CD18 Is Required for Optimal Development and Function of $CD4^+CD25^+$ T Regulatory Cells[1]

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$CD4^+CD25^+$ T regulatory (Treg) cells inhibit immunopathology and autoimmune disease in vivo. $CD4^+CD25^+$ Treg cells' capacity to inhibit conventional T cells in vitro is dependent upon cell-cell contact; however, the cell surface molecules mediating this cell:cell contact have not yet been identified. LFA-1 (CD11a/CD18) is an adhesion molecule that plays an established role in T cell-mediated cell contact and in T cell activation. Although expressed at high levels on murine $CD4^+CD25^+$ Treg cells, the role of LFA-1 in these cells has not been defined previously. We hypothesized that LFA-1 may play a role in murine $CD4^+CD25^+$ Treg function. To evaluate this, we analyzed LFA-1-deficient ($CD18^{-/-}$) $CD4^+CD25^+$ T cells. We show that $CD18^{-/-}$ mice demonstrate a propensity to autoimmunity. Absence of CD18 led to diminished $CD4^+CD25^+$ T cell numbers and affected both thymic and peripheral development of these cells. LFA-1-deficient $CD4^+$ $CD25^+$ T cells were deficient in mediating suppression in vitro and in mediating protection from colitis induced by the transfer of $CD4^+CD25^-$ T cells into lymphopenic hosts. Therefore, we define a crucial role for CD18 in optimal $CD4^+CD25^+$ Treg development and function. *The Journal of Immunology,* **2005, 175: 7889–7897.**

Regulatory $CD4^+CD25^+$ T (Treg)[3] cells are critical for inhibiting immunopathology in vivo. This has been demonstrated in models of colitis, gastritis, diabetes, experimental autoimmune encephalomyelitis, and fetal rejection (1–3). Moreover, either the number or function of these cells is reduced in mouse and human autoimmune diseases (4–6). $CD4^+CD25^+$ Treg cells inhibit conventional T cell proliferation in vitro and control T cell numbers and effector functions in vivo (7). $CD4^+CD25^+$ Treg cells develop in the thymus (8), and they specifically express the transcription factor FoxP3 (9, 10). There is also evidence that either peripheral development or expansion of $CD25^+$ Treg cells occurs (11–13).

The mechanisms wherein $CD4^+CD25^+$ Treg cells mediate suppression of immune responses have not been fully elucidated. Many studies demonstrate a requirement for cell-cell contact (3). However, the molecules mediating this contact have not yet been identified. Potential candidate adhesion molecules expressed on $CD4^+CD25^+$ T cells include ICAM-1, P-selectin, CD18, $\beta7$, CD103, and L-selectin (14), but ICAM-1 and P-selectin were found not to be required for $CD25^+$-suppressive function in a model of experimental autoimmune encephalomyelitis (15), and β_7 integrins were recently found not to be required for $CD25^+$-mediated protection from colitis (16). Although high expression of L-selectin and CD103 on $CD4^+CD25^+$ Treg cells identifies a more potent suppressive subset, a functional role for these adhesion molecules has not been defined (14, 17). Whether CD18 is required for the optimal function of murine $CD4^+CD25^+$ T cells, either in vitro or in vivo, is unknown. In addition, the role of CD18 in the development of $CD4^+CD25^+$ T cells has not been reported.

LFA-1 (α_L/β_2 or CD11a/CD18) is a member of the β_2 integrin family (18). Integrins are a large family of heterodimeric membrane glycoproteins composed of noncovalently linked α- and β-chains that are involved in cell-cell adhesion. LFA-1 is expressed on peripheral blood lymphocytes, neutrophils, monocytes, granulocytes, and NK cells, where it binds multiple ligands, including ICAM-1, ICAM-2, or ICAM-3 (18). LFA-1 is the only member of the β_2 integrin family to be expressed on peripheral $CD4^+$ T cells, where it plays an established role in trafficking and activation (18–22). Consistent with this, LFA-1 is the only β_2 integrin family member expressed on $CD4^+CD25^+$ T cells (data not shown). The physiological importance of LFA-1 is manifest in those individuals lacking the β_2 subunit in a disease known as leukocyte adhesion deficiency (LAD). These patients are characterized by an inability to clear pathogens, recurrent infections, and, frequently, death at an early age (23, 24). Notably, there have been reports of inflammatory bowel disease in patients with LAD (25). $CD11a^{-/-}$ and $CD18^{-/-}$ mice share many of these characteristics, including decreased $CD4^+$ and $CD8^+$ T cells in peripheral lymph nodes (PLN) and in extralymphoid tissue sites (e.g., lung, liver) (19, 22), and in vivo T cell functional defects, such as in tumor rejection, delayed-type hypersensitivity, and infectious responses (26–29). Although the inflammatory manifestations and premature death seen in $CD18^{-/-}$ mice have been presumed to be infectious in nature (30), it is of significance that $CD18^{-/-}$ mice demonstrate a persistence of the inflammatory skin lesions despite being housed in a germfree facility (31). Thus, an infectious etiology is excluded under these conditions and this invokes the possibility of a defect in regulatory immune homeostasis and development of autoimmune disease.

We hypothesized that LFA-1 might play a role in $CD4^+CD25^+$ Treg cell homeostasis. Using $CD18^{-/-}$ mice, we demonstrate that

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[3] Abbreviations used in this paper: Treg, T regulatory; LAD, leukocyte adhesion deficiency; PLN, peripheral lymph node; MLN, mesenteric lymph node; SP, single positive; WT, wild type; LPL, lamina propria lymphocyte; MHC II, MHC class II; DC, dendritic cell.

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CD18 is necessary for the development of optimal numbers of both innate and peripherally induced $CD4^+CD25^+$ Treg cells, as well as for the suppressor function of these cells. Consistent with these defects is the development of autoantibodies, indicating a propensity to autoimmunity in the absence of CD18.

Materials and Methods

Mice

$CD18^{-/-}$ mice on a C57BL/6 background were generously provided by A. Beaudet (Baylor College of Medicine, Houston, TX) (28). $CD18^{-/-}$ mice were backcrossed onto the BALB/c background (nine generations) and in some cases onto DO11.10 TCR transgenic mice. BALB/c.RAG-$1^{-/-}$ and BALB/c mice were obtained from The Jackson Laboratory. T cells were phenotyped through staining of peripheral blood cells with a biotinylated mAb, KJ1-26 (Caltag Laboratories), directed against the TCR clonotype expressed by DO11.10 T cells and by anti-CD18 (BD Pharmingen). Mice were maintained on autoclaved food and irradiated water in a specific pathogen-free facility in microisolator cages with filtered air. Experiments were performed in agreement with our institutional animal care and use committee and according to the National Institutes of Health guidelines for animal use.

Abs and staining reagents

The following Abs and secondary reagents used for flow cytometry were purchased from BD Pharmingen: PE-, FITC-, biotin-, allophycocyanin-labeled anti-CD4, PE- and FITC-labeled anti-CD18 and -CD25, biotin-labeled CD62L, FITC-labeled CD44, PE-labeled CD69, and CyChrome-labeled streptavidin. FITC-labeled FoxP3 was purchased from eBioscience. The following Abs and secondary reagents were purchased from Caltag Laboratories: PE- and biotin-labeled KJ1–26. Alkaline phosphatase-conjugated IgG was purchased from Southern Biotechnology Associates.

Autoimmunity model

RAG-$1^{-/-}$ mice were adoptively transferred with 20×10^6 splenocytes from 5-wk-old $CD18^{+/-}$ or $CD18^{-/-}$ mice. Mice were monitored by serum anti-DNA Abs assessed at weeks 3, 6, and 8 posttransfer. At 8 wk, mice were sacrificed, and tissues were harvested. These tissues were fixed in neutral-buffered formalin and paraffin-embedded by standard methods. Well-oriented 3-μm sections were stained by H&E. These were examined by a gastrointestinal surgical pathologist (J.R.T.) blinded to the experimental treatment.

Measurement of anti-DNA Abs

Ninety-six-well plates (Costar) were coated with sperm DNA (50 μg/ml) (Sigma-Aldrich) in PBS and then blocked with PBS/5% FCS. Duplicates of four to five dilutions of serum (1/200, 1/400, 1/800, 1/1600, and 1/3200) were incubated in the wells at 37°C $\times$ 1 h. Plates were washed and incubated with IgG conjugated with alkaline phosphatase (Southern Biotechnology Associates). Plates were washed and incubated with phosphatase substrate (Sigma-Aldrich), and optical densities were determined at 405 λ.

RNA preparation and real-time RT-PCR

$CD4^+CD25^+$ T cells were isolated by MO-FLO sorting (DakoCytomation) from the thymus and spleen of $CD18^{+/-}$ or $CD18^{-/-}$ mice. RNA was harvested using TRIzol (Invitrogen Life Technologies), and residual DNA was removed with DNaseI treatment (Promega). RNA was reverse transcribed to cDNA with SuperScript II as per manufacturer instructions (Invitrogen Life Technologies). The level of FoxP3 mRNA was determined by real-time PCR using Sybergreen (Molecular Probes) and Taqman Universal PCR Mastermix (Applied Biosystems) on the iCycler (Bio-Rad). GAPDH endogenous control was used to normalize RNA. Primers were as follows: FoxP3 forward: 5′-TCCCAGAGTTCTTCCACAAC-3′, and reverse, 5′-TAAGGGTGGCATAGGTGAAA-3′; GAPDH forward, 5′-CATGGCCTTCCGTGTTCCTA-3′, and GAPDH reverse, 5′-TGTCAT CATACTTGGCAGGTTTCT-3′. $\Delta\Delta C_T$ for relative quantitation was calculated using $CD4^+CD25^-$ T cells as the calibrator.

Induction of peripheral $CD4^+CD25^+$ T cells

Spleen-derived $CD4^+$ T cells were isolated using $CD4^+$ microbeads according to manufacturer instructions (Miltenyi Biotec). Freshly isolated (2.5×10^6) $CD18^{+/-}$ or $CD18^{-/-}$ RAG-$1^{-/-}$ DO11.10 $CD4^+$ T cells were adoptively transferred by i.v. injection into BALB/c mice. Twenty-four hours later, the mice were immunized with 5 μg/ml chicken OVA peptide 323–339 by i.v. injection, and 8 days after immunization, spleen and PLN were harvested, and cells were stained with anti-$CD4^+$, anti-KJ1–26, and anti-$CD25^+$ Abs to assess induction of $CD4^+CD25^+$ T cells.

In vitro suppression of T cell activation

For assessment of $CD18^{+/-}$ or $CD18^{-/-}$ $CD4^+CD25^+$ T cell suppression in vitro of freshly isolated $CD4^+$ T cells, spleen-derived $CD4^+$ T cells were first isolated using $CD4^+$ microbeads (Miltenyi Biotec), and then $CD25^-$ or $CD25^+$ populations were further sorted using the MO-FLO sorter (DakoCytomation). In some cases, $CD25^+$ T cells were additionally stained with CD62L for the isolation of $CD25^+CD62L^{high}$ T cells. A total of 5.0×10^4 $CD4^+$ T cells was incubated with 1 μg/ml anti-CD3 (145-2C11) and 2.5×10^5 irradiated (3000 rad) splenocytes along with increasing concentrations of $CD18^{+/-}$ or $CD18^{-/-}$ $CD4^+CD25^+$ T cells in a 96-well flat-bottom plate. [^{3}H]Thymidine was added to the cultures during the last 18 h of a 72-h assay.

$CD4^+CD25^-$-mediated colitis

Spleen-derived $CD4^+$ T cells were first isolated using $CD4^+$ microbeads (Miltenyi Biotec), and then $CD25^-$ or $CD25^+$ populations were further sorted using the MO-FLO sorter (DakoCytomation). Colitis was induced through the transfer of $CD18^{+/-}CD4^+CD25^-$ T cells (5×10^5) into RAG-$1^{-/-}$ mice, which were placed on a tap water and regular food diet. Protection from colitis was examined through cotransfer of either $CD18^{+/-}$ or $CD18^{-/-}CD4^+CD25^+$ T cells (1×10^5) (from mice 4–5 wk of age) with the $CD4^+CD25^-$ T cells. Mice were monitored over 8–9 wk for weight loss. Mice were sacrificed at 8–9 wk, and colon specimens were fixed in neutral-buffered formalin, paraffin-embedded, sectioned, and stained by H&E. These were examined by a gastrointestinal surgical pathologist (J.R.T.) blinded to the experimental treatment, and activity was scored using a standard five-tier scale of inactive (score of 0), minimal activity (score of 1), mild activity (score of 2), moderate activity (score of 3), and severe activity (score of 4). Minimal activity was defined as an increase in lamina propria neutrophils, lymphocytes, and plasma cells without significant numbers of intraepithelial neutrophils. Mild activity included intraepithelial neutrophils, but no crypt abscesses. Moderate activity was defined by the presence of crypt abscesses. Severe activity included erosions and/or ulceration. Spleen and large bowel lamina propria lymphocytes (LPL) were harvested and examined for $CD4^+$ T cells. LPL were isolated essentially as per Ref. 32. In brief, colons were cut longitudinally and then into ~1-mm pieces. Colonic pieces were washed thoroughly (ice-cold PBS, 5% FCS) and then digested (PBS, 5 mM EDTA, and 5% FCS) at 37°C $\times$ 1 h in a rotating incubator to remove the epithelial cells. The supernatants containing epithelial cells and intraepithelial lymphocytes were discarded, and intestines were washed twice in ice-cold PBS to remove the residual EDTA. The remaining tissue was then incubated for 2 h at 37°C in a rotating incubator in collagenase buffer consisting of RPMI 1640 supplemented with 10% FCS, 200 U/ml collegenase VIII (Sigma-Aldrich), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-ME essential amino acids, 40 μg/ml gentamicin, and 50 U/ml penicillin-50 μg/ml streptomycin. The cells were then filtered through a 40-μm filter (BD Biosciences) and washed twice in medium without collagenase.

Statistical analyses

Statistical comparisons of anti-DNA Abs, cell percentages and numbers, body weights, and pathology scores between treated groups were assessed using a one-tailed Student's t test. Values of $p < 0.05$ were considered significant.

Results

$CD18^{-/-}$ splenocytes transferred into RAG-$1^{-/-}$ mice lead to intestinal inflammation

To evaluate whether defects in regulatory immune homeostasis might contribute to the inflammatory manifestations in $CD18^{-/-}$ mice, we investigated $CD18^{-/-}$ splenocytes in a general model of autoimmunity. Transfer of splenocytes into lymphopenic hosts can lead to selective proliferation of autoreactive T cells (33) and has been used as a means of assessing a propensity toward autoimmunity (34). Both $CD18^{+/+}$ and $CD18^{+/-}$ mice have an identical cell surface expression of CD18 by flow cytometry, and $CD18^{+/-}$ mice are used as littermate controls for the $CD18^{-/-}$ mice. The activation markers on $CD18^{-/-}$ splenocytes are not increased

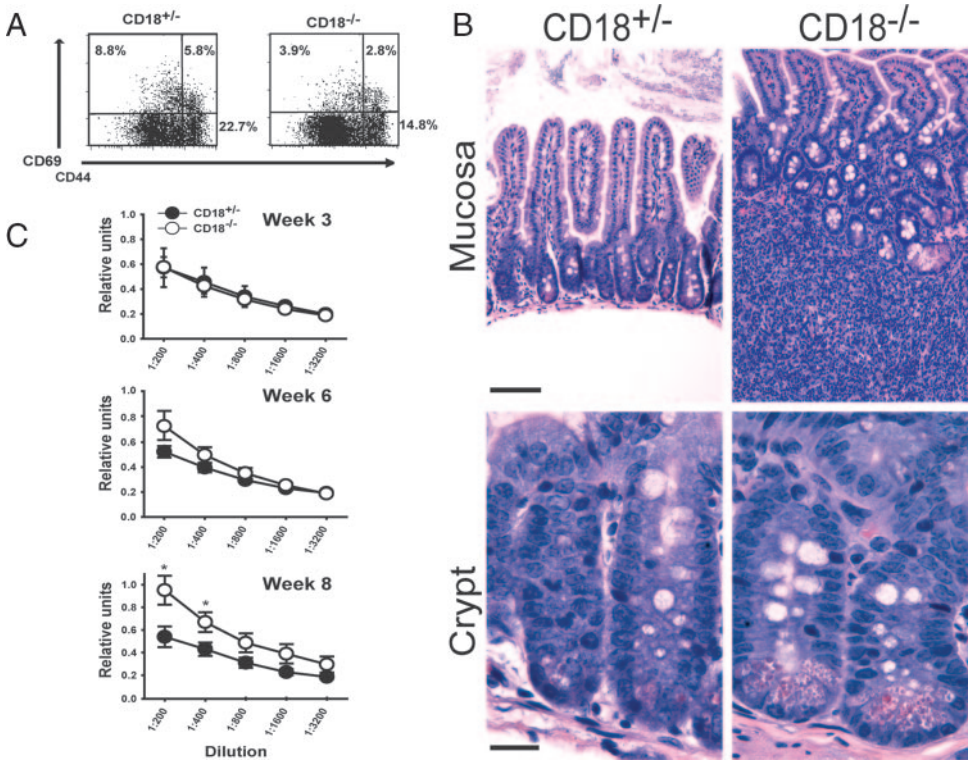

FIGURE 1. CD18$^{-/-}$ splenocyte transfer into RAG-1$^{-/-}$ mice results in a progressive increase in anti-DNA Abs and intestinal inflammation. *A*, Spleen from CD18$^{+/-}$ and CD18$^{-/-}$ mice were stained CD4, CD69, and CD44, and expression of CD69 and CD44 on gated CD4$^+$ T cells is shown. A total of 20×10^6 CD18$^{+/-}$ ($n = 6$) or CD18$^{-/-}$ ($n = 5$) splenocytes was transferred into RAG-1$^{-/-}$ mice. *B*, Eight weeks after transfer, mice were sacrificed, and the ileum was harvested and stained by H&E. CD18$^{+/-}$ splenocyte-transferred mice demonstrate normal ileum (mucosa, scale bar = 100 μM) and crypts with Paneth cells (scale bar = 20 μM). CD18$^{-/-}$ splenocyte-transferred mice (magnifications are identical to CD18$^{+/-}$-transferred mice) demonstrate ileal inflammatory disease with marked wall thickening and increased lamina propria immune cells, decreased goblet cells, and reactive epithelial changes. Examination of crypt epithelium shows increased mitotic activity and expansion of Paneth cells. The average number of Paneth cells increased from 1.1 $\pm$ 0.1 per crypt in CD18$^{+/-}$-transferred mice to 5.5 $\pm$ 0.2 ($p < 0.001$) per crypt in CD18$^{-/-}$-transferred mice. *C*, Serial dilutions of serum anti-DNA Abs were measured by ELISA at weeks 3, 6, and 8 after transfer. The data are represented as relative units = OD ($\pm$ SEM). $*$, $p < 0.05$.

(Fig. 1*A*). Therefore, we transferred equivalent numbers of CD18$^{+/-}$ or CD18$^{-/-}$ splenocytes into RAG-1$^{-/-}$ mice. RAG-1$^{-/-}$ mice transferred with CD18$^{-/-}$ splenocytes uniformly demonstrated an increase of ileal (Fig. 1*B*) and colonic (data not shown) disease. This was characterized by marked wall thickening and transmural inflammation (Fig. 1*B*). Epithelial regeneration, typified by frequent mitotic figures, and evidence of chronic injury, including increased numbers of Paneth cells, were also present.

CD18$^{-/-}$ mice demonstrate high titers of anti-DNA Abs

The development of intestinal inflammation in RAG-1$^{-/-}$ mice transferred with CD18$^{-/-}$ splenocytes was unexpected in light of the known defects in CD18$^{-/-}$ hemopoietic cell trafficking and activation (19, 22, 28, 35). To further study whether these findings were consistent with autoimmunity, we assessed whether recipient mice have increased production of autoantibodies. RAG-1$^{-/-}$ mice transferred with CD18$^{-/-}$ splenocytes demonstrated a progressive increase in serum anti-DNA titers for at least 8 wk after splenocyte transfer (Fig. 1*C*). Similarly, CD18$^{-/-}$ mice had higher titers of anti-DNA Abs than did their CD18$^{+/-}$ littermate controls (Fig. 2).

CD18$^{-/-}$ mice demonstrate defects in thymic and peripheral CD4$^+$CD25$^+$ T cell numbers

The development of intestinal inflammation and anti-DNA Abs in the transfer of CD18$^{-/-}$ splenocytes to RAG-1$^{-/-}$ mice could potentially be explained by increased autoreactive T cells and/or decreased numbers or function of regulatory populations. To evaluate the possibility of autoreactive T cells, we examined the Vβ repertoire in CD18$^{-/-}$ mice as a means of assessing potential defects in thymic selection and T cell repertoire. The Vβ repertoire in CD18$^{-/-}$ mice was equivalent to that of CD18$^{+/-}$ littermate controls, and the deletion of Vβ5 and Vβ8 was observed as expected in BALB/c mice (data not shown). Although other subtleties beyond Vβ repertoire may be present, these data suggest the absence of increased autoreactive T cells.

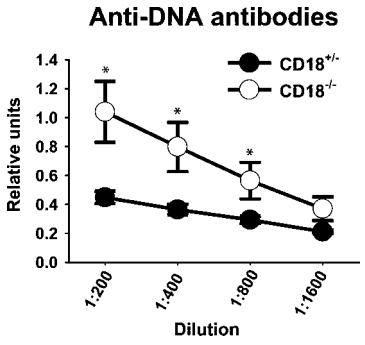

FIGURE 2. CD18$^{-/-}$ mice have elevated titers of anti-DNA Abs. Serial dilutions of serum from CD18$^{+/-}$ ($n = 7$) and CD18$^{-/-}$ ($n = 9$) mice were assessed for anti-DNA Abs by ELISA. The data are represented as relative units = OD ($\pm$ SEM).

Therefore, we next considered defects in regulatory T cell populations. The colonic and ileal inflammation induced by the transfer of $CD18^{-/-}$ splenocytes to RAG-$1^{-/-}$ mice had similarities to the disease observed in the $CD4^+CD45RB^{high}$ transfer model into immunodeficient hosts. $CD4^+CD25^+$ T cells can rescue the intestinal inflammation observed in the $CD4^+CD45RB^{high}$ model (36). Therefore, we examined whether there was a quantitative defect of $CD4^+CD25^+$ T cells in $CD18^{-/-}$ mice. Mice were examined between 4 and 8 wk of age, at which time there is no evidence of spontaneous inflammation of various organs by H&E staining (e.g., kidney, pancreas, liver, stomach, and small and large intestines). In comparison to $CD18^{+/-}$ mice, the percentage of $CD4^+CD25^+$ T cells (Fig. 3, *A* and *C*) is decreased in the mesenteric lymph node (MLN) and spleen, and the absolute number of $CD4^+CD25^+$ T cells (Fig. 3*D*) is decreased in the MLN and PLN of $CD18^{-/-}$ mice. As is observed in the $CD11a^{-/-}$ mice (19), the number of total $CD4^+$ T cells in MLN and PLN of $CD18^{-/-}$ mice is decreased (Fig. 3*B*), accounting for the relative enhancement in the defect in $CD18^{-/-}CD4^+CD25^+$ T cell numbers in these organs. Although the sum total $CD4^+$ T cells in the periphery of $CD18^{-/-}$ mice is not decreased due to the redistribution of $CD4^+$ T cells to the spleen, the total number of $CD4^+CD25^+$ T cells is decreased in these mice. As the most significant defect in $CD4^+CD25^+$ T cells was in the MLN, a lymphoid organ that contains increased populations of effector T cells that can also express $CD25^+$, we assessed the FoxP3-expressing populations in the lymph nodes of $CD18^{-/-}$ mice. The defects in percentage and number of FoxP3-expressing $CD4^+$ T cells in the MLN of $CD18^{-/-}$ mice (Fig. 3, *E* and *F*) parallel that observed with $CD4^+CD25^+$ T cells.

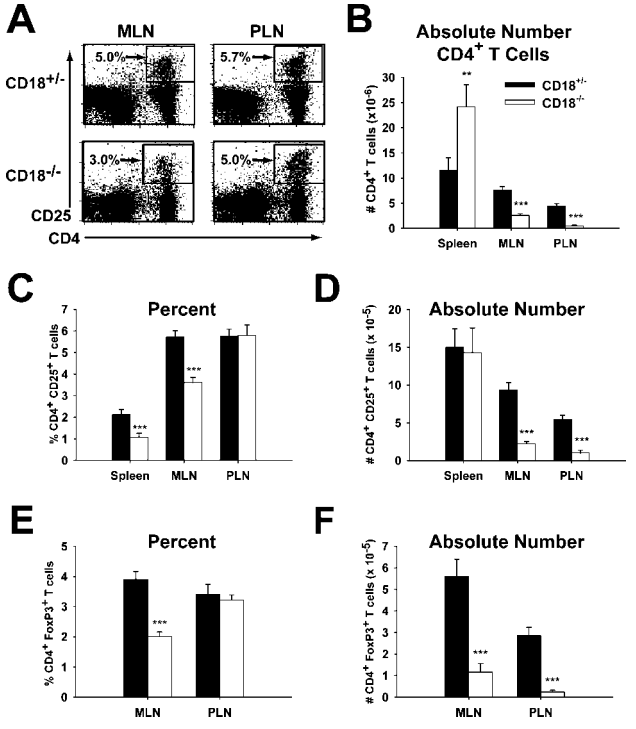

FIGURE 3. $CD18^{-/-}$ mice have a decreased number of $CD4^+CD25^+$ T cells in their secondary lymphoid organs. PLN and MLN from $CD18^{+/-}$ and $CD18^{-/-}$ mice were harvested and stained for expression of CD25 and CD4 (*A*). Bar graphs indicate (*B*) the absolute number of $CD4^+$ T cells and (*C*) the percentage and (*D*) absolute number (= % $CD4^+CD25^+$ T cells × total number of lymphoid cells) of $CD25^+CD4^+$ T cells in each lymphoid organ for $CD18^{+/-}$ ($n = 23$) and $CD18^{-/-}$ ($n = 23$) mice. PLN and MLN were assessed for $CD4^+FoxP3^+$ T cell populations by (*E*) the percentage (% $CD4^+FoxP3^+$ T cells) and (*F*) the absolute number (= % of $CD4^+FoxP3^+$ T cells × total number of lymphoid cells) ± SEM in each lymphoid organ for $CD18^{+/-}$ ($n = 6$) and $CD18^{-/-}$ ($n = 6$) mice. **, $p < 0.01$; ***, $p < 0.001$.

The decrease in peripheral $CD4^+CD25^+$ T cells in $CD18^{-/-}$ mice may be secondary to defects in either thymic or peripheral $CD4^+CD25^+$ T cell development/expansion. To discriminate between these mechanisms, we first assessed defects in thymic $CD4^+CD25^+$ T cells development. The percentage (Fig. 4*A*) and absolute number (Fig. 4*B*) of single positive (SP) $CD4^+CD25^+$ thymocytes was decreased in $CD18^{-/-}$ mice. Notably, the percentage of single and double positive thymic subsets was equivalent in $CD18^{+/-}$ and $CD18^{-/-}$ littermate controls (data not shown). Thus, together with the observed normal Vβ repertoire, these data indicate that there is not a global defect in thymic selection. To determine whether the $CD4^+CD25^+$ thymocytes that do develop in $CD18^{-/-}$ mice activate appropriate transcriptional pathways present in regulatory T cells, we assessed FoxP3 mRNA levels on SP $CD4^+CD25^+$ thymocytes. $CD18^{-/-}$ SP $CD4^+CD25^+$ thymocytes expressed FoxP3 mRNA at levels equal to $CD18^{+/-}$ SP $CD4^+CD25^+$ thymocytes (Fig. 4*C*). We further find that the $CD4^+CD25^+$ T cells in peripheral spleen of $CD18^{-/-}$ mice expressed FoxP3 mRNA (Fig. 4*D*) and protein (Fig. 4, *E* and *F*) at levels equivalent to those of $CD18^{+/-}CD4^+CD25^+$ T cells. Thus, SP $CD4^+CD25^+$ thymocytes, which do develop in $CD18^{-/-}$ mice, have appropriate levels of FoxP3, but the absence of CD18 limits efficient development of the number of SP $CD4^+CD25^+$ thymocytes.

$CD18^{-/-}$ mice demonstrate defects in development of peripheral $CD4^+CD25^+$ T cells to low-dose Ag

The number of $CD4^+CD25^+$ T cells increase in response to low doses of peripheral Ag (10–13, 37). In particular, adoptively transferred DO11.10 $RAG^{-/-}$ $CD4^+$ T cells develop an increased percent of $CD4^+CD25^+$ T cells in response to low-dose OVA peptide in the spleen and PLN, and these cells function as suppressor T cells (11). $CD18^{+/-}$ and $CD18^{-/-}$ DO11.10 RAG-$1^{-/-}$ mice have equivalent and low numbers of $CD4^+CD25^+$ splenocyte T cells (<1.0%) before activation. To assess whether $CD18^{-/-}$ $CD4^+$ T cells can expand the $CD4^+CD25^+$ T cell population after immunization with low-dose Ag, we adoptively transferred $CD18^{+/-}$ or $CD18^{-/-}CD4^+$RAG-$1^{-/-}$ DO11.10 T cells into BALB/c mice. The mice were immunized with 5 μg/ml OVA peptide, and 8 days after immunization, spleen and PLN were harvested. Mice transferred with $CD18^{-/-}CD4^+$RAG-$1^{-/-}$ DO11.10 T cells developed a lower percentage of $CD4^+CD25^+$ T cells in the spleen and PLN upon low-dose OVA immunization in comparison to mice transferred with $CD18^{+/-}$ T cells (Fig. 5*A*). These results correlate to a decreased number of accumulated $CD18^{-/-}$ $CD4^+CD25^+$ T cells in the lymphoid organs (Fig. 5*B*). As we used DO11.10 $CD4^+$ T cells (i.e., not on a RAG-$1^{-/-}$ background) in our previous experiments, we also assessed whether these cells demonstrated a similar defect. We found a defect in the development of $CD4^+CD25^+$ T cells (percentage and absolute number) under low-dose OVA immunization when using $CD18^{-/-}CD4^+$ DO11.10 T cells as well (data not shown).

$CD18^{-/-}CD4^+CD25^+$ Treg cells demonstrate defects in mediating suppression in vitro

To investigate whether CD18 plays a role in the ability of $CD4^+CD25^+$ T cells to mediate suppressive function, we isolated $CD4^+CD25^+$ T cells from the spleen of $CD18^{+/-}$ or $CD18^{-/-}$ mice. In comparison to $CD18^{+/-}CD4^+CD25^+$ T cells, $CD18^{-/-}$

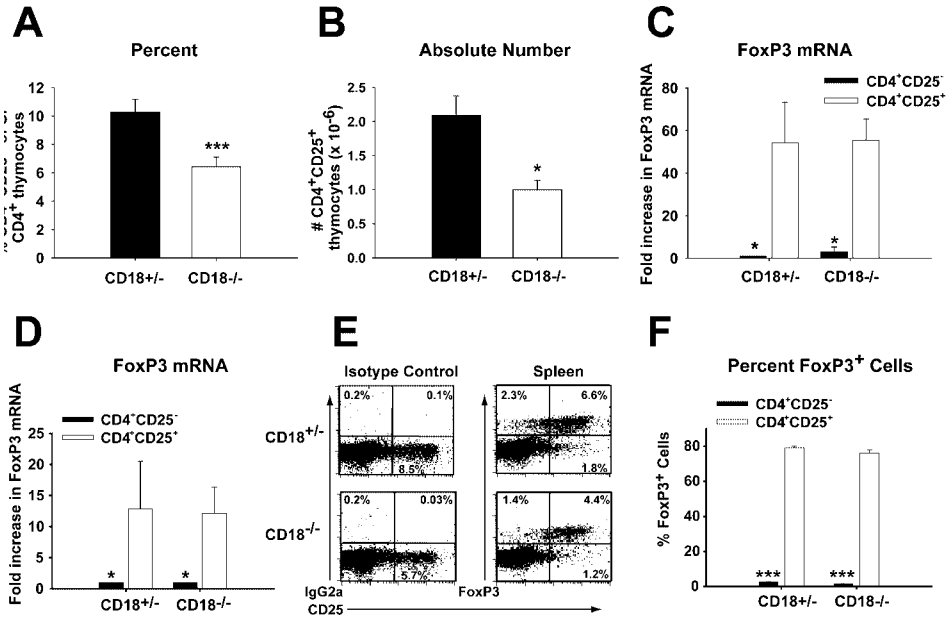

FIGURE 4. $CD18^{-/-}$ mice have a decreased number of SP $CD4^+CD25^+$ thymocytes. Thymocytes from $CD18^{+/-}$ ($n = 25$) or $CD18^{-/-}$ ($n = 25$) mice were assessed for single positive $CD4^+CD25^+$ populations by flow cytometry. The data are indicated as (*A*) a percentage and (*B*) an absolute number (% SP $CD4^+CD25^+$ thymocytes × number of thymocytes) ± SEM. *C*, SP $CD4^+CD25^+$ thymocytes from $CD18^{+/-}$ ($n = 3$) or $CD18^{-/-}$ ($n = 3$) mice or (*D*) $CD4^+CD25^+$ splenocytes from $CD18^{+/-}$ ($n = 6$) or $CD18^{-/-}$ ($n = 6$) mice were sorted by MO-FLO, and FoxP3 mRNA levels were assessed by real-time PCR. The fold increase in FoxP3 levels are calculated relative to $CD4^+CD25^-$ cells, and samples are normalized to GAPDH. Splenocytes from $CD18^{+/-}$ ($n = 6$) or $CD18^{-/-}$ ($n = 6$) mice were assessed for intracellular FoxP3 levels by flow cytometry. The data are indicated as representative flow cytometry gated on $CD4^+$ T cells for expression of $CD25^+$ and FoxP3 (or isotype control) (*E*) and percentage of $CD4^+CD25^+$ T cells expressing FoxP3 ± SEM in comparison to $CD4^+CD25^-$ T cells (*F*). *, $p < 0.05$; ***, $p < 0.001$.

$CD4^+CD25^+$ T cells were less efficient at mediating in vitro suppression of $CD4^+$ T cell activation upon stimulation with anti-CD3 and irradiated wild-type (WT) splenocytes (Fig. 6*A*). The $CD62L^{high}$ subfraction has been demonstrated to contain a greater degree of suppressor activity (17, 38). Therefore, we also assessed whether the defect persisted even when $CD18^{-/-}CD4^+CD25^+CD62L^{high}$ T cells were used as regulatory T cells and found a defect in the suppression of this subset as well (Fig. 6*B*). Thus, $CD18^{-/-}CD4^+CD25^+$ T cells clearly demonstrate a defect in mediating suppression in vitro.

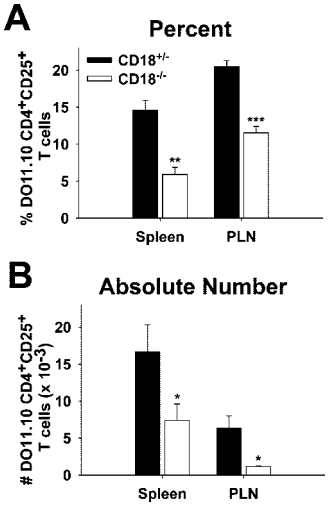

FIGURE 5. $CD18^{-/-}CD4^+$ DO11.10 RAG-$1^{-/-}$ T cells have a defect in the development of $CD4^+CD25^+$ peripheral T cells after low-dose OVA immunization. Freshly isolated RAG-$1^{-/-}$ DO11.10 $CD4^+$ splenocyte T cells from $CD18^{+/-}$ or $CD18^{-/-}$ mice were adoptively transferred into WT BALB/c mice. Mice were immunized 24 h later with i.v. 5 μg of OVA peptide, and 8 days later, spleen and PLN were harvested, and (*A*) the percentage and (*B*) the absolute number (= % $CD4^+CD25^+$ T cells × total number of lymphoid cells) of DO11.10 $CD4^+CD25^+$ T cells were assessed. The data are represented as ± SEM ($n = 3$ mice). The data are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

$CD18^{-/-}CD4^+CD25^+$ T cells have in vivo suppressor function defects

To determine whether the defect in $CD18^{-/-}CD4^+CD25^+$ T cell function was also present in vivo, we used the $CD4^+CD25^-$ T cell transfer into RAG-$1^{-/-}$ mice model of colitis. The cotransfer of $CD4^+CD25^+$ T cells is able to prevent the colitis caused by the transfer of pathogenic T cells into lymphopenic mice (36). Therefore, we are able to investigate the role of CD18 specifically on the $CD4^+CD25^+$ T cell subset in vivo. Consistent with published results, the defect in weight gain observed by the transfer of $CD18^{+/-}CD4^+CD25^-$ T cells was attenuated upon the transfer of $CD18^{+/-}CD4^+CD25^+$ T cells (Fig. 7*A*). Remarkably, $CD18^{-/-}CD4^+CD25^+$ T cells were not able to mediate the same degree of protection in $CD4^+CD25^-$-transferred mice (Fig. 7*A*). The inability of $CD18^{-/-}CD4^+CD25^+$ T cells to prevent disease was confirmed by histopathological examination. These mice exhibited chronic mucosal injury, as evidenced by crypt architectural distortion, surface ulceration, and transmural disease that included wall thickening and inflammatory infiltration of the mucosa and muscularis propria that was similar to mice receiving only $CD4^+CD25^-$ T cells (Fig. 7*B*). In contrast, these changes were diminished significantly by cotransfer of $CD18^{+/-}CD4^+CD25^+$ T cells. When disease activity present in histologic sections was assessed semiquantitatively, it was also apparent that cotransfer of $CD18^{+/-}CD4^+CD25^+$ T cells diminished activity while cotransfer of $CD18^{-/-}CD4^+CD25^+$ T cells did not (Fig. 7*C*). This was

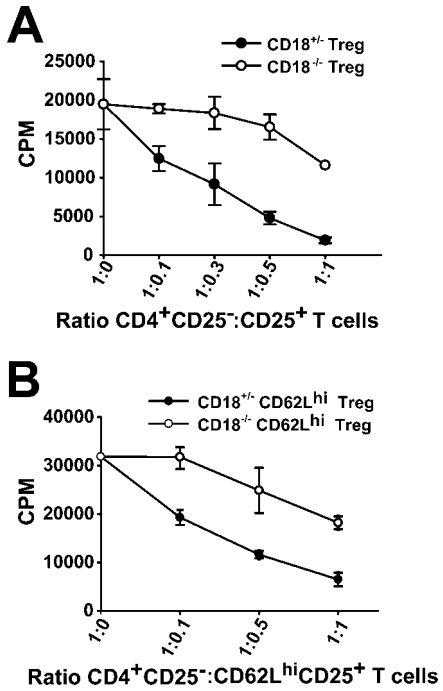

FIGURE 6. $CD18^{-/-}CD4^+CD25^+$ T cells have a defect in suppressor function in vitro. WT $CD4^+CD25^-$ T cells were cocultured with 1 μg/ml anti-CD3 and irradiated splenocytes, along with an increasing number of either $CD18^{+/-}$ or $CD18^{-/-}$ (*A*) $CD4^+CD25^+$ or (*B*) $CD4^+CD25^+CD62L^{high}$ T cells. Thymidine incorporation was measured during the last 18 h of a 72-h assay. The ratio of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T cells is indicated. The data are represented as counts per minute ± SEM. The data are representative of four and two independent experiments, respectively.

further corroborated by the accumulation of colonic $CD4^+$ LPL (Fig. 7*D*) and splenic $CD4^+$ T cells (Fig. 7*E*), which has been previously correlated with the severity of colitis (39). In light of the increased trafficking of $CD18^{-/-}CD4^+$ T cells to the spleen (22), we considered that the increased number of $CD4^+$ T cells in the mice cotransferred with $CD18^{-/-}CD4^+CD25^+$ T cells may be due to the trafficking of these cells to the spleen. However, in mice transferred with $CD18^{+/-}CD4^+CD25^-$ T cells + $CD18^{-/-}$ $CD4^+CD25^+$ T cells, >90% of the $CD4^+$ T cells in the spleen and >95% $CD4^+$ T cells in the colon were $CD18^{+/-}$ (data not shown). As a result, the expansion of $CD4^+$ T cells in the spleen and the colon is due to the disease-inducing $CD18^{+/-}CD4^+CD25^-$-transferred cells. Taken together, these results demonstrate that $CD18^{-/-}CD4^+CD25^+$ T cells are unable to prevent $CD4^+CD25^-$-induced colitis.

Discussion

The inflammatory manifestations observed in CD18-deficient mice have been attributed to the susceptibility of these mice to infection. However, $CD18^{-/-}$ mice demonstrate certain indications of defects in regulatory immune homeostasis, including that some inflammatory disease persists even in germfree facilities (31). Thus, we considered the hypothesis that $CD18^{-/-}$ mice might have defects in Treg function. We find that CD18 deficiency leads to an increased propensity toward autoimmunity. Our data clearly demonstrate that CD18 is required for the development of optimal numbers of innate thymic $CD4^+CD25^+$ T cells and induced peripheral $CD4^+CD25^+$ T cells. In addition, CD18 expression by $CD4^+CD25^+$ T cells is required for optimal suppressor function in vitro and is necessary for the in vivo $CD4^+CD25^+$ T cell-mediated protection of $CD4^+CD25^-$-induced colitis. The development of intestinal inflammation has also been described in humans with CD18 dysfunction in the disease LAD (25).

Our data show that CD18 is required for optimal numbers of $CD4^+CD25^+$ T cells in the thymus. The requirement for CD18 in $CD4^+CD25^+$ thymic development might be on various hematopoietically derived cells within the thymus. IL-2, CD28, B7, and CD40 contribute to thymic development of $CD4^+CD25^+$ Treg cells (4, 40–43). $CD4^+CD25^+$ thymic development also depends on MHC class II (MHC II), and these cells have more efficient interactions with MHC II peptides (8, 40, 44–46). $CD4^+CD25^+$ thymocytes are thought to represent a population of thymocytes, which are selected for their higher affinity for MHC II:self-peptide. This may account for the specific decrease in the SP $CD4^+CD25^+$ thymocyte population, in the context of normal percentages of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ thymocytes (data not shown; Ref. 28) in $CD18^{-/-}$ mice. Numerous studies have demonstrated that LFA-1 enhances the engagement of the TCR for its MHC II:peptide ligand, thereby allowing for a 10–100 times decrease in dose of MHC II:peptide Ag in the activation of mature T cells (20, 47, 48). The absence of CD18 likely reduces the thymocyte affinity/avidity for MHC:self-peptide, which may simply shift the threshold of selection in the total thymocyte population but result in a selective defect in the $CD4^+CD25^+$ T cell population. The equivalent FoxP3 expression on a per cell basis among those SP $CD4^+CD25^+$ thymocytes present argues that those cells selected are, in fact, regulatory cells.

Our data also demonstrate that CD18 is required for induction of optimal numbers of $CD4^+CD25^+$ T cells in the periphery to low-dose Ag. Peripheral expansion of $CD4^+CD25^+$ T cells has been shown to occur under various conditions, including upon immunization with low-dose peptide, upon oral Ag exposure, with lymphopenic proliferation, and upon exposure to TGF-β (11, 37, 49, 50). This peripheral expansion may be due to proliferation of existing $CD4^+CD25^+$ T cells, as well as an education of $CD4^+CD25^-$ T cells (51). Physiological maintenance of peripheral $CD4^+CD25^+$ T cells requires the presence of selecting self-peptide, B7-1/-2 and IL-2 (42, 52). Given the known defect in the activation and IL-2 production of $CD18^{-/-}$ DO11.10 $CD4^+$ T cells upon peripheral immunization (22), possibilities for the defect we observe in the induction of peripheral $CD18^{-/-}$ DO11.10 $CD4^+CD25^+$ T cells include inadequate TCR activation and IL-2 production.

We show that $CD18^{-/-}CD4^+CD25^+$ T cells have a clear defect in suppressor function in vitro. The development of $CD18^{-/-}$ $CD4^+CD25^+$ T cells into Treg cells appears to be intact given the equivalent expression of the essential transcription factor for regulatory function, FoxP3, in $CD18^{+/-}$ and $CD18^{-/-}CD4^+CD25^+$ thymocytes and peripheral $CD4^+CD25^+$ T cells. However, we cannot completely exclude the possibility that the absence of CD18 results in an indirect effect on any number of possible molecules, which, in turn, contributes to defects in suppressor function. Attempts to selectively block LFA-1 on WT $CD4^+CD25^+$ T cells in the in vitro suppression assay and in vivo are confounded by the direct effects of blocking Abs on the proliferation of the responder $CD4^+CD25^-$ T cells, as LFA-1:ICAM-1 interactions are required on this population for optimal proliferation (data not shown). The mechanisms through which $CD4^+CD25^+$ Treg cells mediate their functions are not completely understood. However, studies in human and murine $CD4^+CD25^+$ T cells have identified prevention of dendritic cell (DC) maturation, interactions through latent Ag gene of TGF-β, lysis of autologous cells, and the induction of IDO in DC as putative mechanisms whereby these cells contribute to suppression of T cell activation (53–56). A recent

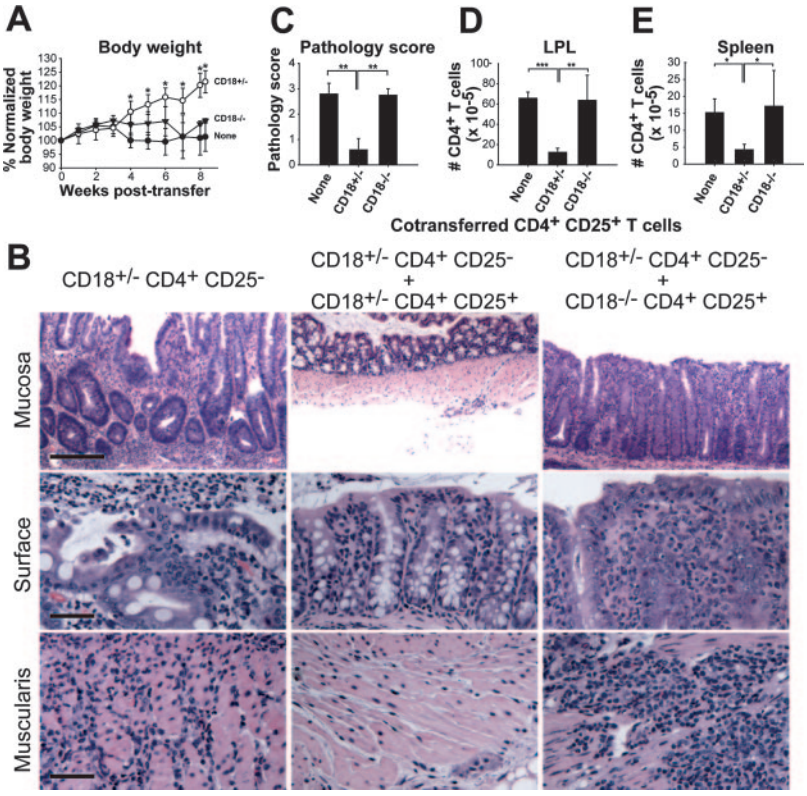

FIGURE 7. $CD18^{-/-}CD4^+CD25^+$ T cells from $CD18^{-/-}$ demonstrate a defect in protection from WT $CD4^+CD25^-$-induced colitis after transfer into RAG-$1^{-/-}$ mice. A total of 5×10^5 $CD18^{+/-}$ CD^+CD25^- T cells were transferred into RAG-$1^{-/-}$ mice alone or with 1×10^5 $CD18^{+/-}$ or $CD18^{-/-}$ $CD4^+CD25^+$ T cells. *A*, Weekly weights were measured. Line labels indicate the cotransferred $CD4^+CD25^+$ T cells. *B*, Mice were sacrificed at 8–9 wk, and H&E staining of the colon specimens was performed. A low power exam of the mucosa demonstrates that $CD18^{+/-}CD25^-$ T cells transferred alone show transmural inflammation, intestinal wall thickening, and crypt architectural distortion (scale bar = 200 μM). Higher power examination of the mucosal surface (scale bar = 50 μM) demonstrates intraepithelial neutrophils and sheets of overlying neutrophils, while examination of the muscularis propria (scale bar = 50 μM) reveals extensive inflammatory infiltration. Mice in which $CD18^{+/-}CD25^-$ T cells were cotransferred with $CD18^{+/-}CD25^+$ T cells demonstrate amelioration of all aspects of disease (note that the intestinal wall is substantially thinner at the same magnification as the diseased colon), whereas cotransfer with $CD18^{-/-}CD25^+$ offered no significant protection. *C*, Pathology scores are indicated. Numbers of $CD4^+$ T cells in (*D*) the LPL and (*E*) the spleen were assessed. Of note is that one mouse in the group transferred with $CD18^{+/-}CD25^-$ T cells alone, and two mice in the group cotransferred with $CD18^{+/-}CD25^-$ and $CD18^{-/-}CD25^+CD4^+$ T cells died before completion of the study. These mice demonstrated weight loss. Data are represented as $CD4^+$ T cell numbers ± SEM. $n = 4–6$ for each condition assessed. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

report describes the ability of human Treg cells to mediate autologous target cell death and that this process depends on CD18 (57). It has not yet been reported whether this process also occurs in murine systems. LFA-1 is expressed at high levels on $CD4^+CD25^+$ T cells (14). The deficiency of LFA-1 on $CD4^+CD25^+$ T cells may result in defective regulatory function through a variety of mechanisms, including a defect in: 1) the cell-cell contact required by $CD4^+CD25^+$ T cells to mediate their function; and 2) adequate engagement of the TCR on $CD4^+CD25^+$ T cells. The defect in cell-cell contact could result in suboptimal effects on target cells, such as DC, with a defect in the generation of suppressor DC (55) (i.e., induction of IDO, down-regulation of costimulatory molecules on DC) or in the interaction with surface TGF-β. Regulatory activity of T cells has been shown to correlate with possession of high avidity for MHC:self-peptide complexes (28). LFA-1 on the cell surface of $CD4^+CD25^+$ T cells may be providing for enhanced avidity, leading to improved competition of $CD4^+CD25^+$ T cells for their ligand. In addition, as engagement of the TCR on $CD4^+CD25^+$ T cells is required for their function, a defect in this engagement in the absence of CD18 may result in a defect in the regulatory function of the Treg cells.

The disruption of LFA-1:ICAM-1 interactions in vivo affects a wide variety of cell:cell interactions such that the impact on a given process is ultimately an integration of these effects. In fact, given the importance of LFA-1:ICAM-1 interactions in the proper activation of effector T cells, inhibition of these interactions has resulted in the amelioration of a number of inflammatory diseases, including various Th1-mediated diseases and murine models of lupus (58–61). A similar complexity exists in $CD28^{-/-}$ mice. $CD28^{-/-}$ plays a critical role for effector T cell responses, and $CD28^{-/-}$ mice demonstrate numerous defects in T cell activation, function, and amelioration of various inflammatory diseases (62). However, careful studies revealed a reduced number of $CD4^+CD25^+$ T cells with increased autoimmunity under specific circumstances, such as in the NOD background (4) and a failure of $CD28^{-/-}CD4^+CD25^+$ thymocytes to demonstrate suppressor function (43). The defect in the ability of $CD18^{-/-}CD4^+CD25^+$ T cells to protect from $CD4^+CD25^-$-mediated colitis may be due to mechanisms other than those addressed in vitro and unique to the in vivo conditions of this model. Upon $CD4^+CD25^+$ T cell transfer into RAG-$1^{-/-}$ mice, the cells undergo lymphopenic proliferation, and $CD4^+CD25^+$ T cells, which have undergone lymphopenic proliferation, develop enhanced regulatory function (63). LFA-1 may be playing a role in this process. Furthermore, LFA-1 is critical for optimal trafficking of $CD4^+$ T cells to the PLN, MLN, and various peripheral tissues, including the intestine (19, 22, 64). The trafficking patterns of $CD4^+CD25^+$ T cells have not been clearly defined. There have been certain studies examining

specific chemokine receptors and the capacity to migrate to various chemokines under particular conditions (14, 65). In addition, the expression of a host of different adhesion molecules have been identified on the surface of $CD4^+CD25^+$ Treg cells (14). However, the necessity of these trafficking molecules for trafficking to specific lymphoid organs and tissues under various conditions has not been fully defined.

In conclusion, these studies demonstrate that CD18 is required for the development of optimal numbers of thymic and peripheral $CD4^+CD25^+$ T cells, as well as the optimal function of murine $CD4^+CD25^+$ T cells in vitro and in vivo. The absence of CD18 on $CD4^+CD25^+$ T cells may lead to defects in immune homeostasis and contribute to the development of autoimmune diseases. In addition, in the use of blocking anti-CD18 or anti-LFA-1 Abs in clinical disease, one needs to consider potential consequences on $CD4^+CD25^+$ T cells.

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Disclosures

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References

1. Shih, F. F., L. Mandik-Nayak, B. T. Wipke, and P. M. Allen. 2004. Massive thymic deletion results in systemic autoimmunity through elimination of $CD4^+CD25^+$ T regulatory cells. *J. Exp. Med.* 199: 323–335.
2. Aluvihare, V. R., M. Kallikourdis, and A. G. Betz. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nat. Immunol.* 5: 266–271.
3. Sakaguchi, S. 2004. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.
4. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431–440.
5. Kriegel, M. A., T. Lohmann, C. Gabler, N. Blank, J. R. Kalden, and H. M. Lorenz. 2004. Defective suppressor function of human $CD4^+CD25^+$ regulatory T cells in autoimmune polyglandular syndrome type II. *J. Exp. Med.* 199: 1285–1291.
6. Viglietta, V., C. Baecher-Allan, H. L. Weiner, and D. A. Hafler. 2004. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* 199: 971–979.
7. Oldenhove, G., M. de Heusch, G. Urbain-Vansanten, J. Urbain, C. Maliszewski, O. Leo, and M. Moser. 2003. $CD4^+CD25^+$ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. *J. Exp. Med.* 198: 259–266.
8. Bensinger, S. J., A. Bandeira, M. S. Jordan, A. J. Caton, and T. M. Laufer. 2001. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of $CD4^+25^+$ immunoregulatory T cells. *J. Exp. Med.* 194: 427–438.
9. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
10. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *J. Clin. Invest.* 112: 1437–1443.
11. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory $CD25^+CD4$ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 167: 188–195.
12. Green, E. A., Y. Choi, and R. A. Flavell. 2002. Pancreatic lymph node-derived $CD4^+CD25^+$ Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. *Immunity* 16: 183–191.
13. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199: 1401–1408.
14. Huehn, J., K. Siegmund, J. C. Lehmann, C. Siewert, U. Haubold, M. Feuerer, G. F. Debes, J. Lauber, O. Frey, G. K. Przybylski, et al. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like $CD4^+$ regulatory T cells. *J. Exp. Med.* 199: 303–313.
15. Kohm, A. P., and S. D. Miller. 2003. Role of ICAM-1 and P-selectin expression in the development and effector function of $CD4^+CD25^+$ regulatory T cells. *J. Autoimmun.* 21: 261–271.
16. Denning, T. L., G. Kim, and M. Kronenberg. 2005. Cutting edge: $CD4^+CD25^+$ regulatory T cells impaired for intestinal homing can prevent colitis. *J. Immunol.* 174: 7487–7491.
17. Taylor, P. A., A. Panoskaltsis-Mortari, J. M. Swedin, P. J. Lucas, R. E. Gress, B. L. Levine, C. H. June, J. S. Serody, and B. R. Blazar. 2004. L-Selectin^{hi} but not the L-selectin$^{lo}CD4^+25^+$ T regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* 104: 3804–3812.
18. Lub, M., Y. van Kooyk, and C. G. Figdor. 1995. Ins and outs of LFA-1. *Immunol. Today* 16: 479–483.
19. Berlin-Rufenach, C., F. Otto, M. Mathies, J. Westermann, M. J. Owen, A. Hamann, and N. Hogg. 1999. Lymphocytes migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *J. Exp. Med.* 189: 1467–1478.
20. Abraham, C., J. Griffith, and J. Miller. 1999. The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J. Immunol.* 162: 4399–4405.
21. Abraham, C., and J. Miller. 2001. Molecular mechanisms of IL-2 gene regulation following costimulation through LFA-1. *J. Immunol.* 167: 5193–5201.
22. Kandula, S., and C. Abraham. 2004. LFA-1 on $CD4^+$ T cells is required for optimal antigen-dependent activation in vivo. *J. Immunol.* 173: 4443–4451.
23. Larson, R. S., and T. A. Springer. 1990. Structure and function of leukocyte integrins. *Immunol. Rev.* 114: 181–217.
24. Bunting, M., E. S. Harris, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 2002. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving β_2 integrins and selectin ligands. *Curr. Opin. Hematol.* 9: 30–35.
25. Uzel, G., D. E. Kleiner, D. B. Kuhns, and S. M. Holland. 2001. Dysfunctional LAD-1 neutrophils and colitis. *Gastroenterology* 121: 958–964.
26. Schmits, R., T. M. Kundig, D. M. Baker, G. Shumaker, J. J. Simard, G. Duncan, A. Wakeham, A. Shahinian, A. van der Heiden, M. F. Bachmann, et al. 1996. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. *J. Exp. Med.* 183: 1415–1426.
27. Schonlau, F., K. Scharffetter-Kochanek, S. Grabbe, B. Pietz, C. Sorg, and C. Sunderkotter. 2000. In experimental leishmaniasis deficiency of CD18 results in parasite dissemination associated with altered macrophage functions and incomplete Th1 cell response. *Eur. J. Immunol.* 30: 2729–2740.
28. Scharffetter-Kochanek, K., H. Lu, K. Norman, N. van Nood, F. Munoz, S. Grabbe, M. McArthur, I. Lorenzo, S. Kaplan, K. Ley, et al. 1998. Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J. Exp. Med.* 188: 119–131.
29. Andrew, D. P., J. P. Spellberg, H. Takimoto, R. Schmits, T. W. Mak, and M. M. Zukowski. 1998. Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. *Eur. J. Immunol.* 28: 1959–1969.
30. Scharffetter-Kochanek, K., K. Norman, D. C. Bullard, I. Lorenzo, N. P. van Nood, S. Rich, W. Smith, and A. L. Beaudet. 1996. Generation and characterization of a CD18 complete knock-out mouse: new insights in the function of the CD18 molecule? *J. Invest. Dermatol.* 106: 809.
31. Barlow, S. C., H. Xu, C. T. Weaver, J. R. Lindsey, T. R. Schoeb, and D. C. Bullard. 2004. Development of dermatitis in CD18-deficient PL/J mice is not dependent on bacterial flora, and requires both $CD4^+$ and $CD8^+$ T lymphocytes. *Int. Immunol.* 16: 345–351.
32. Newberry, R. D., W. F. Stenson, and R. G. Lorenz. 1999. Cyclooxygenase-2-dependent arachidonic acid metabolites are essential modulators of the intestinal immune response to dietary antigen. *Nat. Med.* 5: 900–906.
33. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117: 265–277.
34. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395–1401.
35. Lee, S. H., J. E. Prince, M. Rais, F. Kheradmand, F. Shardonofsky, H. Lu, A. L. Beaudet, C. W. Smith, L. Soong, and D. B. Corry. 2003. Differential requirement for CD18 in T helper effector homing. *Nat. Med.* 9: 1281–1286.
36. Mottet, C., H. H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 170: 3939–3943.
37. Zhang, X., L. Izikson, L. Liu, and H. L. Weiner. 2001. Activation of $CD25^+CD4^+$ regulatory T cells by oral antigen administration. *J. Immunol.* 167: 4245–4253.
38. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, M. Mao, Y. Ding, and J. S. Bromberg. 2004. $CD4^+CD25^+CD62^+$ T regulatory cell subset has optimal suppressive and proliferative potential. *Am. J. Transplant.* 4: 65–78.
39. Barthlott, T., G. Kassiotis, and B. Stockinger. 2003. T cell regulation as a side effect of homeostasis and competition. *J. Exp. Med.* 197: 451–460.
40. Fehervari, Z., and S. Sakaguchi. 2004. $CD4^+$ Tregs and immune control. *J. Clin. Invest.* 114: 1209–1217.
41. Liang, S., P. Alard, Y. Zhao, S. Parnell, S. L. Clark, and M. M. Kosiewicz. 2005. Conversion of $CD4^+CD25^-$ cells into $CD4^+CD25^+$ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J. Exp. Med.* 201: 127–137.
42. Bayer, A. L., A. Yu, D. Adeegbe, and T. R. Malek. 2005. Essential role for interleukin-2 for $CD4^+CD25^+$ T regulatory cell development during the neonatal period. *J. Exp. Med.* 201: 769–777.
43. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat. Immunol.* 6: 152–162.
44. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
45. Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising $CD25^+CD4^+$ T cell receptors. *Immunity* 21: 267–277.

46. van Santen, H. M., C. Benoist, and D. Mathis. 2004. Number of Treg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J. Exp. Med.* 200: 1221–1230.
47. Kuhlman, P., V. T. Moy, B. A. Lollo, and A. A. Brian. 1991. The accessory function of murine intercellular adhesion molecule-1 in T lymphocyte activation: contributions of adhesion and co-activation. *J. Immunol.* 146: 1773–1782.
48. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* 7: 549–557.
49. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3: 756–763.
50. Peng, Y., Y. Laouar, M. O. Li, E. A. Green, and R. A. Flavell. 2004. TGF-β regulates in vivo expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA* 101: 4572–4577.
51. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced $CD4^+CD25^+$ cells educate $CD4^+CD25^-$ cells to develop suppressive activity: the role of IL-2, TGF-β, and IL-10. *J. Immunol.* 172: 5213–5221.
52. Curotto de Lafaille, M. A., A. C. Lino, N. Kutchukhidze, and J. J. Lafaille. 2004. $CD25^-$ T cells generate $CD25^+Foxp3^+$ regulatory T cells by peripheral expansion. *J. Immunol.* 173: 7259–7268.
53. Mellor, A. L., B. Baban, P. Chandler, B. Marshall, K. Jhaver, A. Hansen, P. A. Koni, M. Iwashima, and D. H. Munn. 2003. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J. Immunol.* 171: 1652–1655.
54. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat. Immunol.* 3: 1097–1101.
55. Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting edge: human $CD4^+CD25^+$ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J. Immunol.* 172: 4676–4680.
56. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med.* 194: 629–644.
57. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589–601.
58. Watanabe, T., and J. Fan. 1998. Atherosclerosis and inflammation mononuclear cell recruitment and adhesion molecules with reference to the implication of ICAM-1/LFA-1 pathway in atherogenesis. *Int. J. Cardiol.* 66(Suppl. 1): S45–S53; discussion S55.
59. Moriyama, H., K. Yokono, K. Amano, M. Nagata, Y. Hasegawa, N. Okamoto, K. Tsukamoto, M. Miki, R. Yoneda, N. Yagi, et al. 1996. Induction of tolerance in murine autoimmune diabetes by transient blockade of leukocyte function-associated antigen-1/intercellular adhesion molecule-1 pathway. *J. Immunol.* 157: 3737–3743.
60. Taniguchi, T., H. Tsukada, H. Nakamura, and Y. Seino. 1996. The effects of the anti-ICAM-1 monoclonal antibody on acute DSS colitis. *Gastroenterology* 110: A1025.
61. Sykulev, Y., Y. Vugmeyster, A. Brunmark, H. L. Ploegh, and H. N. Eisen. 1998. Peptide antagonism and T cell receptor interactions with peptide-MHC complexes. *Immunity* 9: 475–483.
62. Lenschow, D. J., K. C. Herold, L. Rhee, B. Patel, A. Koons, H.-Y. Qin, E. Fuchs, B. Singh, C. B. Thompson, and J. A. Bluestone. 1996. CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. *Immunity* 5: 285–293.
63. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells in vivo. *Nat. Immunol.* 3: 33–41.
64. Huleatt, J. W., and L. Lefrancois. 1996. β_2 integrins and ICAM-1 are involved in establishment of the intestinal mucosal T cell compartment. *Immunity* 5: 263–273.
65. Lim, H. W., P. Hillsamer, and C. H. Kim. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J. Clin. Invest.* 114: 1640–1649.