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Aberrant TGF-β signaling reduces T regulatory cells in ICAM-1-deficient mice, increasing the inflammatory response to *Mycobacterium tuberculosis*

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ABSTRACT

Foxp3+ T regulatory cells are required to prevent autoimmune disease, but also prevent clearance of some chronic infections. While natural T regulatory cells are produced in the thymus, TGF-β1 signaling combined with T-cell receptor signaling induces the expression of Foxp3 in CD4+ T cells in the periphery. We found that ICAM-1−/− mice have fewer T regulatory cells in the periphery than WT controls, due to a role for ICAM-1 in induction of Foxp3 expression in response to TGF-β1. Further investigation revealed a functional deficiency in the TGF-β1-induced translocation of phosphorylated Smad3 from the cytoplasmic compartment to the nucleus in ICAM-1-deficient mice. This impairment in the TGF-β1 signaling pathway is most likely responsible for the decrease in T regulatory cell induction in the absence of ICAM-1. We hypothesized that in the presence of an inflammatory response, reduced production of inducible T regulatory cells would be evident in ICAM-1−/− mice. Indeed, following *Mycobacterium tuberculosis* infection, ICAM-1−/− mice had a pronounced reduction in T regulatory cells in the lungs compared with control mice. Consequently, the effector T-cell response and inflammation were greater in the lungs of ICAM-1−/− mice, resulting in morbidity due to overwhelming pathology. *J. Leukoc. Biol.* 86: 713–725; 2009.

Introduction

T regulatory cells play a key role in suppressing autoimmunity [1]. During chronic infections with pathogens, such as *Leishmania major* and *Helicobacter pylori*, T regulatory cell function inhibits pathogen clearance and dampens the immune response [2–4]; thus, these cells are partially responsible for persistence of certain pathogens. CD4+ T regulatory cells are identified most reliably by expression of Foxp3 [5–7] but were originally identified by high expression of CD25.

Natural CD4+CD25+Foxp3+ T regulatory cells are produced in the thymus. A very strong T-cell receptor signaling response could turn a developing T cell down the path to become a T regulatory cell [8], and it has been suggested that these cells play a strong role in control over autoimmunity due to thymic expression of self-antigens [9]. Naïve CD4+ T cells in the periphery can also become Foxp3+ through T-cell receptor stimulation in conjunction with activated TGF-β1 cytokine in vitro and in vivo [10–14]. Inducible T regulatory cells have suppressive functions in response to specific non-self-antigens [10, 11, 14]. Since natural and TGF-β1-induced T regulatory cells have the same phenotype in vivo during an inflammatory response (CD4+CD25+Foxp3+), it is difficult to discriminate between the roles of these two phenotypes or whether it is natural or inducible T regulatory cells that inhibit pathogen clearance.

The role of T regulatory cells during *Mycobacterium tuberculosis* infection has been investigated previously. Depletion of CD4+CD25+ T cells ex vivo increased human and murine effector T-cell production of IFN-γ, a proinflammatory cytokine required for control over *M. tuberculosis* infection [15–17], supporting a suppressive role for T regulatory cells in tuberculosis. While in vivo depletion of CD25+ cells in *M. tuberculosis*-infected mice did not reduce bacterial burden [17], specific depletion of Foxp3+ cells or reconstitution of RAG−/− mice with leukocytes lacking CD4+CD25+ cells did improve control of infection [18, 19]. Thus, the available data support that, as in other infectious diseases, T reg-

Abbreviations: EAE=experimental autoimmune encephalon myelitis, ICAM=intercellular adhesion molecule, WT=wild type

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ulatory cells dampen the immune response to *M. tuberculosis* infection and inhibit clearance of the infection. However, another role for regulatory T cells may be the inhibition of inflammatory responses and subsequent pathology associated with infections.

The intercellular adhesion molecule-1 (ICAM-1), expressed not only on endothelium but also on leukocytes, can mediate extravasation of leukocytes into the lung [20]. ICAM-1 binding to LFA-1 allows T cells to adhere to thymic stroma during development [21] and assists in thymic positive selection of CD4+ or CD8+ T lymphocytes [21, 22]. ICAM-1 can also be found in association with LFA-1 at the immunological synapse [23–25] and transmits costimulatory signals to T lymphocytes [26–30]. Sakaguchi and colleagues reported in a review article (as unpublished data) that ICAM-1-deficient mice develop fewer T regulatory cells in the periphery than controls [31], but the etiology of the deficiency has not completely examined. It has been shown that ICAM-1−/− CD4+CD25+ T cells adoptively transferred into mice immunized with MOG peptide [which induces experimental autoimmune encephalomyelitis (EAE)] could depress proliferation of T cells specific for MOG peptide, but like wild-type (WT) T regulatory cells, they could not prevent onset of EAE [32]. As the expression of Foxp3 was not assessed in adoptively transferred CD4+CD25+ T cells and the number of T regulatory cells from ICAM-1−/− and WT mice was not compared, the role of ICAM-1 in T regulatory cell development and function has yet to be established.

Two studies addressing the role of ICAM-1 in *M. tuberculosis* infection gave conflicting results. One study reported that mice required ICAM-1 to form granulomas, with higher bacterial burdens and lower survival in ICAM-1−/− mice [33]. Another study indicated that ICAM-1 is not required for survival of mycobacterial infection [34]. Deficiency in the LFA-1 ICAM-1 ligand results in decreased T-cell priming, and aberrant granuloma formation [35], while another ligand for ICAM-1, Mac-1 (CD11b/CD18), is not required to control *M. tuberculosis* [35]. The immunologic function of ICAM-1 during *M. tuberculosis* infection, thus, remains unclear.

In this study, we investigated the role of ICAM-1 in the production of T regulatory cells. In spite of similar Foxp3 expression in the thymus, ICAM-1−/− mice had significantly fewer T regulatory cells in peripheral organs such as the lung and spleen. Further investigation showed that ICAM-1 is required for the induction of CD4+CD25+Foxp3+ T regulatory cells in response to active TGF-β1 and T-cell receptor stimulation and that this was due to a functional defect in the TGF-β1 signaling pathway. In response to low-dose aerosol *Mycobacterium tuberculosis* infection, the deficit in expression of Foxp3+ T regulatory cells in ICAM-1−/− mice resulted in a more robust effector T lymphocyte response and greater inflammation compared with control mice. During high-dose *M. tuberculosis* infection, inflammation became overwhelming, and ICAM-1−/− mice became moribund more rapidly than wild-type mice, in spite of having a bacterial burden below that usually considered lethal.

MATERIALS AND METHODS

Animals

Breeding pairs of B6.129S4-*ICAM-1*^{tm1jcgr}/J (Generation N10F21) (ICAM-1−/−) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). ICAM-1−/− (Generation N10F21) and control mice (Jackson Laboratory) were housed in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Mycobacteria and infection of mice

Low-dose aerosol infection of mice (20–100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been described previously [36]. The bacterial burden was determined as described previously [37].

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI, USA).

IFN-γ detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay, performed as described previously [38, 39], using bone marrow-derived dendritic cells infected with *M. tuberculosis* (MOI 3) for stimulation of lung and lymph node cells.

Production of bone marrow-derived antigen-presenting cells

Preparation of bone marrow-derived dendritic cells used as antigen-presenting cells in the ELISpot assay was as described previously [38, 39].

Flow cytometry

Phenotyping of leukocytes has been described previously [40, 41]. Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70), anti-GR1 (clone RB6-8C5), anti-CD25 (clone PC61), anti-KI-67 (clone B56) and anti-Annexin V (BD PharMingen). Data were collected on a BD FACSCalibur, and analyses were performed in FlowJo software (Tree Star, Inc., Ashland, OR, USA). Activated caspase 3 (clone information not provided) (Cell Technology Inc., Mountainview, CA, USA), phosphorylated Smad3 (clone information not provided) (Cell Signaling Technology, Danvers, MA, USA), and Foxp3 (clone FKJ-16 s) (eBioscience, San Diego, CA, USA) were detected according to the manufacturer's protocols.

Histopathology

Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. 5- to 6-μm sections were H&E stained and examined in a blinded fashion with 2-3 fields per lung section imaged at ×4 or ×10 magnification using a Zeiss Axiovert 200 microscope. Randomly selected fields that did not overlap, in which lung tissue filled the entire image, and cross sections of large airways were avoided, were selected for analysis. The amount of airspace in each image was quantified as pixels with ImageJ (National Institutes of Health, available at http://rsb.info.nih.gov/ij/). Images were analyzed by thresholding the image, applying a median filter set to a 1 pixel diameter, inverting the image so that the airspaces were highlighted and using the Analyze Particle feature to measure amount of airspace present in each image. The percent airspace per image was determined by dividing the area in the image occupied by airspace by the total image area. Mean percent airspace for all images was calculated for each mouse, and data are presented as mean percent airspace for all mice within each treatment group.

In vivo migration assays and adoptive transfer of T regulatory cells

CD4+CD25+ T lymphocytes from *M. tuberculosis* infected ICAM-1−/− and WT spleens were separated with MACS Regulatory T cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's protocol. For adoptive transfer, 8×10^6 T regulatory cells were intravenously injected into recipient ICAM-1−/− or WT mice. T regulatory cells for migration assays were counted, labeled using PKH26 (red, ICAM-1−/−) or PKH67 (green, WT) (Sigma-Aldrich, St. Louis, MO), and combined in a 1:1 ratio (ICAM-1−/−:WT). Eighteen-hour postintravenous injection of donor cells, lung perfusion, and bronchoalveolar lavage (BAL) were performed on *M. tuberculosis*-infected ICAM-1−/− and WT mice (following euthanasia). Tissue from the lung, liver, lymph nodes, and spleen was used for flow cytometry.

In vivo survival assays

CD4+ and CD8+ T lymphocytes were enriched using MACS beads (Miltenyi Biotec). WT or ICAM-1−/− T lymphocytes were combined in a 1:1 ratio with WT or ICAM-1−/− non-T lymphocytes and incubated with 30 units/ml IL-2 (Peprotec, Rocky Hill, NJ, USA). At predetermined time points, cells were collected for flow cytometry.

In vitro induction of Foxp3 expression in CD4+CD25-T cells

CD4+CD25- splenocytes were separated from naïve ICAM-1−/− or WT spleens using MACS CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotech). Enriched T cells were incubated at 37°C for up to 72 h with 100 U/ml IL-2 (PeproTech), 5 ng/ml active TGF-β1 (R&D Systems, Minneapolis, MN, USA), anti-CD3 and anti-CD28 antibodies (BD PharMingen, San Jose, CA, USA) in a protocol adapted from those described previously by other groups [12, 14, 15, 42]. At predetermined time points, aliquots of T cells were taken for flow cytometry staining or immunoblotting. To add back ICAM-1, 12 μg/ml ICAM-1-Fc chimera (R&D Systems), was added to ICAM-1−/− cultures.

Cell lysates for immunoblots

Smads and β-actin were detected by immunoblotting. Collected lymphocytes were counted using Trypan blue exclusion and lysed in cell lysis buffer (Tris, 1 NaCl, EDTA, NP-40) for 10 min on ice with phosphatase and protease inhibitors: leupeptin, aprotinin, pepstatin A, AEBSF, β-GLP, and orthvan. Cell lysates were centrifuged at high speed for 10 min. The supernatant (cytoplasmic extract) was removed from the pellet (nuclear extract). Sample running buffer (Tris-Cl, SDS, glycerol, DTT, bromophenol blue) was added to each sample, and boiled for 5 min prior to loading on the gel.

Immunoblotting

Cell lysates were loaded on a 10% acrylamide mini-gel next to Bio-Rad Precision Protein Dual Color Standard (Bio-Rad, Hercules, CA, USA). Proteins were wet transferred onto a 0.4-μm PVDF membrane (Pall Life Sciences, East Hills, NY, USA) in tris-glycine-methanol buffer. Membranes were blocked in 4% bovine serum albumin. Primary antibody against specific Smads or β-actin (Cell Signaling Technology, Boston, MA, USA) were diluted in 4% bovine serum albumin in TBS/Tween 20 and incubated at 4°C overnight. After washing the immunoblot, we incubated the secondary antibody conjugated to HRP at room temperature for at least 1 h. Bands were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA), according to the manufacturer instructions, and images were captured and quantified using a Kodak Image station.

Immunoprecipitation

Specific monoclonal antibody against Smad4 (Abcam, Cambridge, MA, USA) and L-Sepharose beads (Fisher Scientific, Pittsburgh, PA, USA) were added to nuclear or cytoplasmic lysates rocked at 4°C overnight. The following morning, beads were centrifuged out of solution, and the supernatant was removed. Western blot sample running buffer was added to the beads to detach them from antibody, and beads were centrifuged out of solution. Samples were then analyzed by immunoblotting.

Statistical analysis

Statistical significance between two groups was compared by a two-sided Student's *t* test when data were normally distributed. Bacterial burden was normalized by log transformation prior to statistical analysis. A value of $P < 0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

RESULTS

Fewer Foxp3+ T regulatory cells are in the periphery of ICAM-1−/− mice

A review suggested that fewer T regulatory cells could be found in the absence of ICAM-1 expression [31], but the supporting data were not published. To explore whether ICAM-1 is required for production of T regulatory cells, the presence of Foxp3+CD4+ T regulatory cells was assessed by flow cytometry in the periphery of C57BL/6 ICAM-1−/− and wild-type (WT) control mice (**Fig. 1A**). Significantly fewer Foxp3+ T regulatory cells were detected in the lungs and spleens of ICAM-1−/− mice compared with WT controls. In contrast, in the thymi of ICAM-1−/− and WT mice, Foxp3 expression was similar, suggesting that ICAM-1 is not required for thymic production of T regulatory cells.

ICAM-1 might play several roles in localizing T regulatory cells in peripheral organs. First, because both ICAM-1 and its lymphocyte ligand, LFA-1 can produce internal signals that cause proliferation, apoptosis, or costimulation [26–30], ICAM-1 ligation may be a required survival in the T regulatory cell. Next, ICAM-1 might be required for firm adhesion that allows migration of T regulatory cells into the peripheral organs. ICAM-1 may also provide a signal supporting proliferation of T regulatory cells. Finally, although natural T regulatory cells are produced equivalently in the thymi of ICAM-1−/− and control mice, ICAM-1 might be required to produce inducible T regulatory cells in the periphery.

ICAM-1 is not required for the survival of T regulatory cells

To test the hypothesis that ICAM-1 is required to send survival signals to regulatory T cells, flow cytometry staining of activated caspase 3 was used as a marker for apoptosis. Apoptosis of Foxp3+ T regulatory cells in the thymi of ICAM-1−/− and WT mice was similar (Fig. 1B), suggesting that ICAM-1 is not required for natural T regulatory cells to survive within the thymus. Foxp3+ T regulatory cells in the lungs and in the spleens of ICAM-1−/− and WT mice showed similar percentages of activated caspase 3+ cells within the Foxp3 population (Fig. 1B), suggesting that Foxp3+ T regulatory cells do not require ICAM-1 ligation to survive.

To confirm that ICAM-1 is not required for survival of T regulatory cells in the periphery, an in vivo T regulatory cell survival assay was performed by injecting congenic marker dis-

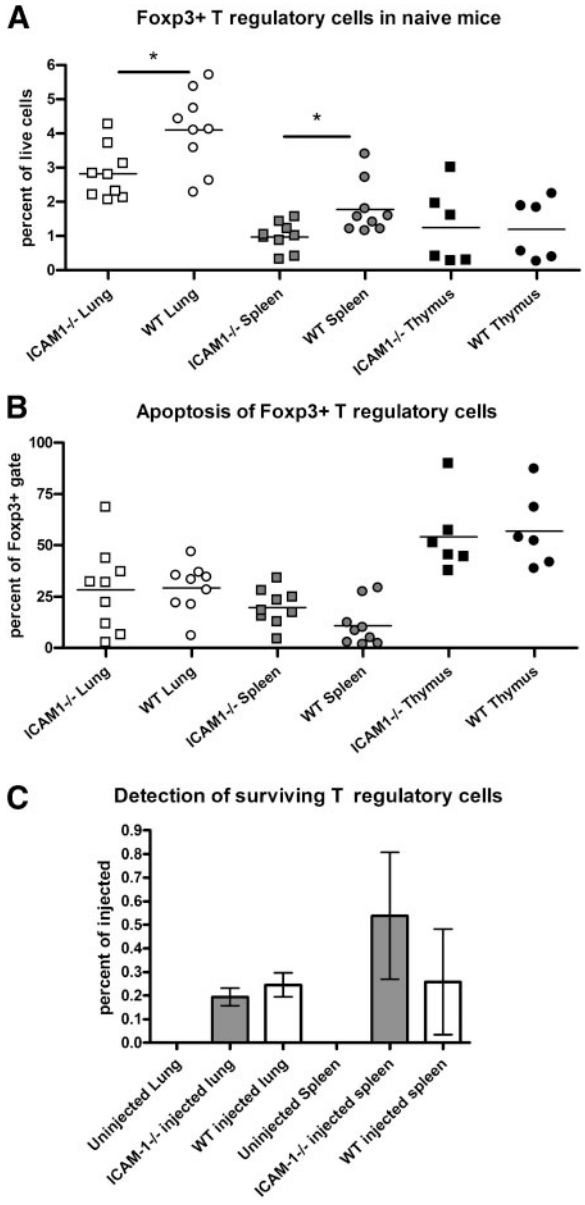

Figure 1. Foxp3 expression is significantly reduced in ICAM-1−/− mice. (A) Expression of Foxp3 was determined in naïve mice by flow cytometry and reported as a percentage of CD4+ T cells. *, $P < 0.05$. (B) The percentage of T regulatory cells undergoing apoptosis was determined by flow cytometry. The CD4+ Foxp3+ T regulatory cells were gated on within the live cell and lymphocyte gates. The total caspase 3+ cells within the Foxp3+ gate are reported. (C) The percentage of surviving injected T regulatory cells (Thy1.2+) in the recipient (Thy1.1+) organs was determined by flow cytometry of the lung cells and gating on the live cells and CD4+ lymphocytes. The total Thy1.2+ cells detected in the lungs are shown as a percentage of the total Thy1.2+ T regulatory cells that were injected.

crepant T regulatory cells into recipients and comparing survival of ICAM-1−/− and WT cells. Thy1.2+CD4+CD25+ T regulatory cells were enriched from ICAM-1−/− and WT controls. 5×10^6 T regulatory cells were adoptively transferred into WT Thy1.1+ recipients. Two weeks following transfer, lungs and spleens of Thy1.1 recipients were harvested, and the survival of injected ICAM-1−/− or WT T regulatory cells was assessed. Similar percentages of input Thy1.2+ donor T regulatory cells were detected in the lungs and spleens of WT recipients (Fig. 1C), confirming that ICAM-1 is not required on T regulatory cells for their survival in the periphery.

ICAM-1 is not required for the migration of T regulatory cells in the periphery

T regulatory cells could be dependent on ICAM-1 for migration in the periphery. To test this hypothesis, we used an antigen-specific system to follow the migration of T regulatory cells into the tissues during an inflammatory response. ICAM-1−/− or WT mice were infected with *M. tuberculosis* for at least 4 wk. CD4+CD25+ T regulatory cells from infected ICAM-1−/− or WT spleens were enriched using MACS columns. To observe migration of T regulatory cells, ICAM-1−/− and WT CD4+CD25+ T cells were differentially labeled red (PKH26) or green (PKH67), combined in equal numbers and adoptively transferred into recipients that had been infected with *M. tuberculosis* for at least 4 wk. In this way, each recipient received equal numbers of adoptively transferred ICAM-1−/− and WT cells, and their migration could be directly compared. Donor CD4+CD25+ T cells were allowed to migrate for 18 h, at which point, recipient organs were harvested to assess the migration of ICAM-1−/− and WT T regulatory cells. **Figure 2A** shows representative dot plots of flow cytometry detection of labeled T regulatory cells in recipient lungs. Although it appeared that fewer ICAM-1−/− T regulatory cells migrated into ICAM-1−/− lungs when comparing dot plots, the differences were not statistically significant by Student's *t* test, even when 15 mice per group were compared (Fig. 1A and data not shown). WT T regulatory cells were equally capable of migrating into the lungs (Fig. 2B) and spleens (data not shown) of ICAM-1−/− and WT recipient mice, suggesting that ICAM-1 expression is not required on the endothelium in order for T regulatory cells to migrate into infected tissues. Similar percentages of ICAM-1−/− and WT T regulatory cells were detected in the lungs (Fig. 2C) and spleens (data not shown) of WT mice, suggesting that ICAM-1 is not required on the T regulatory cell for the migration to tissues from the periphery. Similar experiments performed with total CD4 T cell populations demonstrated that ICAM-1 expression on T cells or endothelium was not required for normal migration of cells into the lungs of infected mice, nor was there an increase in migration into ICAM1−/− lungs of total CD4 T cells (data not shown).

Induction of regulatory T cells by TGF-β1 partially requires ICAM-1 expression

Foxp3 expression that results in functional T regulatory cells can be induced in peripheral CD4+ T cells by TGF-β1 stimulation in vitro [12, 14, 43] and in vivo [11, 13]. To determine whether ICAM-1 is required to induce Foxp3 expression, CD4+CD25- (non-T regulatory) cells were enriched from naïve ICAM-1−/− or WT spleens using MACS columns. It is possible that Foxp3+ T regulatory cells could be CD25 low, allow-

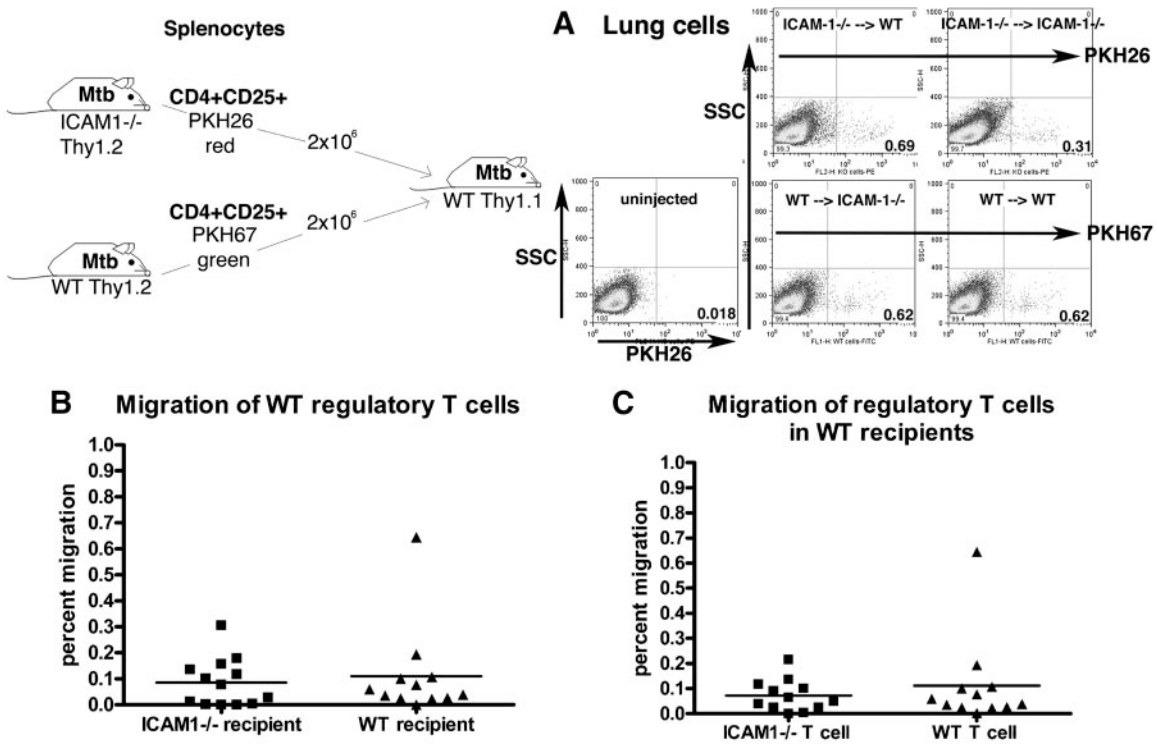

Figure 2. ICAM-1 expression is not required for migration of T regulatory cells in the periphery. The in vivo migration of T regulatory cells from *M. tuberculosis*-infected mice stained red (ICAM-1−/−) or green (WT) in *M. tuberculosis*-infected recipients following adoptive transfer by intravenous injection. (A) Representative dot plots: detection of ICAM1−/−1 (PKH26, red) or WT (PKH67, green) transferred cells in the lungs of recipient mice by flow cytometry. The dot plots shown here were gated on CD4+ T cells within the live cell and lymphocyte gates. (B) To determine whether migration requires ICAM-1 on the endothelium, the percentages of injected WT T regulatory cells detected in lungs of ICAM-1−/− (squares) or WT (triangles) recipients (cells detected in organs/injected cells•100) were compared. No significant differences were observed. (C) To determine whether migration requires ICAM-1 on the T regulatory cells, the percentages of transferred WT (triangles) or ICAM-1−/− (squares) T regulatory cells detected in the lungs of WT mice were compared (cells detected in organs/injected cells•100). No significant differences were found.

ing some T regulatory cells to be present in these cultures. However, flow cytometry analysis of Foxp3 expression after enrichment showed no expression of Foxp3 in CD4+CD25- T cells (**Fig. 3A**, time 0 and Supplemental Fig. 1), supporting that the vast majority of T regulatory cells had been removed with this treatment. CD4+CD25-T cells were incubated for up to 72 h with recombinant active TGF-β1, IL-2, anti-CD3, and anti-CD28 antibodies, as described by others as a method of inducing T regulatory cells [12, 14, 15, 42]. CD25 expression was up-regulated on 100% of ICAM-1−/− and WT T cells by 48 h of incubation and maintained through 72 h (data not shown). After 72 h, Foxp3 expression was detected in significantly fewer ICAM-1−/− CD4+ T cells compared with WT (Fig. 3A and Supplemental Fig. 1), suggesting a functional difference between ICAM-1−/− and WT CD4+ T cells in their ability to respond to TGF-β1. The addition of soluble ICAM-1-Fc recovered Foxp3 induction, supporting a role for ICAM-1 in Foxp3 induction (Fig. 3A).

To confirm that ICAM-1−/− and WT-inducible T regulatory cells were functionally active, we collected activated leukocytes from the lungs of *M. tuberculosis*-infected mice. Lung leukocytes were added to an IFN-γ ELISpot, in which bone marrow derived dendritic cells presented *M. tuberculosis* antigens. Inducible T regulatory cells were added in a 1:1 ratio with lung leukocytes to determine whether IFN-γ production could be suppressed by inducible T regulatory cells. IFN-γ production by WT T cells from *M. tuberculosis*-infected mice was suppressed by both ICAM-1−/− and WT T regulatory cells (Supplemental Fig. 2).

Active TGF-β1 interacts with TGF-βRII, which phosphorylates TGF-βRI. In turn, TGF-βR1 recruits and phosphorylates Smads [44, 45] that translocate to the nucleus to act as transcription factors [46]. To assess whether TGF-β1 signaling was equivalent in ICAM-1−/− and WT CD4+ T cells, phosphorylation and translocation of Smad2 and Smad3 was compared by immunoblotting. Protein was collected from the cytoplasmic and nuclear fractions of T cells in the in vitro culture system described above, with representative blots shown in Fig. 3B. Early expression of phosphorylated Smad2 during TGF-β1 stimulation was similar in ICAM-1−/− and WT T cells (data not shown). ICAM-1−/− and WT T cells also had similar levels of total Smad3 protein prior to treatment with TGF-β1 (data not shown), and similar levels of phosphorylated Smad3 (pSmad3) were detected in the cytoplasm of ICAM-1−/− and WT CD4+ T cells during in vitro culture (Fig. 3C). However, significantly more pSmad3 was detected in the nucleus of WT CD4+ T cells compared with ICAM-1−/− CD4+ T cells (Fig. 3D). More total pSmad3 (determined by adding

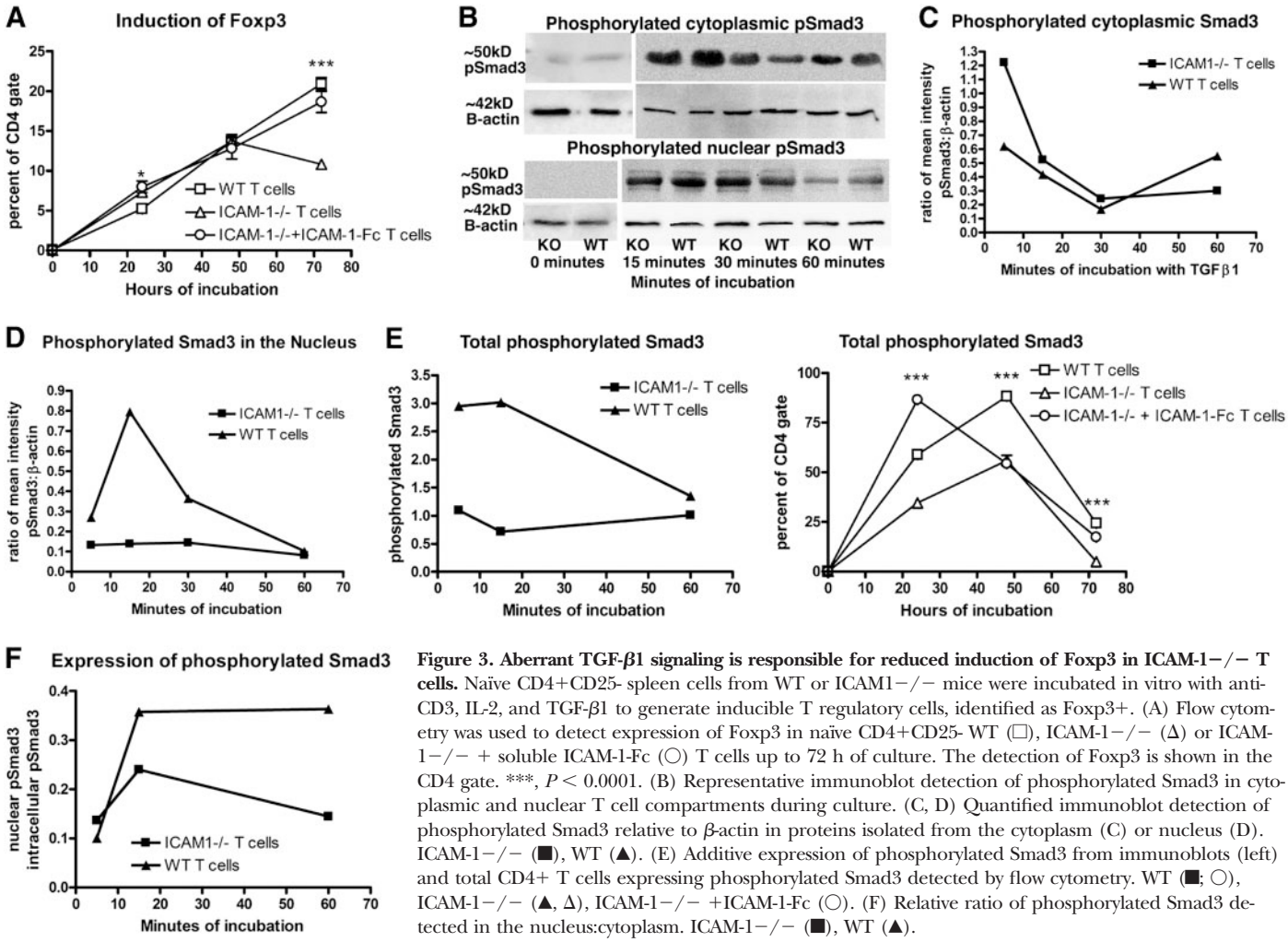

Figure 3. Aberrant TGF-β1 signaling is responsible for reduced induction of Foxp3 in ICAM-1−/− T cells. Naïve CD4+CD25- spleen cells from WT or ICAM1−/− mice were incubated in vitro with anti-CD3, IL-2, and TGF-β1 to generate inducible T regulatory cells, identified as Foxp3+. (A) Flow cytometry was used to detect expression of Foxp3 in naïve CD4+CD25- WT (□), ICAM-1−/− (Δ) or ICAM-1−/− + soluble ICAM-1-Fc (○) T cells up to 72 h of culture. The detection of Foxp3 is shown in the CD4 gate. ***, $P < 0.0001$. (B) Representative immunoblot detection of phosphorylated Smad3 in cytoplasmic and nuclear T cell compartments during culture. (C, D) Quantified immunoblot detection of phosphorylated Smad3 relative to β-actin in proteins isolated from the cytoplasm (C) or nucleus (D). ICAM-1−/− (■), WT (▲). (E) Additive expression of phosphorylated Smad3 from immunoblots (left) and total CD4+ T cells expressing phosphorylated Smad3 detected by flow cytometry. WT (■; ○), ICAM-1−/− (▲, Δ), ICAM-1−/− +ICAM-1-Fc (○). (F) Relative ratio of phosphorylated Smad3 detected in the nucleus:cytoplasm. ICAM-1−/− (■), WT (▲).

relative nuclear and cytoplasmic expression in immunoblots, and by flow cytometry) was seen in WT compared with ICAM-1−/− CD4+ T cells (Fig. 3E). The addition of soluble ICAM-1-Fc to ICAM-1−/− CD4+ T cells resulted in a significant increase in pSmad3 (Fig. 3E), which correlated with recovery of WT levels of Foxp3 expression. In WT CD4+ T cells, the relative ratio of pSmad3 in the nucleus compared with the cytoplasm was higher than in ICAM-1−/− CD4+ T cells (Fig. 3F), supporting that there is a defect in translocation of pSmad3 into the nucleus of ICAM-1−/− T cells.

Optimal translocation of pSmad3 in association with Smad4 requires ICAM-1

The requirement of ICAM-1 in translocation of pSmad3 from the cytoplasm into the nucleus was surprising. After being phosphorylated, Smad transcription factors associate with Smad4, a Smad shuttle protein that facilitates movement from the cytoplasm into the nucleus (reviewed in [47]). We hypothesized that ICAM-1 might be required for association of pSmad3 with Smad4 following phosphorylation, so that ICAM-1 deficiency prevents pSmad3 from binding to Smad4, causing pSmad3 to remain in the cytoplasm. To test this hypothesis, Smad4 was immunoprecipitated from cytoplasmic and nuclear cell lysates, and coimmunoprecipitation of pSmad3 was assessed by immunoblotting. Representative immunoblots are shown in **Fig. 4A**. The mean intensity of the detected ICAM-1−/− pSmad3 bands was much lower than pSmad3 from WT T cells in the nucleus (Fig. 4B), while similar pSmad3 was detected in ICAM-1−/− and WT cytoplasmic immunoprecipitates (Fig. 4C). pSmad3 in nuclear or cytoplasmic lysates was normalized to Smad4 in immunoprecipitates, and this ratio supported a greater detection of pSmad3 in the nuclear lysastes of WT cells, while similar levels of pSmad3 could be detected in the cytoplasm (Fig. 4D). This suggested ICAM-1 may play a role in pSmad3 translocation downstream of its association of Smad4.

ICAM-1−/− T regulatory cell deficit results in more severe inflammation

We hypothesized that during an inflammatory response, there would be a more pronounced difference due to the ICAM-1−/− defect in induction of Foxp3 in CD4 T cells. To test this, ICAM-1−/− and WT mice were infected with *M. tuberculosis* (either ~25 or ~100 colony forming units). After 7 wk of infection, the total number of Foxp3+ T regulatory cells in-

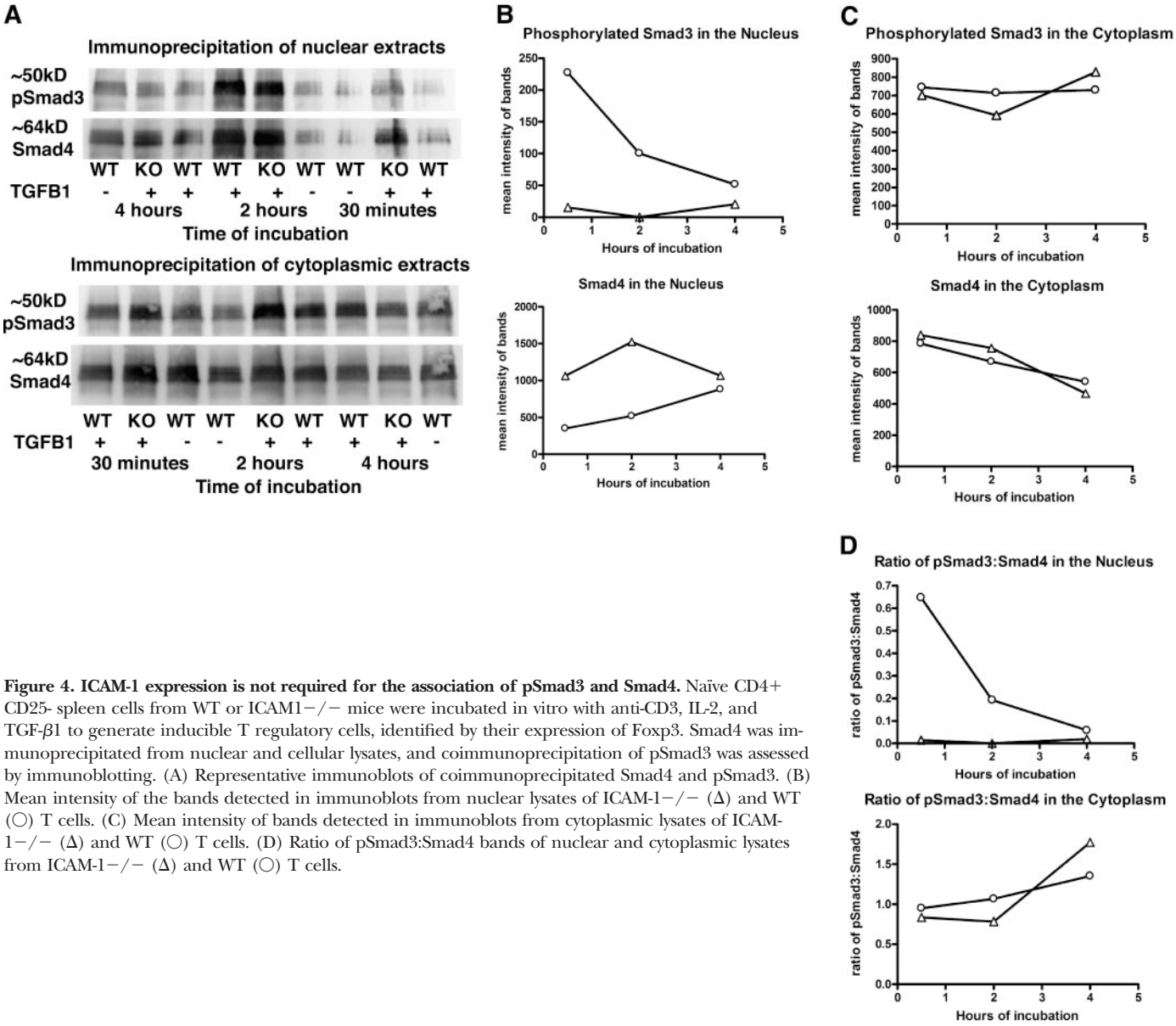

Figure 4. ICAM-1 expression is not required for the association of pSmad3 and Smad4. Naïve CD4+ CD25- spleen cells from WT or ICAM1−/− mice were incubated in vitro with anti-CD3, IL-2, and TGF-β1 to generate inducible T regulatory cells, identified by their expression of Foxp3. Smad4 was immunoprecipitated from nuclear and cellular lysates, and coimmunoprecipitation of pSmad3 was assessed by immunoblotting. (A) Representative immunoblots of coimmunoprecipitated Smad4 and pSmad3. (B) Mean intensity of the bands detected in immunoblots from nuclear lysates of ICAM-1−/− (Δ) and WT (○) T cells. (C) Mean intensity of bands detected in immunoblots from cytoplasmic lysates of ICAM-1−/− (Δ) and WT (○) T cells. (D) Ratio of pSmad3:Smad4 bands of nuclear and cytoplasmic lysates from ICAM-1−/− (Δ) and WT (○) T cells.

creased in both WT and ICAM-1−/− mice, but there were significantly fewer T regulatory cells in the lungs of ICAM-1−/− mice than in WT lungs by 7 wk postinfection, which is the beginning of the chronic phase of infection (**Fig. 5A**). At this time point, Foxp3+ T regulatory cells in the lungs of WT mice represented 7.26 ± 1.06% of CD4+ T cells, while Foxp3+ T regulatory cells in ICAM-1−/− mice represented only 3.09% ± 0.89% of CD4+ T cells ($P<0.0001$). We hypothesized that ICAM-1 signals proliferation of T regulatory cells during an inflammatory response to account for the reduction in T regulatory cells in ICAM-1−/− mice. However, experiments throughout acute and chronic infection comparing expression of cell cycle antigen KI67 and lymphocyte proliferation assays early in infection indicated that there was no difference in the proliferation of T regulatory cells during *M. tuberculosis* infection (data not shown).

Recent studies suggest that in the absence of T regulatory cells, the cytokine response to *M. tuberculosis* is enhanced [15, 17–19, 48], and bacterial control is improved [18, 19]. There was no difference in survival of ICAM-1−/− and WT mice infected with ~25 CFU of *M. tuberculosis* (both strains survived more than a year postinfection), and bacterial numbers in the lungs were similar over the course of infection (Fig. 5B). Histological examination of the lungs of ICAM-1−/− and WT mice infected with *M. tuberculosis* revealed that organized granulomas were detectable in the lungs by 4 wk postinfection (**Fig. 6A**). However, after 16 wk of infection, lung infiltration appeared much higher by histological observation in ICAM-1−/− animals than controls (Fig. 6B). Computational comparison of the available airspace in random histological samples revealed that ICAM-1−/− mice had significantly less airspace available than WT mice ($P<0.0001$) (Fig. 6C),

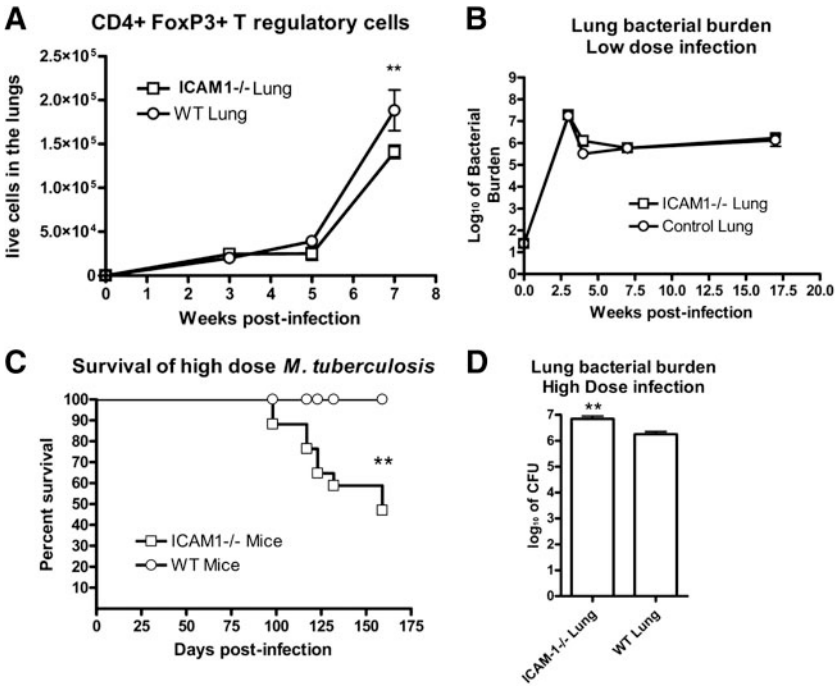

Figure 5. Fewer T regulatory cells are found in the lungs in *M. tuberculosis*-infected ICAM1−/− mice, and at higher inoculum survival of the ICAM-1−/− mice is impaired. C56BL/6 mice were aerosol infected with low dose (A, B) or high dose (C, D) *M. tuberculosis*, and the immune response was followed over the course of infection. (A) Total Foxp3+ T regulatory cells in ICAM-1−/− (□) or WT (▲) lungs determined by multiplying the total number of cells in the lungs by the fraction of live cells that were CD4+ Foxp3+ by flow cytometry; **, $P < 0.001$ (representative of 8 separate experiments). The percentage of Foxp3+ T cells in the CD4+ gate was consistently lower in ICAM1−/− mice compared with WT mice, and the total number of cells is reflected to show that although there are more total cells in the ICAM1−/− mice, there are fewer total T regulatory cells. (B) Bacterial numbers in the lungs of ICAM1−/− (square) and WT (triangle) mice infected with a low dose of *M. tuberculosis* (~25 CFU) are not substantially different over the course of infection. Experiment was repeated 8 times. (C) Survival of ICAM-1−/− (squares) and WT (circles) aerosol infected with higher dose (~100 CFU/mouse). $P < 0.001$. Experiment was repeated twice. (D) Bacterial numbers were slightly higher in ICAM1−/− lungs compared with WT lungs at 18 wk postinfection when mice were infected with high dose (~100 CFU/lungs) *M. tuberculosis* ($P<0.001$).

suggesting that the higher lung infiltrate compromised lung capacity in ICAM-1−/− mice.

In light of previous publications [33, 34] in which ICAM-1−/− mice were more susceptible to *M. tuberculosis* infection, we infected ICAM-1−/− and WT mice with a higher dose of infection (~100 CFU), and followed survival. ICAM-1−/− mice began to succumb to infection by 14 wk postinfection (Fig. 5C). Further investigation revealed that while bacterial burden was slightly higher in ICAM-1−/− mice than WT mice (~5 fold in the lungs) (Fig. 5D), it was not at a level that is usually fatal. Histological examination revealed substantially greater inflammation and pathology in the lungs of ICAM-1−/−, compared with WT mice. The lack of effector T-cell regulation in lungs of ICAM-1−/− mice likely contributed to overwhelming inflammation and increased morbidity in the absence of ICAM-1.

Flow cytometry analysis confirmed that significantly higher numbers of CD4+ and CD8+ T lymphocytes were present in the lungs of ICAM-1−/− mice infected with both doses of *M. tuberculosis* beginning ~7 wk postinfection (**Fig. 7, A** and **B**).

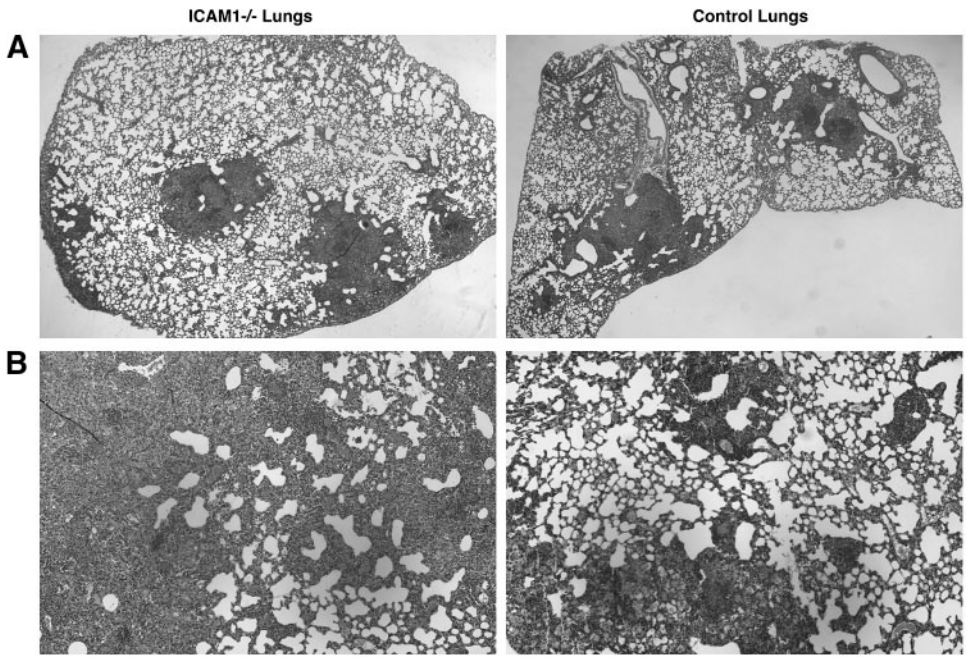

Figure 6. ICAM-1 is not required for granuloma formation, but in absence of ICAM-1, inflammation is increased in *M. tuberculosis*-infected lungs. Representative sections of H&E-stained ICAM-1−/− (left) or WT (right) lungs from mice infected with low-dose aerosol *M. tuberculosis*. (A) ×4 magnification of lung sections from mice infected 4 wk with *M. tuberculosis* (B) ×10 magnification of lung sections from mice infected 16 wk with *M. tuberculosis*. (C) The pixels of airspace were calculated using ImageJ software in the randomly selected, nonoverlapping fields 7 wk postinfection (details of analysis described in Materials and Methods). ICAM-1−/− lungs had significantly less airspace than WT mice ($P<0.05$).

Interestingly, levels of CD4+Foxp3-T cells tended to be higher in naïve ICAM-1−/− lungs and spleen compared with WT (data not shown). Overall, in the ICAM-1−/− mice in organs where fewer T regulatory cells were present, more nonregulatory CD4+ T cells could be detected, both in naïve mice, and during *M. tuberculosis* infection, suggesting that Foxp3+ T regulatory cells are required to maintain T-cell homeostasis.

The increased numbers of T cells in the lungs of ICAM-1−/− mice accordingly led to higher IFN-γ production 7 wk postinfection in an ELISpot assay (data not shown). Flow cytometry confirmed that the percentage of CD4+ and CD8+ T cells producing IFN-γ were similar, but the numbers of these cells in ICAM-1−/− lungs were increased (data not shown). At all time points, lung-infiltrating macrophages, neutrophils, and dendritic cells were equivalent between ICAM-1−/− and control mice (data not shown). No differences in any cell populations were detected in the lung draining lymph nodes (data not shown), suggesting that similar numbers of T lymphocytes were produced in the secondary lymphoid organs.

ICAM-1 may be required for T lymphocyte apoptosis in the lungs of *M. tuberculosis* infected mice

As similar T-cell populations were detected in the lymph nodes of ICAM-1−/− and control mice (data not shown), differences in priming the total CD4+ and CD8+ lymphocyte population is unlikely. Increased effector T lymphocytes detected in ICAM-1−/− lungs could be due to greater proliferation or decreased apoptosis. Expression of the nuclear antigen Ki67 was used to compare proliferation of T lymphocytes in the lungs of *M. tuberculosis*-infected mice by flow cytometry; there was no difference in proliferation of ICAM-1−/− and WT lung T cells (data not shown). A significant decrease in ICAM-1−/− T lymphocyte apoptosis was consistently detected 7 wk postinfection ICAM-1−/− lungs (Fig. 7C), detected by Annexin V staining and confirmed by activated caspase 3 staining (data not shown). These data suggest that optimal contraction of the effector T lymphocyte population in the lungs during *M. tuberculosis* infection requires ICAM-1 expression. Alternatively, the decrease in apoptosis of effector T cells may be due to the increased inflammation in the absence of T regulatory cells, instead of by a direct T regulatory cell-mediated mechanism.

Adoptive transfer of naïve CD4+ CD25+ T regulatory cells prior to *M. tuberculosis* infection decreases total effector T cells in the lungs over time

In naïve and *M. tuberculosis*-infected ICAM-1−/− mice, fewer T regulatory cells are present than in WT mice (Fig. 1). The total number of T lymphocytes in naïve spleen and *M. tuberculosis*-infected lung was also higher in ICAM-1−/− mice com-

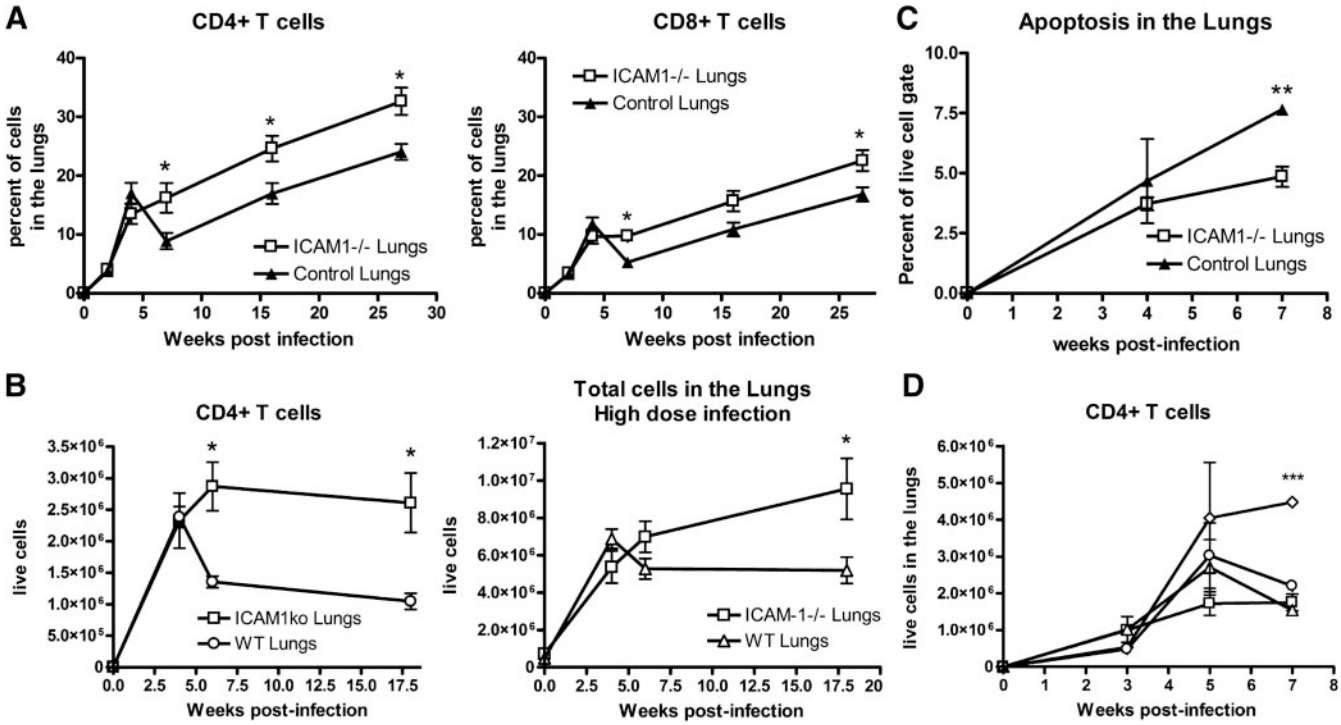

Figure 7. ICAM-1-deficient mice infected with *M. tuberculosis* have increased functional effector T cells in the lungs despite a similar bacterial burden to WT. (A) ICAM-1−/− (□) or WT (▲) CD4+ and CD8+ T lymphocytes in the lungs were detected by flow cytometry during low dose *M. tuberculosis* infection. $*P < 0.05$. Representative of 8 experiments. (B) Total cells and CD4+ T cells in the lungs increase in response to high dose (~100 bacteria/lung) *M. tuberculosis* infection; $*P < 0.05$. (C) Apoptosis of ICAM-1−/− (□) or WT (▲) T lymphocytes was detected by Annexin V staining in CD3+ gated T cells by flow cytometry; **, $P<0.001$. (D) Mice received 8×10^6 adoptively transferred naïve splenic WT CD4+CD25+ cells. CD4+ T lymphocytes in the lungs of ICAM-1−/− (◊), WT (○), adoptive transfer (AT) recipient ICAM-1−/− (□), and recipient WT (○) mice following *M. tuberculosis* infection. Transfer of T regulatory cells corrected the increased inflammation in ICAM1−/− lungs.

pared with WT mice (data not shown). To determine whether these phenotypes are related, naïve CD4+CD25+ T cells were enriched from WT spleens (99% were also Foxp3+) and adoptively transferred into ICAM-1−/− or WT mice one day prior to *M. tuberculosis* infection. We hypothesized supplemental WT CD4+CD25+ T cells would decrease the ICAM-1−/− adaptive response to *M. tuberculosis* infection. At 7 wk postinfection, the total number of CD4+ (Fig. 7D) and CD8+ (data not shown) T cells in lungs of ICAM1−/− mice that received WT CD4+CD25+ cells was reduced significantly compared with nonrecipient ICAM-1−/− mice. In addition, the inflammation of the lungs was markedly reduced in ICAM-1−/− mice adoptively transferred with WT CD4+CD25+ T cells (**Fig. 8**). Analysis of available airspace showed that ICAM-1−/− mice had significantly increased available airspace (49.02±5.49%) compared with ICAM-1−/− mice that did not receive adoptive transfers (38.73±8.06) ($P<0.05$). These data support that WT regulatory T cells complement the defect in ICAM-1−/− lungs, and restore normal T-cell and inflammatory responses to *M. tuberculosis*.

DISCUSSION

The data presented here demonstrate that ICAM-1 is required for the induction of Foxp3+ T regulatory cells in the periphery, which impacts T-cell homeostasis. The reduced responsiveness to TGF-β1 signaling in ICAM-1-deficient CD4+ T cells resulted in impaired Foxp3 expression, and a subsequent reduction in T regulatory cells in the tissues of ICAM-1−/− mice. Reduction of T regulatory cells was more pronounced during an inflammatory response to *Mycobacterium tuberculosis* infection and was the cause of increased effector T lymphocytes in infected lungs, as addition of regulatory T cells to the ICAM1−/− mice restored normal numbers of T cells and reduced inflammation in the lungs. Rather than controlling proliferation, T regulatory cells may play a role in contraction of T lymphocytes, as apoptosis was reduced during the contraction phase of the immune response. Contrary to previous reports [33, 34], in the absence of ICAM-1, granulomas formed in response to low-dose *M. tuberculosis* infection, allowing mice to control bacterial burden and survive equivalently to WT mice. However, in mice infected with a higher dose of *M. tuberculosis*, lung inflammation and pathology increased substantially late in infection, contributing to the decreased survival of ICAM1−/− mice. The data presented here support that the reduction in regulatory T cells in ICAM1−/− mice contributed to increased inflammation and pathology, and a worsened outcome in *M. tuberculosis*-infected mice. This suggests a previously unappreciated role for ICAM-1 in regulation of immune responses.

Although there was a significant reduction in T regulatory cells in peripheral organs in naïve ICAM-1−/− mice, similar expression of Foxp3 in the thymus of ICAM-1−/− and WT mice was observed, suggesting that thymic production of natural T regulatory cells does not require ICAM-1. Fewer Foxp3+ T regulatory cells in the tissues of naïve ICAM-1−/− mice and in lungs of ICAM-1−/− *M. tuberculosis* infected mice support a requirement for ICAM-1 for peripheral T regulatory cell induction, particularly because a difference in T regulatory cell proliferation was not detected in the presence or absence of ICAM-1. Survival or migration of T regulatory cells, or migration of all T cells, were not dependent on ICAM-1. ICAM-1−/−

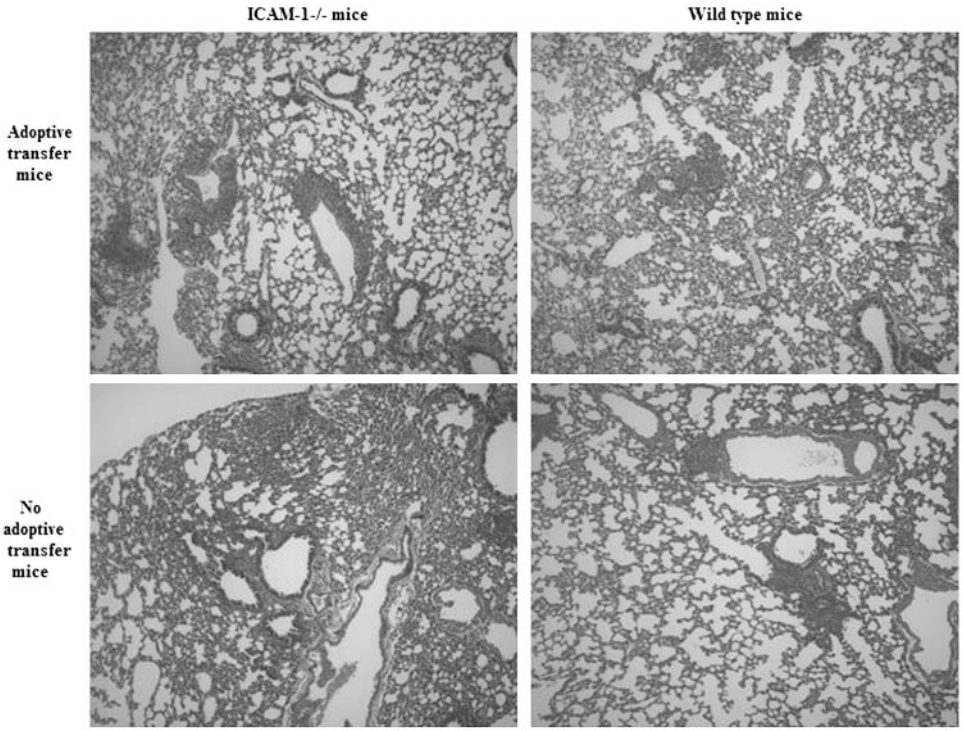

Figure 8. Improved lung pathology in ICAM-1−/− mice by adoptively transferred WT T regulatory cells. Representative sections of H&E stained ICAM-1−/− (left) or WT (right) lungs from mice infected with low-dose aerosol *M. tuberculosis*. Mice that received adoptive transfer of wild-type CD25+ CD4+ T regulatory cells are shown in the top panels, while mice that did not receive adoptively transferred T regulatory cells are shown in the bottom panels.

CD4+CD25- splenocytes cultured in vitro had significantly reduced induction of Foxp3 in response to TGF-β1 compared with WT cells, suggesting that postthymic induction of T regulatory cells is impaired in the absence of ICAM-1.

During *M. tuberculosis* infection in WT mice, Foxp3 expression increased into the chronic phase of infection. Foxp3 expression in ICAM-1−/− CD4+ T cells remained much lower in vitro and in vivo, which suggests that the same mechanism could be responsible for this deviation from WT responses. The expression of activated TGF-β1 was very similar in the lungs and lymph nodes of ICAM-1−/− and WT mice throughout infection (data not shown), suggesting that it is the induction of Foxp3 that is defective in vivo, and not availability of active TGF-β1. Although the phenotype of natural and inducible T regulatory cells in vivo is indistinguishable (CD4+ CD25+Foxp3+), it seems more likely that inducible T regulatory cells are produced during the inflammatory response, rather than increased production of natural T regulatory cells. T regulatory cells may also be proliferating in vivo during *M. tuberculosis* infection, but Ki67 staining of T cells from lungs of ICAM-1−/− and WT mice was similar, supporting that de novo expression of Foxp3 in peripheral CD4+ T cells is responsible for increased T regulatory cell expression. Transferring additional T regulatory cells to ICAM-1−/− mice reduced the inflammation in the lungs during *M. tuberculosis* infection to wild-type levels, strongly implicating the reduction in T regulatory cells in the ICAM-1−/− mice as the responsible factor for increased numbers of lymphocytes in the lungs of these mice.

We have observed that during low dose *M. tuberculosis* infection in ICAM-1−/− mice, granuloma formation occurs with similar kinetics seen in WT mice. Bacterial burden was maintained at a similar level to WT, and ICAM-1−/− mice are able to survive low-dose *M. tuberculosis* infection equivalently. However, ICAM-1−/− mice succumb to higher-dose infection at ~14 wk, most likely because of the increased pathology observed in the lungs. Although LFA-1 deficiency resulted in insufficient granuloma formation due to decreased T lymphocyte priming [35], ICAM-1−/− antigen-presenting cells clearly primed effector cells efficiently and mounted a sufficient immune response. The findings reported here show that ICAM-1 expression is not required to control *M. tuberculosis* infection, although it is involved in control of inflammation.

TGF-β1 signaling requires transport of phosphorylated Smads into the nucleus, where transcription responses rely on prolonged TGF-β-receptor signaling [49, 50]. TGF-β1 induces Foxp3 expression requisite for CD4+CD25+ T regulatory cells [14]. In the absence of ICAM-1 expression, induction of Foxp3 through TGF-β receptor signaling was reduced. A defect in the TGF-β receptor signaling machinery caused reduced translocation of phosphorylated Smad3, but not phosphorylated Smad2, from the cytoplasm into the nucleus. These results complement findings in Cbl-b-deficient mice, in which Cbl-b was required for phosphorylation of Smad2, but not phosphorylated Smad3 [51]. Together, the findings reported here and previously suggest that regulation of TGF-β1 signaling is mediated on several levels by molecules as diverse as ubiquitin ligases and adhesion molecules.

The relationship between ICAM-1 expression and TGF-β receptor signaling is not intuitive. ICAM-1 expression was not required for the association of phosphorylated Smad3 with Smad4, but it was required for the movement of pSmad3 (presumably complexed with Smad4) into the nucleus. This may indicate a requirement for signaling between ICAM-1 and LFA-1 during TGF-β1 stimulation. The addition of soluble ICAM-1 to the in vitro cultures restored the ability of ICAM-1−/− cells to express Foxp3 (Fig. 3A). The mechanism through which ICAM-1 plays a role in pSmad3 translocation into the nucleus has not been defined and is beyond the scope of this study. However, we can hypothesize the following. Although we were unable to detect ICAM-1 expression on the vast majority of WT T regulatory cells (data not shown), ICAM-1 shedding and/or secretion has been well documented [52–55] and could play a role in LFA-1 ligation. Soluble ICAM-1 does not lose its ability to bind LFA-1 [52], and, if present, could transmit a signal through LFA-1 on CD4+ T cells that assists in TGF-β1 signaling. LFA-1 ligation activates actin polymerization [56], myosin motor activity [57], and rearrangement of the microtubule network [58, 59]. The microtubule network is responsible for controlling Smad localization in the cytoplasm, such that destabilization of microtubules allows the release of Smads into the nucleus [60]. The role of LFA-1 in rearranging the microtubule network might, therefore, allow phosphorylated Smads to be released from the cytoplasm into the nucleus. Loss of ICAM-1/LFA-1 interaction could inhibit cytoskeletal rearrangements required for translocation of nucleus-bound transcription factors, such as phosphorylated Smad3 from the cytoplasm. We attempted to test this hypothesis by comparing the rearrangement of the microtubule network by confocal microscopy but were unable to detect changes on the macroscopic level (data not shown). However, we cannot rule out this as the reason for the requirement of ICAM-1 for translocation of pSmad3 into the nucleus that is required for Foxp3 expression.

Further studies are required to investigate the role of ICAM-1/LFA-1 signaling in microtubule reorganization that may allow translocation of transcription factors into the nucleus. It is of interest to determine whether it is only TGF-β receptor signaling that is affected by the absence of ICAM-1, or whether there are additional signaling pathways that are negatively affected by ICAM-1 deficiency. Finally, TGF-β1 signaling may be impaired in somatic cells other than T lymphocytes, and this has not yet been investigated.

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KEY WORDS:
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