of T regulatory cells in CD7/CD28-deficient animals resulted in the spontaneous development of autoimmune disease. Compared with age-matched wild-type and single gene-deficient animals, there were no outward signs of autoimmune disease such as hair loss, rashes, weight loss, decreased reproduction, etc. However, we observed significant thyroiditis with T and B cell infiltrate in aged CD7/CD28-double-deficient mice that was not seen in age-matched control mice. Thus, the combined deficiency of CD7 and CD28 resulted in the loss of T regulatory cells and allowed for the spontaneous development of thyroiditis in animals >1 year of age. Seddon and Mason (35) have previously shown in a rat thymectomy/irradiation model of autoimmune thyroiditis that disease can be suppressed by transfer of T regulatory phenotype cells (35). Together, these findings suggest that in vivo loss of peripheral T regulatory cells due to deletion of both CD7 and CD28 allows for the development of spontaneous thyroiditis.

In addition to the development of thyroiditis only in CD7/CD28-double-deficient mice, the other key differences between CD28-deficient and CD7/CD28-double-deficient mice in our study were that the CD7/CD28-double-deficient mice had a more profound deficiency in absolute numbers of peripheral $CD4^+CD25^+$ T cells

compared with CD28-deficient mice and they had low numbers of thymus $CD4^+CD25^+$ T cells, whereas CD28-deficient mice had normal numbers of these cells in the thymus. We also observed a significant enhancement of in vivo cytokine responses to LPS in CD7/CD28-double-deficient mice. It will be important to determine whether this enhancement is related to the deficiency of $CD4^+CD25^+$ T cells in these mice.

In summary, our data provide new evidence for a role for CD7-CD7 ligand interactions in thymic and peripheral homeostasis of $CD4^+CD25^+$ regulatory T cells. Understanding the roles of CD7 and CD28/CTLA-4 in regulating T regulatory cell growth and persistence in this novel model should provide important insight into the molecular mechanisms of regulation of normal immune responses and autoimmune disease.

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