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Ulcerative colitis and autoimmunity induced by loss of myeloid αv integrins

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The gastrointestinal tract is constantly challenged by foreign antigens and commensal bacteria but nonetheless is able to maintain a state of immunological quiescence. Recent advances have highlighted the importance of active suppression by regulatory lymphocytes and immunosuppressive cytokines in controlling mucosal immunity. Failures of these mechanisms contribute to the development of inflammatory bowel disease, but how these regulatory networks are established remains unclear. Here, we demonstrate key roles for αv integrins in regulation of mucosal immunity. We report that deletion of αv in the immune system causes severe colitis, autoimmunity, and cancer. Mice lacking immune cell αv have fewer regulatory T (Treg) cells in the colon and corresponding increases in activated T cells and T cell cytokine production, leading to colitis. Using conditional gene targeting, we demonstrate that this is specifically attributable to loss of αv from myeloid cells. Furthermore, we show that gut-associated macrophages and dendritic cells fail both to remove apoptotic cells efficiently and to induce Treg cells. Our results identify a vital role for myeloid αv integrins in generating mucosal Treg cells and emphasize the importance of antigen-presenting cells in establishing immune tolerance.

immunoregulation | phagocytosis | apoptotic | dendritic cell | regulatory T cell

Integrins are dimeric cell-surface receptors composed of α and β subunits (1). αv is the most promiscuous α subunit, associating with five different β subunits (β1, β3, β5, β6, and β8) and participates in many important cellular processes, including cell adhesion, migration, survival, and growth factor signaling. αv integrins are expressed in many tissues throughout development, and αv knockout mice die at midgestation from placental deficiencies or, immediately after birth, with cerebral and intestinal hemorrhage and cleft palate (2). The lethality of αv−/− mice is attributable mainly to loss of αvβ8, because β8−/− mice show similar placental and neural vasculature abnormalities (3), and these defects are principally attributable to loss of αvβ8 from parenchymal tissues supporting blood vessels (4, 5). Knockouts of most other αv-associated β integrins (β3, β5, and β6) are viable and reveal roles for αv in repair of tissue injury, inflammation, and immune responses (1, 6–8). αvβ6 and αvβ8 are expressed in epithelium, where their functions include binding and activating TGF-β (9), and αvβ8 is also expressed by myeloid cells and T cells (10, 11). αvβ3 and αvβ5 are more widely expressed and are up-regulated by many cells after tissue injury. In the immune system, αvβ3 and αvβ5 are expressed by many different cell types and promote cell adhesion, migration, and survival. αv integrins also mediate the uptake of apoptotic cells (12–14), a process central to immune regulation and inflammation resolution (15).

Here we report that αv integrins are central to the normal regulation of immune responses in the intestine and that deletion of αv in the immune system leads to spontaneous colitis, wasting, and autoimmunity. Colitis is associated with activated T cells, high expression of T cell cytokines, and corresponding loss of tissue-resident regulatory T (Treg) cells. We dissect the contribution of αv on different immune cell populations, identifying myeloid cells [macrophages, dendritic cells (DCs), and/or neutrophils] as the critical expressers of αv integrins. We further show that deletion of αv leads to impaired removal of apoptotic cells and failure of mesenteric lymph node (mLN) DCs to generate Treg cells.

Results

Mice Lacking αv Integrins Develop Colitis. Determining the precise contributions of αv integrins to immune functions in vivo has been limited by the lethal phenotype of αv knockout mice, which die from vascular and developmental defects (2). To circumvent this problem, we generated a conditional knockout of αv (αv\textsubscript{flox/flox} mice) [supporting information (SI) Fig. 7], which was crossed with αv-knockout heterozygous mice (αv\textsuperscript{+/−}) and tie2-CRE transgenic mice (16) to generate αv-tie2 and control mice (αv\textsubscript{flox};tie2-cre+ and αv\textsubscript{flox};tie2-cre−, respectively). Tie2-CRE transgenic mice express CRE in endothelial cells and hemangioblasts, leading to gene deletion specifically in endothelial and hematopoietic cells (17) (Fig. 1 a and b and SI Fig. 7), which was confirmed for both the floxed αv allele and αv protein (Fig. 1 c and d).

αv-tie2 mice were born at expected frequency and developed normally with no evidence of defects in angiogenesis or development of hematopoietic cells (4) (SI Figs. 8 and 9). However, from 12 weeks onward, αv-tie2 mice lost weight and body condition compared with controls and died prematurely (median lifespan of 44 weeks, n = 42, compared with >80 weeks for littermates, n = 58) (Fig. 2 a and b). Many αv-tie2 mice died after acute constriction of the intestine (Fig. 2d), suggesting that defects in intestinal immunity underlie the wasting and death. Despite these areas of constriction appearing throughout the intestine, no inflammatory infiltrates were seen in the small intestine. However, inflammation was seen in the colon and cecum of all αv-tie2 mice from 14 weeks onward, with infiltrates


The authors declare no conflict of interest.

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Abbreviations: aTreg, adaptive regulatory T; DC, dendritic cell; mLN, mesenteric lymph node; RGD, arginine-glycine-aspartic acid, Treg, regulatory T.

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Inflammation was chronic and progressive, leading to ulcers, blood vessel from reporter mice at higher magnification (Fig. 1a). PCR across exon 4 of TIE2 gene in DNA from sorted lung endothelial cells (CD31+ cells) or splenocytes from mice carrying one floxed and one nonfloxed allele for TIE2 show that the floxed allele is lost when mice also carry the TIE2-CRE transgene. (Fig. 1b) Sections of stained tissue show CRE-mediated deletion in blood vessels and immune organs, indicated by an arrowhead and arrow, respectively. (Fig. 1c) CRE-mediated deletion in endothelial cells in vessels from control and reporter mice (Upper) and a single blood vessel from reporter mice at higher magnification (Lower). (Fig. 1d) FACS analysis of macrophages and total splenocytes from control (TIE2 flox/-; TIE2-CRE+) or αv-tie2 mice stained for surface expression of αv. Red lines show staining with isotype control antibody, and blue-filled histograms show staining for αv.

Immune Cell Activation in αv-tie2 Mice. Histology of the colon suggested that the main effector cells mediating inflammation were T cells, and hence we looked for evidence of T cell activation. The mLN s and Peyer’s patches of αv-tie2 mice were enlarged compared with controls (Fig. 3a) and contained significantly higher proportions of activated (CD69high CD44high) CD4+ T cells (Fig. 3b; Table 1, and SI Fig. 11). The spleen and peripheral lymph nodes (PLNs) also contained higher numbers of activated CD4+ cells in older mice (>12 weeks) (SI Fig. 11), although they did not demonstrate gross enlargement. Expression of the T cell cytokines IL4 and IFN-γ was increased both systemically and in the colon of αv-tie2 mice (Fig. 3c and Table 2). Other cytokines were also increased in the colon, including IL5, IL6, and TNF-α but not IL12 and IL23 (SI Fig. 11). Notably, T cell activation preceded inflammation, with enlargement of mLN s, T cell activation, and increased expression of IL4 and IFN-γ evident even at weaning (3 weeks; Tables 1 and 2), suggesting colitis arose from early loss of regulation of T cell responses.

Changes in Treg Cells in αv-tie2 Mice. The central importance of Treg cells in controlling mucosal inflammation is demonstrated in the many studies showing that, in their absence, effector T cells rapidly induce colitis and wasting (18). We therefore examined whether Treg cells were present in αv-tie2 mice. Two principal pathways give rise to Treg cells: “natural” Treg (nTreg) cells arise in the thymus, are found in lymphoid organs, and mediate tolerance to self-antigen, whereas “adaptive” Treg (iTreg) cells arise in the periphery and are thought to be the main mechanism for regulating responses to tissue-specific or foreign antigens, such as those derived from commensal bacteria (19). As determined by CD4+ CD25+ FoxP3+, equivalent numbers of Treg cells were found in spleens of controls and αv-tie2 mice, whereas the mLN of αv-tie2 mice contained increased proportions of Tregs as compared with controls (Fig. 3d). These cells are likely to be predominantly natural Tregs and appear to be generated in the absence of αv. In contrast with the results for spleen and mLN, the colons of αv-tie2 mice had significantly decreased numbers (50% reduction) of Treg cells compared with
controls (Fig. 3d). These Treg cells are probably aTreg cells, suggesting that failure to generate these cells leads to loss of mucosal tolerance in αv-tie2 mice.

Colitis Is Caused by Loss of αv from Myeloid Cells. The above data indicate that T cells are the main effectors of inflammation in αv-tie2 mice and suggest that this may be attributable to loss of colon Treg cells. However, because Tie2-CRE causes deletion in both endothelial and hematopoietic cells (Fig. 1), these changes in T cells could either be attributable to direct effects of loss of αv from T cells or indirect effects after loss of αv from other cell types. We therefore carried out a series of experiments to test directly the importance of αv on T cells and other immune cells.

Transplant of an αv-deficient immune system to lethally irradiated control mice resulted in weight loss and colitis (Fig. 4a and b), and, conversely, reconstitution of old (24-week-old) αv-tie2 mice with control bone marrow reversed ongoing inflammation with restoration of normal colon histology (Fig. 4a and b). Therefore, inflammation and colonic damage did not arise from defects in intestinal development or from loss of αv from endothelial cells, but were directly attributable to deletion of αv in the immune system.

We then generated four additional αv-flox CRE mouse lines to analyze contributions of αv on immune cell subpopulations. Mice lacking αv on T cells, B cells, or both T and B cells did not develop colitis despite efficient deletion of αv (Fig. 4c and d and SI Fig. 12). In contrast, mice lacking αv on macrophages, neutrophils, and DCs (αv-LysM mice (20)) (SI Fig. 11) developed colitis closely resembling that from αv-tie2 mice, with similar histology and proinflammatory cytokine expression (Fig. 4c–f). The incidence and severity of disease were lower in these mice than in αv-tie2 mice of the same age (30% severe colitis in αv-LysM at 40 weeks compared with 100% in αv-tie2), probably as a consequence of incomplete gene deletion (20) (50–80% gene deletion in αv-LysM mice compared with 100% in αv-tie2) (SI Fig. 12). Taken together, these data show that inflammation does not arise from loss of αv on adaptive immune cells but instead indicate an important role for αv on myeloid cells in immune regulation.

Impaired Function of mLN DCs in αv-tie2 Mice. The dependence of colitis on deletion of αv from myeloid cells raised the possibility that the loss of colon Treg cells was attributable to defects in DCs. We therefore tested the ability of αv-deficient DCs to generate aTreg cells. Culture of bone marrow DCs with CD4+ CD25+ T cells generated ~5% Treg cells regardless of αv expression by the DCs (Fig. 5a). However, DCs from mLNs of αv-tie2 mice generated fewer (~1% compared with 4–5%) CD4+ CD25+ FoxP3+ T cells than DCs from control mice (Fig. 5a). These results show that mucosal DCs but not bone marrow-derived DCs from αv-tie2 mice are impaired in their ability to generate Treg cells. It is recognized that certain mLN DC subsets induce differentiation of Th1 cells into Th2 cells and that a specific population, marked by expression of CD103 (αEβ7 integrin), is implicated in imprinting gut-homing (21). CD11c<sup>high</sup> CD103+ DCs were reduced in mLNs of αv-tie2 mice (Fig. 5b), providing a potential explanation for the reduction in intestinal Treg cells.

### Table 1. Comparison of T cell activation in mLNs of αv-tie2 mice before (3 weeks) and after (12 weeks) onset of histologically evident colitis

<table>
<thead>
<tr>
<th>Cell number × 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Activated CD4&lt;sup&gt;+&lt;/sup&gt; T cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>3 weeks</td>
<td>12 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>7.1 ± 0.23 (P = 0.001)</td>
</tr>
<tr>
<td>αv-tie2</td>
<td>15 ± 1.1 (P = 0.001)</td>
</tr>
</tbody>
</table>

T cell activation was assessed by CD44 and L-selectin staining. P values are from Student’s t test.

### Table 2. Proinflammatory cytokine expression in colon of αv-tie2 mice before (3 weeks) and after (18 weeks) onset of histologically evident colitis

<table>
<thead>
<tr>
<th>IFN-γ</th>
<th>IL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26 ± 4.7 (P = 0.017)</td>
</tr>
<tr>
<td>αv-tie2</td>
<td>9.6 ± 1.3 (P = 0.086)</td>
</tr>
</tbody>
</table>

Gene expression is relative to GAPDH, × 10<sup>3</sup>. P values are from Student’s t test.
more, macrophages lacking αv were no longer sensitive to the inhibitory effects of RGD peptides (Fig. 6b). Similar phagocytic defects were seen in DCs derived from αv-tie2 mice (Fig. 6c). To determine whether deletion of αv affected phagocytosis in vivo, we carried out two further experiments. First, apoptotic cells were injected into the peritoneal cavity, and their internalization by macrophages were measured. Reduced phagocytosis was seen in both αv-tie2 and αv-LysM mice (Fig. 6d). Second, to assess the rate of removal of apoptotic cells from the intestine, we measured the number of uncleared TUNEL+ cells in the colons of control and αv-tie2 mice at 6 weeks of age (before the onset of colitis). Twice as many apoptotic cells were seen in αv-tie2 mice, consistent with reduced phagocytosis and apoptotic cell removal (Fig. 6e–f). A well recognized consequence of persistent apoptotic cells is the generation of autoantigens that, in turn, drive autoantibody production (23). We therefore determined whether mice lacking αv had increased levels of autoantibodies. Serum from both αv-tie2 and αv-LysM mice contained high levels of autoantibodies, including antibodies to tropomyosin, an autoantigen associated with colitis in humans (24), and antibodies to prominent components of dying cells, such as phosphatidylserine, dsDNA, and anti-nuclear antibodies (Fig. 6g–i).

Discussion

In summary, we have shown that αv integrins are central to intestinal immune regulation and that deletion of αv leads to colitis, autoimmunity, and cancer, thus establishing a mouse model for inflammatory bowel disease. The maintenance of mucosal immune homeostasis relies on the sampling of intestinal antigens by DCs and the subsequent production of Treg cells that home to the intestine and regulate immune responses. Colitis in αv-tie2 mice is likely to be caused by loss of these colon aTreg cells and corresponding increases in activated T cells and T cell cytokine production. This phenotype is attributable to loss of αv from macrophages and DCs, which reduces phagocytosis of apoptotic cells and inhibits the generation of mucosal aTreg cells.

The generation and function of aTreg cells requires TGF-β signaling in and between T cells, and mice lacking TGF-β or TGF-β signaling components specifically in T cells have phenotypes resembling those of αv-tie2 mice (25, 26). It is pertinent that αv, when dimerized with β6 and β8, binds and activates TGF-β (9). Loss of αv-mediated TGF-β activation may therefore contribute to the αv-tie2 phenotype, and we propose that DC-expressed αv integrins locally activate TGF-β, which acts in a paracrine fashion to initiate Treg differentiation in T cells. Furthermore, our data suggest that TGF-β production by T cells is insufficient to regulate immune responses in the absence of TGF-β activation by antigen-presenting cells, providing a mechanism by which antigen specificity is retained in a Treg cells. The DCs that inhabit mucosal tissues appear particularly sensitive to the loss of αv integrins because bone marrow-derived DCs generate Tregs normally in the absence of αv. TGF-β is present at high levels in the intestine, and it is likely that αv also contributes to TGF-β signaling during DC maturation, because TGF-β conditions DCs to generate aTreg cells. Furthermore, fewer mLN DCs from αv-tie2 mice express CD103+ (αE integrin), which is stimulated by TGF-β. We propose that absence of these DCs reflects, in part, failure of macrophages and DCs in the intestine to activate TGF-β.

Colitis is not seen in mice lacking β3, β5, or β6 integrins or in hematopoietic β1 knockouts (27), heavily implicating β8 in control of mucosal immunity. Recent complementary condi-
tional knockout studies confirm this, showing that loss of αvβ8 integrins from DCs but not from T cells also causes colitis (11). However, the phenotype of these mice is less severe than that seen in αv-tie2 mice, suggesting that loss of other αv integrins contributes to the severity of colitis. αvβ3 and αvβ5 have been implicated in the removal of apoptotic cells by macrophages and DCs, and αv-deficient mice have impaired phagocytosis of apoptotic cells, leading to persistence of dying cells in the colon. Apoptotic epithelial cells are thought to provide an important source of intestinal self-antigen, and DCs that exit the intestine bearing apoptotic cells express low levels of costimulatory molecules and are poor stimulators of T cell responses (28). Such “functionally immature” DCs have been shown to preferentially induce Treg differentiation when compared with classically activated DCs, and similar DCs can be induced in vitro by culture with apoptotic cells (29). Phagocytosis of apoptotic cells may therefore provide intestinal DCs with both antigen and the appropriate conditioning to generate Tregs. Impaired phagocytosis would lead to fewer DCs bearing apoptotic cells in the lymph nodes and potentially fewer Treg cells. The importance of this process in maintaining immune tolerance is demonstrated by genetic deletion of key components of the apoptotic cell-recognition machinery, which leads to persistent apoptotic cells, chronic inflammation, and development of autoimmune disease resembling systemic lupus erythematosus (15). These include mice lacking the serum protein MFGE8/Lactadherin, which mediates apoptotic cell removal through αv integrins, implicating αv-dependent removal of apoptotic cells in the induction of immune tolerance (30). Hence, although phagocytosis defects may not be sufficient to induce the colitis seen in αv-tie2 and αv-LysM mice, they are likely to contribute to autoimmunity and inflammation. Intriguingly, many of the effects of apoptotic cell uptake have been attributed to the release of TGF-β, and it is tempting to speculate that uptake of apoptotic cells, synthesis of active TGF-β, and generation of Tregs are causally linked (31). In support of this possibility, systemic administration of large numbers of apoptotic cells promotes Treg expansion through TGF-β production (22), a process critically dependent on both macrophages and DCs.

We therefore propose a model of mucosal tolerance in which αv integrins have a dual role: αvβ3 and αvβ5 would mediate uptake of apoptotic cells by macrophages and DCs, thus providing self antigen and modifying the extent of inflammation, and αvβ8 would generate Treg cells through local activation of TGF-β and conditioning of DCs (SI Fig. 14). The results presented here and our proposed model emphasize the vital role of antigen-presenting cells in regulation of inflammatory responses and maintenance of immune tolerance. Furthermore, these studies demonstrate the pleiotrophic role of αv integrins in the regulation of mucosal immune responses and provide insights into mechanisms that control inflammatory bowel disease.

Methods
Mice. Generation of mice is described in SI Text. All mice used in the study were of mixed 129:C57BL/6/FVB background and
were housed under specific pathogen free conditions at Massachusetts Institute of Technology. Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

Analysis of Colitis. Representative longitudinal and cross-sections of large intestine were stained with hematoxylin and eosin and graded by using a scheme based on that described by Powrie and colleagues (32), as follows: 0 = normal; 1 = mild epithelial hyperplasia, mild mucosal inflammation; 2 = moderate hyperplasia, mucin depletion, moderate inflammation; 3 = severe hyperplasia, transmural inflammation, ulceration. The highest score seen in sections from the length of the colon and cecum was reported for each mouse. All scoring was on coded samples and independently confirmed by two pathologists (M.B. and R.T.B.).

Immune Analysis. Cell preparation and staining are described in SI Text. Serum cytokines were measured by in vivo cytokine capture assay. All autoantibody titers were determined by ELISA (SI Text) except antinuclear antibodies, which were from staining of HEP-G2 cells.

Treg Cell Generation. CD11c+ DCs were sorted from mLN or spleen by using CD11c microbeads (Miltenyi Biotech, Auburn, CA) and were routinely >85% CD11c+ cells after sorting. CD4+ CD25− T cells were sorted from spleen suspensions by enrichment of CD4+ cells followed by depletion of CD25+ cells, using MACs microbeads (Miltenyi Biotech). A total of 105 T cells were cultured with 2.5 × 104 DCs in the presence of 0.5 μg/ml anti-CD3 antibody for 3 days, and the proportion of CD4+ CD25+ FoxP3+ cells was measured by FACS.

Phagocytosis Assays. Bone marrow-derived macrophage and DC culture, apoptotic cell generation, and phagocytosis assays are described in refs. 29 and 33. For ov blockade, anti-ov (H9–2B8), control Ig (E36–239) (50 μg/ml; both BD Biosciences Pharmingen, San Diego, CA), glycine-RGD-serine, or glycine-RAD-serine (0.5 mg/ml; both Calbiochem, San Diego, CA) were incubated with macrophages for 20–30 min before and throughout phagocytosis assays. For in vivo phagocytosis, 107 fluorescently labeled apoptotic thymocytes were injected into the peritoneal cavity, and phagocytosis were assayed after 20 min by FACS of F4/80-stained lavages.

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