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LFA-1 is Critical for Regulatory T cell Homeostasis and Function

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Abstract

Cellular adhesion molecules involved in cell-to-cell mediated suppression by Tregs are not well characterized. We found that the majority of Tregs expressed LFA-1 but most strikingly that the frequency of Tregs in LFA-1^{-/-} mice was significantly lower (~50%) in the spleen, lymph nodes, and Peyer's Patches compared to wild type controls. The reduction in LFA-1^{-/-} Treg cells appears due in part to a reduced capacity of LFA-1^{-/-} CD4⁺CD25⁻ cells to be induced to become Tregs in the lymph nodes. Importantly, we found that LFA-1^{-/-} Tregs fail to suppress T cell responses *in vitro* and have reduced function *in vivo*. Treg mediated-suppression does not depend on LFA-1 interactions with ICAM-1 on the surface of responder cells. Our data demonstrate that LFA-1 plays a critical role in regulatory T cell homeostasis and function.

Keywords

adhesion molecules; T cells; immunosuppression

1. Introduction

Regulatory T cells (Tregs) are critical to maintaining peripheral tolerance, through the ability to suppress T cells at all stages of an immune response (DiPaolo et al., 2005; Oldenhove et al., 2003; Sarween et al., 2004; Sojka et al., 2005; Stassen et al., 2004; Sukiennicki and Fowell, 2006). Many immunosuppressive mechanisms have been attributed to Tregs including inhibition through cell-to-cell contact, release of soluble factors, and competition for growth factors (Scheffold et al., 2007; Sojka et al., 2008). Migration of Tregs to sites of inflammation is critical to their function and they express higher levels of CD103, L-selectin, P-selectin, and ICAM-1 than effector T cells (Kohm et al., 2002). Some adhesion molecules, such as CD103 and L-selectin, define Treg subsets based on immunosuppressive function, while others including ICAM-1 and P-selectin, do not appear to be critical to the suppressive mechanisms of Treg cells (Kohm and Miller, 2003). CD103 and L-selection appear to be important markers of Treg subsets rather than directly

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Conflict of Interest

The authors have no financial conflicts to disclose.

participating in effector T cell suppression. Although little is known about cellular adhesion molecules utilized by Tregs for contact-mediated effector T cell suppression, β_2 -integrins have been implicated in this function. CD18-deficient mice, the common β -chain for β_2 -integrins, have reduced Treg populations and CD18^{-/-} Tregs fail to suppress T cell responses *in vivo* (Marski et al., 2005). However, CD18^{-/-} mice do not express any of the β_2 -integrins (LFA-1, Mac-1, p150,95, and α_D) and thus which family member or members contributed to the observed phenotype remains unclear. It has recently been shown that LFA-1 is critical to the development and progression of EAE (Dugger et al., 2009; Wang et al., 2007), however transfer of wild type encephalitogenic T cells to LFA-1^{-/-} mice, unexpectedly resulted in exacerbated disease (Dugger et al., 2009). These results suggested the possibility of defective Tregs in LFA-1^{-/-} recipients and that LFA-1 expression on Tregs is important for their suppressive functions. We now report that LFA-1^{-/-} mice have significantly reduced levels of Tregs throughout their secondary lymphoid system, but elevated numbers in the thymus. In addition, unlike wild type, naïve CD4⁺CD25⁻ T cells, LFA-1^{-/-} T cells do not readily convert to CD4⁺CD25⁺Foxp3⁺ T cells. Importantly, LFA-1^{-/-} Tregs are nonfunctional in *in vitro* suppression assays and poorly immunosuppressive in experimental colitis. These data demonstrate that LFA-1 plays a major role in Treg function and may be a central receptor in cell-to-cell contact mechanisms utilized by Tregs to suppress effector cells.

2. Materials and Methods

2.1 Mice

CD45.1 mice were obtained from Jackson labs (Bar Harbor, ME). CD45.2 and RAG1^{-/-} mice were provided by the genomic core facilities at the University of Alabama, Birmingham. CD11a^{-/-}, CD11b^{-/-}, CD11c^{-/-}, and ICAM-1^{null} mice were generated as previously described (Bullard et al., 2007; Ding et al., 1999; Lu et al., 1997; Wu et al., 2004). C57BL/6 mice were used as controls for all experiments. All studies were performed with approval from the UAB IACUC.

2.2 Flow Cytometry

Cells obtained from lymph nodes, spleens, blood, thymus, and Peyer's patches were stained with anti-CD4-FITC and anti-CD25-PE (eBioscience). Cells were permeabilized using the eBioscience regulatory T cell staining kit and then stained with anti-Foxp3-APC. Stained cells were analyzed using a FACSCalibur and data was analyzed using CellQuest software (BD Biosciences).

2.3 Treg Isolation

CD4⁺CD25⁺ cells were isolated or depleted from thymus, spleens, lymph nodes and Peyer's patches of wild type, CD11a^{-/-}, CD11b^{-/-}, CD11c^{-/-}, and ICAM^{null} mice using the Miltenyi regulatory T cell isolation kit, as per the manufacturer's instructions. Isolated CD4⁺CD25⁺ populations contained greater than 95% Foxp3 expressing cells.

2.4 Treg Suppression Assay

CD4⁺CD25⁻ responder T cells (5×10^4) from control mice were stimulated in round bottom 96-well plates using anti-CD3 and CD28 antibodies along with CD4⁺CD25⁺ Tregs from wild type mice (1×10^5) or LFA-1^{-/-} mice (1×10^5) as previously described (Pandiyar et al., 2007). Cells were pulsed after 48 hr with ³H-thymidine (1 μ Ci) for an additional 18 hrs, harvested and thymidine incorporation determined.

2.5 Peripheral Treg Generation

CD4⁺CD25⁻ cells were isolated from wild type and LFA-1^{-/-} CD45.2 mice and then retro-orbitally injected (2×10^6) into irradiated (500R) CD45.1 recipients. In some experiments CD45.1 CD4⁺CD25⁻ cells were isolated from wild type mice and retro-orbitally injected (2×10^6) into CD45.2 wild type or LFA-1^{-/-} mice. Six weeks later spleens and lymph nodes were harvested and the percentage of CD45.2 or CD45.1 Treg cells were measured using FACS analysis.

2.6 Colitis

CD45RB^{hi} and CD45RB^{lo} cells were sorted from CD4⁺ cells isolated from spleens and lymph nodes of wild type or LFA-1^{-/-} mice with MACS beads. CD45RB^{hi} cells (5×10^6) were injected (i.p.) into RAG1^{-/-} recipients colonized with *h. hepaticus*. In some experiments, wild type or LFA-1^{-/-} CD45RB^{lo} cells were co-injected with wild type CD45RB^{hi} cells. Six weeks post-injection colons were prepared for histological analysis.

3. Results and Discussion

3.1 Altered Treg populations in secondary lymphoid tissues and the thymus

In recent studies, we found that transfer of wild type encephalitogenic T cells to LFA-1^{-/-} recipients resulted in significantly exacerbated EAE compared to wild type-to-wild type transfers (Dugger et al., 2009). T cells in these transfer experiments expanded dramatically and were readily detected in cervical and axillary lymph nodes, spinal cord and brains. These data raised the possibility that LFA-1^{-/-} mice have an immune regulatory defect, potentially in the Treg compartment. To address this question, we first examined blood, thymus, and secondary lymphoid organs including lymph nodes, spleen and Peyer's patches for the frequency of Tregs in wild type and LFA-1^{-/-} mice. LFA-1^{-/-} mice had significantly lower Treg populations in the spleen, lymph nodes, and Peyer's Patches, but surprisingly had increased numbers of Tregs in the thymus (Fig. 1A). The increased thymic Treg population in LFA-1^{-/-} mice is unique in the β_2 -integrin adhesion molecule family as no difference was observed in the number of thymic Tregs in CD11b^{-/-} or CD11c^{-/-} mice compared to wild type mice (Fig. 1B). We did not find any differences in the double or single positive CD4/CD8 profile of regulatory T cells in the thymus of LFA-1^{-/-} mice compared to wild type mice, suggesting that the absence of LFA-1 does not delay Treg development in the thymus (data not shown). We also did not observe sexual dimorphism or age-related effects that might account for the higher Treg population in the thymus. The elevated thymic Treg population in LFA-1^{-/-} mice may be due in part to a compensatory mechanism to repopulate the periphery where Treg numbers are reduced or an LFA-1-dependent emigration defect. Interestingly, our results contrast with those of Marski and

colleagues who reported reduced numbers of Tregs in the thymus of CD18^{-/-} mice (Marski et al., 2005). The reasons for this are unclear given our observations in CD11b- and CD11c-deficient mice (Fig. 1B), however the absence of all β_2 -integrins in CD18^{-/-} mice may lead to altered Treg thymic development or perhaps accelerated Treg thymic emigration.

3.2 *In vivo* conversion efficiency of LFA-1^{-/-} CD4⁺CD25⁻ T cells to Tregs depends on the presence or absence of LFA-1 in the recipient

The reduced Treg populations in LFA-1^{-/-} mice could be attributed in part to a reduced capacity of inducible Tregs in the periphery to mature to Foxp3⁺ cells (Chen et al., 2003; Liang et al., 2005). To assess this possibility, we performed allogeneic transfers to examine the ability of naïve LFA-1^{-/-} effector CD4 T cells to induce Foxp3 expression. In control experiments, we found that 17% of wild type CD4⁺CD25⁻CD45.2⁺ cells converted to CD4⁺CD25⁺Foxp3⁺ Tregs in the spleen and lymph nodes of the wild type CD45.1 recipient mice within six weeks after transfer (Fig. 2A). A similar level of peripheral conversion to Tregs (15%) was observed in the spleen using LFA-1^{-/-} CD45.2 effector T cells. In contrast, a significantly lower proportion of LFA-1^{-/-} CD4⁺CD25⁻CD45.2⁺ cells (11%) were able to convert into CD4⁺CD25⁺Foxp3⁺ cells in lymph nodes after transfer to wild type CD45.1 recipients (Fig. 2A). To ensure that the data represented a reduction in the induction of Foxp3 rather than a defect in trafficking of LFA-1^{-/-} T cells we calculated numbers of wild type or LFA-1^{-/-} CD45.2 cells recovered from the lymph nodes and found no differences (Fig. 2B). These data indicate that LFA-1^{-/-} T cells are able to turn on Foxp3 expression in the periphery, but that the efficiency of conversion maybe organ specific and this may account, in part, for the reduced Treg populations in lymph nodes of LFA-1^{-/-} mice. We next examined whether LFA-1 expression in the recipient mouse was required for conversion of naïve CD4⁺CD25⁻ T cells to Tregs. We found comparable conversion of wild type CD4⁺CD25⁻CD45.1⁺ cells to CD4⁺CD25⁺Foxp3⁺ Tregs in the spleen and lymph nodes of the wild type CD45.2 recipients and in the spleen of LFA-1^{-/-} CD45.2 recipients (Fig. 2C). Unexpectedly, we observed a two-fold higher conversion of WT CD4⁺CD25⁻CD45.1⁺ cells to Tregs in the lymph nodes of LFA-1^{-/-} CD45.2 recipients (9.1% vs. 5% p=0.006), a finding that may suggest a compensatory mechanism to increase Treg numbers in the LFA-1-deficient background. Again these results are not driven by trafficking defects, given the CD45.1 cell numbers recovered from lymph nodes of recipient mice were equivalent (Fig. 2D).

3.3 LFA-1^{-/-} Tregs are unable to suppress wild type CD4⁺proliferation *in vitro*

Onishi and colleagues (Onishi et al., 2008) demonstrated that LFA-1^{-/-} Tregs could not suppress naïve T cell proliferation in the presence of dendritic cells, but did not determine if LFA-1^{-/-} Tregs could directly suppress effector T cell proliferation. As expected, wild type Tregs readily suppressed effector T cell proliferation in response to antigen-independent stimulation using anti-CD3 and anti-CD28 antibodies. In contrast, LFA-1^{-/-} CD4⁺CD25⁺ cells completely failed to suppress wild type effector T cell proliferation (Fig. 3A). A predominant ligand for LFA-1 is ICAM-1 and interactions between LFA-1 and ICAM-1 are critical to the formation of the immune synapse and in providing co-stimulation during T cell activation (Pribila et al., 2004). To determine if ICAM-1 is critical for Treg-mediated suppression, we stimulated ICAM-1^{null} effector T cells, *in vitro*, in the presence of wild type

Tregs and analyzed proliferation levels. To our surprise, wild type Tregs were capable of suppressing ICAM-1^{null} effector T cells (Fig. 3B), suggesting that LFA-1 interaction with ICAM-1 is not critical to the of function of Tregs.

3.4 LFA-1^{-/-} Tregs poorly suppress inflammation in experimental colitis

To test the ability of LFA-1^{-/-} Tregs to suppress effector T cell responses *in vivo*, we examined the ability of LFA-1^{-/-} Tregs to prevent development of experimental colitis. Six weeks after transfer of wild type CD4⁺CD45RB^{hi} cells into RAG-1^{-/-} mice, severe colitis characterized by inflammatory cell infiltration, epithelial hyperplasia, and goblet cell loss, had developed in the recipient mice (Fig. 4). Co-transfer of wild type CD4⁺CD45RB^{lo} cells with wild type CD4⁺CD45RB^{hi} cells prevented colitis development. LFA-1^{-/-}CD4⁺CD45RB^{lo} cells were not able to prevent disease development. Although not completely non-functional *in vivo*, these cells were significantly less effective at suppressing disease than WT CD4⁺CD45RB^{lo} cells (Fig. 4). Previous studies using CD18^{-/-} mice and a similar colitis model, demonstrated that CD18^{-/-} Tregs (which do not express any β_2 -integrins) could not suppress disease development (Marski et al., 2005). The loss of Treg suppressive function in CD18^{-/-} mice was attributed to the absence of LFA-1, however studies with LFA-1^{-/-} mice were not performed to substantiate this claim. The fact that we did not see similar levels of suppression with LFA-1^{-/-} Tregs, suggests that another β_2 -integrin (or integrins) also contributes to Treg immunosuppressive functions.

Mechanisms of Treg function have been broadly categorized to include suppression by inhibitory cytokines (IL-10 and TGF- β), cytotoxicity in a perforin and granzyme-dependent fashion, metabolic disruption either by IL-2 sequestration or generation of adenosine, and targeting dendritic cell function and/or maturation (reviewed in (Vignali et al., 2008)). LFA-1 could potentially contribute to any one of these functions either through adhesive mechanisms (cytotoxicity and dendritic cell maturation) or through appropriate co-stimulation that may modulate inhibitory cytokine production or the adenosine suppressive pathway. We examined for changes in the expression of CD39 and CD73 on LFA-1^{-/-} Tregs, seeking a link between LFA-1 and the adenosine suppression pathway but found no difference compared to wild type Tregs (data not shown). Our study demonstrates that LFA-1 alone does not appear to be the key β_2 -integrin playing a role in Treg-mediated immunosuppression. Utilization of multiple β_2 -integrins by Tregs to modulate their function could operate at several levels, including 1) increasing the ligand/receptor pairings between Tregs and thymic epithelium, potentially contributing to various stages in Treg development, 2) aid in controlling their emigration from the thymus into the periphery and 3) allow differential co-stimulation during Treg-mediated regulation of immune responses and perhaps contribute to the fine-tuning of the overall response. Our findings point to unique roles not only for LFA-1 in Treg biology, but for other β_2 -integrin families as well.

Acknowledgments

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Figure 1.

LFA-1^{-/-} mice have reduced frequencies of regulatory T cells. A, Cells isolated from control or LFA-1^{-/-} blood, spleen, lymph node, Peyer's patch, and thymus using a Miltenyo regulatory T cell kit were stained with CD4, CD25, and Foxp3 antibodies, as described in materials and methods. Data shown is gated on CD4⁺ cells. Statistical analysis was performed for each wild type and LFA-1^{-/-} tissue using the student's t-test (**, p 0.01 and ***, p 0.001). B, The thymus of LFA-1^{-/-} mice has elevated frequency of Tregs compared to other β_2 -integrin-deficient mice. Cells were isolated from thymus of control, CD11a^{-/-}, CD11b^{-/-} and CD11c mice and immunostained as described in A. Data shown is gated on CD4⁺ cells. Statistical analysis was performed using Anova (p 0.001).

Figure 2.

LFA-1 deficiency prevents peripheral Treg generation from CD4⁺CD25⁻ T cells. A, CD4⁺CD25⁻ cells were isolated from CD45.2 wild type or LFA-1^{-/-} mice and injected into wild type CD45.1 recipients as described in materials or methods. B, CD4⁺CD25⁻ cells were isolated from wild type CD45.1 mice and injected into wild type or LFA-1^{-/-} CD45.2 recipients. Six weeks post-injection the generation of peripheral Tregs was measured in the spleen and lymph node by FACS analysis. Cells were stained for CD4, CD25, CD45.1 or CD45.2, and Foxp3. Numbers of transferred cells recovered from the lymph nodes of experiments in A and B are shown in C and D, respectively. Data shown is gated on CD4 and CD45.2 (A) or CD45.1 (B) cells. Statistical analysis was performed for each tissue measured using the student's t-test (**, $p < 0.01$ and ***, $p < 0.001$).

Figure 3.

LFA-1^{-/-} Tregs fail to suppress CD4⁺ effector cell proliferation. *In vitro* suppression assays were performed as described in materials and methods using wild type or ICAM^{null} CD4⁺CD25⁻ responder cells and either control CD4⁺CD25⁺ or LFA-1^{-/-} CD4⁺CD25⁺ regulatory T cells. Cultures were stimulated with soluble anti-CD3 and anti-CD28 antibodies for 48 hours and were pulsed with ³H-thymidine for the last 18 hours. A, The results are shown as fold-increase over baseline controls for WT CD4⁺CD25⁻ cells alone (n=6), control CD4⁺CD25⁻ cells with wild type CD4⁺CD25⁺ cells (n=7), and for wild type CD4⁺CD25⁻ cells with LFA-1^{-/-} CD4⁺CD25⁺ cells (n=5). B, wild type Treg cells are able to suppress proliferation of ICAM^{null} CD4⁺ T cells. The results shown are fold-increase over baseline controls for wild type CD4⁺CD25⁻ cells alone (n=6), wild type CD4⁺CD25⁻ cells with wild type CD4⁺CD25⁺ cells (n=7), ICAM^{null} CD4⁺CD25⁻ cells alone (n=5), and for ICAM^{null} CD4⁺CD25⁻ cells with wild type CD4⁺CD25⁺ cells (n=5). Statistical analysis was performed using Anova (**, p 0.01 and ***, p 0.001).

Figure 4.

LFA-1^{-/-} Tregs are less efficient at suppressing colitis development compared to wild type Tregs. RAG-1^{-/-} mice colonized with *H. hepaticus* were injected with CD4⁺CD45RB^{hi} cells (n=9) alone or with wild type (n=10) or LFA-1^{-/-} (n=10) CD4⁺CD45RB^{lo} cells as described in materials and methods. Six weeks post-injection colitis development was measured by histopathological analysis of colons. Colons were scored to assess the inflammation and tissue disruption throughout the tissue. LFA-1^{-/-} CD4⁺CD45RB^{lo} cells did suppress colitis development significantly less than wild type CD4⁺CD45RB^{lo} cells (p = 0.005, student's t-test).