CD11b facilitates the development of peripheral tolerance by suppressing Th17 differentiation

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The induction, execution, and maintenance of immune tolerance requires direct cell–cell contact mediated by specific surface molecules on APCs, notably MHC-II, B7.1, B7.2, and ICOSL, and their corresponding partners on T lymphocytes (1–5). As these molecules also involved in T cell activation, it is unclear how APCs function to facilitate both immune tolerance and activation. One potential mechanism that controls such opposite effects is the current dogma on APC maturation status, which is that immature DCs and nonprofessional APCs that lack sufficient surface expression of costimulatory molecules induce T cell apoptosis, anergy, or differentiation of suppressor T cells, whereas mature DCs that express high levels of costimulatory molecules support immune activation (2).

Another mechanism by which APCs control immune activation versus suppression involves their ability to modulate the production of proinflammatory cytokines (IL–10 and TGF-β) (1, 6–8). In particular, it was recently reported that IL-6, together with TGF-β, promotes the generation of Th17 cells, which are a distinct T cell lineage characterized by their production of IL-17 (9, 10). Interestingly, despite the abundant expression of costimulatory molecules such as CD80 and CD40, certain differentiated DCs are fully capable of suppressing T cell activation (7, 11). Thus, the mechanism that dictates the ability of APCs to induce immune activation versus immune tolerance cannot be explained only by the levels of costimulatory molecules.

One of the highly expressed molecules on APCs is integrin CD11b/CD18 (αM, Mac-1, and CR3). Based on the observation that C3bi, which is a specific ligand of CD11b/CD18 (12), functions critically in the development of specific forms of immune suppression (13, 14), we hypothesize that the development of peripheral immune tolerance is dependent on CD11b/CD18. In this study, we tested the role of CD11b in orally induced peripheral immune tolerance.
(oral tolerance) using CD11b−/− mice. Our data show that genetic inactivation of CD11b does not significantly affect the maturation of APCs or the cellular compositions of the draining LNs. Rather, CD11b deficiency leads to increased expression of IL-6, preferential immune deviation toward the Th17 pathway, and enhanced production of IL-17, which interferes with the establishment of oral tolerance. Together, this study identifies CD11b/CD18 as an important player in the development of antigen-induced immune tolerance, at least in part because of its ability to suppress Th17 differentiation.

RESULTS AND DISCUSSION
CD11b−/− mice exhibit defective antigen-induced oral tolerance
Recent studies demonstrate that unlike its homologue receptor CD11a, genetic inactivation of CD11b does not protect mice from the development of autoimmune diseases. On the contrary, CD11b deficiency worsens the inflammation and disease progression in several autoimmune disease models, including systemic lupus erythematosus, asthma, and arthritis (15–17), suggesting that CD11b/CD18 is potentially involved in immune suppression rather than immune activation. To test our hypothesis that CD11b is required for peripheral immune tolerance, we studied the role of CD11b in low-dose antigen-induced oral tolerance, based on a commonly used feeding regimen (18). Thus, wild-type (WT) and CD11b−/− mice were fed with 1 mg OVA in PBS or PBS alone daily for 7 d, and then immunized with OVA emulsified in complete Freund’s adjuvant (CFA). 7 d later, OVA-specific delayed-type hypersensitivity (DTH) was determined. In both WT and CD11b−/− mice that received PBS only, immunization with OVA induced strong immune responses, as indicated by increase in footpad thickness upon challenge with particulate OVA (Fig. 1 a). Thus, CD11b deficiency does not affect immune activation. On the other hand, feeding WT mice with low-dose OVA strongly suppressed the subsequent immune response, as no significant footpad swelling developed in the fed WT mice. In contrast, similarly fed CD11b−/− mice still developed strong DTH responses (Fig. 1 a), demonstrating that CD11b−/− mice are defective in developing immune suppression upon low-dose antigen feeding.

The lack of immune suppression in CD11b−/− mice was directly verified by in vitro antigen-induced lymphocyte proliferation assays. Single-cell suspensions prepared from the draining LNs of immunized mice with or without low-dose antigen feeding were restimulated in vitro with graded concentrations of OVA in 96-well plates for 72 h, and cell proliferation was measured on day 3 by [3H]thymidine incorporation. Lymphocytes from immunized WT and CD11b−/− mice showed similarly strong antigen-dependent proliferation (Fig. 1 b), again demonstrating that CD11b deficiency does not adversely affect antigen-specific immune responses. Low-dose oral OVA administration significantly suppressed the resultant proliferation of lymphocytes from WT mice, but did not affect the proliferation of lymphocytes from similarly fed CD11b−/− mice (Fig. 1 b). Together, these results revealed that unlike other known costimulatory molecules present on APCs, such as B7.1, B7.2, and ICOSL, which support both activation and suppression (4, 5, 19), CD11b/CD18 is uniquely involved in oral tolerance, but not in immune activation.

The defective immune tolerance in CD11b−/− mice can be restored by passive transfer of WT APCs
Professional APCs, which express high levels of CD11b, may depend on this molecule to support oral tolerance. In support of this hypothesis, adoptive transfer of total splenocytes or adhesion-enriched APCs from naïve WT mice into CD11b−/− mice, followed by antigen feeding, restored oral tolerance in CD11b−/− mice, as indicated by the substantial reduction in OVA-induced footpad swelling (Fig. 1 c), strongly arguing that...
CD11b expressed on APCs is likely involved in the process of oral tolerance in OVA-fed mice.

Given a potential role of CD11b/CD18 in leukocyte trafficking, CD11b deficiency may change the cellular composition of the lymphoid organs in response to antigen feeding, thus abolishing oral tolerance. However, no significant differences in either the percentage or the total number of CD19+ (B cells), CD3+ (T cells), and MHC-II+ (APC) cells within the draining mesenteric LNs (mLNs) were observed between OVA-fed WT and CD11b−/− mice (Table S1, available at http://www.jem.org/cgi/content/full/jem.20062292/DC1). CD11b deficiency may also interfere with DC maturation within the draining LNs in response to antigen feeding, which is very unlikely given the normal immune response observed in Fig. 1a. Indeed, two-color flow cytometric analysis of the DC population within the draining mLNs, using CD11c as a marker, showed similar surface expression of MHC-II, CD40, and CD86 between OVA-fed WT or CD11b−/− mice (Fig. S1).

**CD11b deficiency promotes Th17 immune deviation under the oral tolerance condition**

To understand the cellular mechanism by which CD11b deficiency abolished the establishment of oral tolerance, we analyzed cytokine production by draining LN cells in response to antigen restimulation. Single-cell suspension was prepared from the inguinal LNs (iLNs) of PBS- or OVA-fed and immunized WT and CD11b−/− mice for 3 d, and their supernatants were analyzed for cytokine production using multiplex microbead-based cytokine assay kit. Compared with naive mice (not depicted), immunization increased the production of IL-2 and -6 similarly in WT and CD11b−/− mice (Fig. 2a). Under antigen-feeding conditions, CD11b−/− mice exhibited approximately sixfold increased cytokine production when compared with similarly fed WT mice (P < 0.001). Production of IL-17 was only observed in CD11b−/− mice.

A major source of IL-17 expression has been attributed to a newly described Th17 cell population (20, 21). Thus, the presence of large quantities of IL-17 suggested that CD11b deficiency may result in Th17 immune deviation in vivo. In support of this hypothesis, IL-6, which is another cytokine that is associated with the development of Th17 cells (9, 10), was also detected in large quantities in the supernatants from antigen-fed and immunized CD11b−/− mice (Fig. 2a). To directly test our hypothesis, we examined whether CD11b−/− mice contained a Th17 cell population under the condition of oral tolerance induction. Thus, single-cell suspension was obtained from the draining iLNs of PBS- or OVA-fed and immunized WT or CD11b−/− mice, and the presence of Th17 cells within CD4+ population, as characterized by expression of IL-17, but not IFNγ, was determined by intracellular staining with their corresponding antibodies according to an established protocol (20, 21). Indeed, flow cytometric analyses revealed fivefold more IL-17+ IFNγ− cells within the gated CD4+ cells (i.e., Th17 cells) obtained from OVA-fed CD11b−/− mice than that from OVA-fed WT mice (Fig. 2b).

**Figure 2.** CD11b deficiency leads to Th17 immune deviation under the condition of oral tolerance induction. (a) Cytokine production in response to antigen restimulation. Single-cell suspension, obtained from the iLNs of PBS- or OVA-fed and immunized WT (open bar) or CD11b−/− (shaded bar) mice, were restimulated in vitro with 100 μg/ml OVA for 72 h, and the cytokine concentrations in the supernatants were determined by microbead multiplex assay.* P < 0.001, WT versus CD11b−/−, n = 4. (b) Identification of Th17 cells by intracellular staining. Total CD4+ T cells obtained from PBS- or OVA-fed and immunized mice were stimulated with PMA and ionomycin in the presence of GolgiPlug for 5 h, and then analyzed for intracellular expression of IL-17 and IFNγ by three-color flow cytometry. Plots were gated on CD4+ cells (top, gate R3) and the percentages of cells staining positive for IL-17 or IFNγ were shown. (c) Existence of a Th17 cell population in CD11b−/− mice under conditions of oral tolerance induction. The percentages of Th17 cells, identified by their production of IL-17, but not IFNγ, within CD4+ subpopulation (gate R3), were determined by flow cytometry. Data shown are the means ± the SEM and are representative of three independent experiments.* P = 0.0001, WT versus CD11b−/−; n = 12.

Statistical analysis showed a significant increase in the percentage of Th17 cells within the draining LNs of CD11b−/− mice compared with WT mice (P = 0.0001; n = 12; Fig. 2c). No detectable Th17 population was observed in either naive WT or CD11b−/− mice (unpublished data).

To verify if the IL-17–expressing T cells represented a mature and stable Th17 population, we tested whether they could respond to restimulation by TGFβ, which is a cytokine that promotes the differentiation of Th17 cells (9, 10), and by IL-23, which is a cytokine that promotes their proliferation (9, 22). Thus, total CD4+ T cells were isolated from OVA-fed and immunized WT and CD11b−/− mice, and then restimulated with
Suppression of IL-17 production by CD11b is critical to the development of oral tolerance

To test our hypothesis, we evaluated whether administration of exogenous IL-17 could block the establishment of oral tolerance in WT mice. Thus, WT mice were injected i.v. with IL-17 (60 pg/mouse/d) and simultaneously fed with low-dose OVA for 7 d. The development of OVA-specific immune tolerance was assessed by the DTH response, as described in Fig. 1 a. Low-dose IL-17 injections did not significantly affect the total blood leukocyte counts (unpublished data), but completely abrogated OVA-specific immune tolerance, leading to the development of OVA-specific DTH response in WT mice, when compared with the PBS-injected and antigen-fed WT control mice (Fig. 4). Based on the above results, we propose that genetic inactivation of CD11b leads to enhanced production of IL-17 by APCs, which, in the presence of TGF-β, promote Th17 immune deviation (9, 10). The presence of Th17 cells and their production of IL-17 within the draining LNs interfere with oral tolerance in CD11b−/− mice.

Th17 cells and the cytokine IL-17 they produce are associated with a variety of allergic and autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and asthma (23-26). Our observation that skewed differentiation toward Th17 pathway in CD11b−/− mice blocks the establishment of oral tolerance is consistent with these clinical observations. Thus, the information provided from this study not only helps us better understand the function of APCs in oral tolerance but also may have clinical implications for patients with autoimmune diseases.

MATERIALS AND METHODS

Mice. WT and CD11b−/− strains were all in the C57BL/6j background, used at 8–13 wk old. WT mice were purchased from the National Cancer Institute; CD11b−/− mice were provided by C.M. Ballantyne (Baylor College of Medicine, Houston, TX) (27) and have been backcrossed to the C57BL/6j background for more than seven generations. Animals were housed in a pathogen-free facility, and all procedures were performed in accordance with the Institutional Animal Care and Use Committee approval.

Antibodies and reagents. FITC–anti–mouse CD4 (clone GK1.5) was purchased from Miltenyi Biotec Inc. FITC–anti–mouse CD25 (clone 7D4), PE–anti–CD3 (clone 17A2), PerCP–anti–CD4 (clone L3T4), FITC–anti–CD86
SEM and are representative of two independent experiments.

5% contaminating T cells based on flow cytometry. The WT splenocytes (enriched APCs) were detached mechanically without trypsin, which contained nonadherent cells were removed by washing. The adherent cells (i.e., adhesion-

μg/ml penicillin (100 U/ml streptomycin [100 μg/ml]) and 10% FBS in RPMI-1640. The cultures were maintained in a 5% CO₂ environment at 37°C. The plates were washed with sterile phosphate buffered saline (PBS) prior to experimentation, and all secretions were harvested in the presence of 10 μg/ml GolgiPlug (BD Biosciences) in complete media (RPMI-1640, 10% FBS, 10 mM Hepes, 1 μM sodium pyruvate, 1X nonessential amino acids, 50 μM 2-mercaptoethanol, and penicillin [100 U/ml streptomycin [100 μg/ml]) at 37°C for 20 h later with a caliper, and the thickness increment was calculated as the difference between the left and right footpad measurements.

Adoptive cell transfer. Single-cell suspensions were prepared from spleens harvested from naive WT mice and allowed to adhere to tissue culture dishes in complete media (RPMI-1640, 10% FBS, 10 μM Hepes, 1 mM sodium pyruvate, 1X nonessential amino acids, 50 μM 2-mercaptoethanol, and penicillin [100 U/ml streptomycin [100 μg/ml]) at 37°C for 30 min, washed, and analyzed by two- or three-color flow cytometry (FACScan; BD Biosciences) using CellQuest software (BD Biosciences). The cell mixtures obtained from the draining LNs of different mice or from the in vitro cultures were restimulated with 50 ng/ml PMA and 2 μM ionophore in the presence of 1 μg GolgiPlug (BD Biosciences) in complete media at 37°C for 5 h. These cells were then incubated with Fe Blocker (clone 2.4G2; BD Biosciences) at 4°C, stained with a first set of fluorescence-labeled (FITC, PE, or PerCP) antibodies for 25 min, and then washed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min. These cells were then incubated with a second set of antibodies at 4°C for 30 min, washed, and analyzed by two- or three-color flow cytometry (FACScan; BD Biosciences) using CellQuest software (BD Biosciences).

Statistical analysis. Statistical analyses were performed using Student’s t test (Systat Software, Inc.). P values <0.05 were considered significant.

Online supplemental materials. Fig. S1 shows that CD11b deficiency did not compromise DC maturation in response to antigen feeding. Table S1 shows that CD11b deficiency did not affect leukocyte trafficking into the draining LNs. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062292/DC1.

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BRIEF DEFINITIVE REPORT

Figure 4. IL-17 administration in WT mice interferes with the establishment of oral tolerance. WT mice were injected i.v. with PBS or IL-17 (60 pg/mouse) during OVA feeding, immunized with OVA/CFA, and challenged. Development of immune tolerance was determined by inhibition of footpad swelling in the DTH response, as in Fig. 1 a. * P = 0.003, PBS versus IL-17. n = 5–6 mice per group. Data shown are means ± the SEM and are representative of two independent experiments.

In vitro differentiation/expansion of Th17 cells. Total CD4⁺ T cells were prepared using CD4⁺ T cell isolation kit (Miltenyi Biotech) from the iLNs of WT or CD11b⁻/⁻ mice that were fed with OVA for 7 d and immunized with OVA/CFA at the tail base for 7 d. Isolated CD4⁺ T cells (10⁵ cells/well) were cocultured with their corresponding irradiated APCs (3 × 10⁶ cells/well; adhesion-enriched) in 200 μl/well of complete media plus 10 μg/ml anti-CD3 (mAb 1452C11) and different sets of cytokines and antibodies, as follows: 20 ng/ml IFNγ plus 10 μg/ml anti-IL-4 (for Th1 polarization); 20 ng/ml IL-4 plus 10 μg/ml anti-IFNγ (for Th2 polarization); or 2 ng/ml TGFB plus 8 ng/ml IL-6 (for Th17 polarization) at 37°C for 3 d. Differentiation/expansion of Th17 cells was determined by intracellular staining using anti–IL-17 and anti-IFNγ, and the Th17 cells were identified as IL-17⁺IFNγ⁺ CD4⁺ T cells by three-color flow cytometry.

Intracellular cytokine staining. To retain the cytokines intracellularly, the cell mixtures obtained from the draining LNs of different mice or from the in vitro cultures were restimulated with 50 ng/ml PMA and 2 μM ionophore in the presence of 1 μg GolgiPlug (BD Biosciences) in complete media at 37°C for 5 h. These cells were then incubated with Fe Blocker (clone 2.4G2; BD Biosciences) at 4°C, stained with a first set of fluorescence-labeled (FITC, PE, or PerCP) antibodies for 25 min, and then washed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min. These cells were then incubated with a second set of antibodies at 4°C for 30 min, washed, and analyzed by two- or three-color flow cytometry (FACScan; BD Biosciences) using CellQuest software (BD Biosciences).

In brief, low-dose oral tolerance was induced by feeding 1 mg OVA per day via drinking water for 7 d. In certain experiments, wild-type mice (5–6 mice per group) were injected i.v. daily with either IL-17 (60 pg/mouse) or PBS, while being fed with 1 mg OVA for 7 d. The extent of tolerance induced by these methods was measured by DTH in response to OVA immunization. Table S1 shows that CD11b deficiency did not compromise DC maturation in response to antigen feeding. Figure S1 shows that CD11b deficiency did not affect leukocyte trafficking into the draining LNs. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062292/DC1.

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