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Effect of $\alpha 4\beta 7$ Blockade on Intestinal Lymphocyte Subsets and Lymphoid Tissue Development

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Abstract

Background—Blockade of the integrin $\alpha 4\beta 7$ has promise as a therapy for inflammatory bowel disease. $\alpha 4\beta 7$ plays diverse roles in the intestinal immune system, including lymphocyte homing and lymphoid tissue formation; however, the effects of $\alpha 4\beta 7$ blockade on these processes during inflammation and their relationship to the efficacy of $\alpha 4\beta 7$ blockade and its potential untoward effects are largely unknown.

Methods— $\alpha 4\beta 7$ function was inhibited by genetic manipulation or antibody blockade. The effects of these manipulations on lymphoid tissues and the presence of lymphocyte subpopulations in the murine small intestine and colon were evaluated in the unchallenged state, during the acute injury dextran sodium sulfate model, and during the splenocyte transfer chronic inflammation model.

Results— $\alpha 4\beta 7$ inhibition resulted in a decrease in the B-lymphocyte population in the diffuse lamina propria and a decrease in the number of lymphoid aggregates in the uninflamed intestine and in the acute injury model. $\alpha 4\beta 7$ blockade did not reduce the Foxp3[−] T-lymphocyte population but did decrease the Foxp3⁺ T-lymphocyte population located selectively within the lymphoid aggregates in the uninflamed intestine and in the acute injury model. In contrast, $\alpha 4\beta 7$ blockade reduced the intestinal T-lymphocyte population and decreased the production of inflammatory cytokines in the T-lymphocyte mediated chronic inflammation model.

Conclusions—These findings demonstrate differential use of $\alpha 4\beta 7$ by B-lymphocytes, Foxp3[−] T-lymphocytes, and Foxp3⁺ T-lymphocytes to home to the gut, and suggest that $\alpha 4\beta 7$ blockade may serve as a targeted therapy that selectively inhibits the accumulation of pathogenic T-lymphocyte populations in the chronically inflamed intestine.

Keywords

integrin; T-lymphocyte; murine

Integrins are dimeric cell surface molecules composed of α and β subunits that promote cell–cell interactions and play important roles in the immune system. Within the integrin family, $\alpha 4\beta 7$ displays relative specificity for the mucosal immunity and has multiple functions including lymphocyte homing, intestinal lymphoid tissue formation, and promoting inflammatory responses.^{1–6} Blockade of $\alpha 4\beta 7$ is an appealing therapy for intestinal inflammatory diseases, as the effects of blocking this pathway will largely be limited to mucosal sites. While diverse functions for $\alpha 4\beta 7$ in intestinal immunity are well described, the essential nature of $\alpha 4\beta 7$ in these roles is less clear, and blockade of these essential

functions could result in untoward effects of this therapy including inhibiting the homing of nonpathogenic lymphocytes and the formation of homeostatic lymphoid tissues.

Mucosal vascular addressin cell adhesion molecule (MAdCAM-1) is the binding partner for $\alpha 4\beta 7$,¹ and is expressed on high endothelial venules (HEVs) and postcapillary vessels in the intestine. In contrast, $\alpha 4\beta 7$ is expressed by diverse populations of hematopoietic cells including lymphocytes, natural killer cells, monocytes, and lymphoid tissue inducer (LTi) cells.^{5–9} A role for $\alpha 4\beta 7$ /MAdCAM-1 interactions in lymphocyte trafficking to the intestine is widely accepted; however, the lymphocyte subsets, cellular compartments, and conditions in which $\alpha 4\beta 7$ plays an essential role, and therefore would be affected by $\alpha 4\beta 7$ blockade, are less clear. Early studies using adoptive transfer of labeled lymphocytes identified a role for $\alpha 4\beta 7$ in lymphocyte homing to Peyer's patches and mucosa in the uninflamed intestine.² Later studies of using genetically modified mice confirmed that in the absence of $\beta 7$ in the uninflamed intestine, Peyer's patches had diminished cellularity and mucosal lymphocyte populations were decreased.¹⁰ However, these studies were performed prior to the appreciation of the diversity of lymphocyte subsets, and consequently information regarding the essential nature of $\alpha 4\beta 7$ for homing of specific lymphocyte populations in the uninflamed intestine is largely lacking. Moreover, blockade of the $\alpha 4\beta 7$ /MAdCAM-1 pathway has had variable effectiveness in animal models of intestinal inflammation,^{3,11–14} which could be explained by differences in the specificity of the blockade strategy, or the homing mechanisms used by lymphocyte subpopulations playing a pathogenic or protective role in each model.

In addition to the above effects, $\alpha 4\beta 7$ blockade may affect the development of intestinal lymphoid tissues during inflammation. $\alpha 4\beta 7$ /MAdCAM-1 interactions play a critical role in the development of isolated lymphoid follicles (ILFs),⁶ or intestinal lymphoid aggregates, in the uninflamed adult intestine. During pathogenic inflammatory diseases, including inflammatory bowel disease (IBD), increased numbers of lymphoid aggregates are also seen,^{15–18} and the above observations suggest that their development may also be dependent on $\alpha 4\beta 7$ /MAdCAM-1 interactions. During physiologic inflammation, ILFs mediate homeostatic functions in intestinal immunity; conversely, the function of lymphoid aggregates during chronic intestinal inflammation is less clear and may be pathogenic, as these lymphoid aggregates have been proposed to be the sites containing the earliest manifestations of IBD.^{19,20} Thus, $\alpha 4\beta 7$ blockade could affect lymphoid aggregate development during uncontrolled intestinal inflammation, resulting in either detrimental or beneficial effects.

In order to better understand the processes lost in the absence of $\alpha 4\beta 7$ function we investigated the effect of $\alpha 4\beta 7$ blockade and $\beta 7$ deficiency on lymphoid tissue development and lymphocyte subpopulations in the uninflamed and inflamed intestine. In contrast to current perceptions, we observed that T-lymphocyte populations in the diffuse lamina propria and lymphoid aggregates were unaffected in the absence of $\alpha 4\beta 7$ function in normal mice and in the acute injury dextran sodium sulfate (DSS) colitis model. Concordant with prior observations we observed that lamina propria B-lymphocyte populations and the development of B-lymphocyte containing lymphoid aggregates were both affected by loss of $\alpha 4\beta 7$ function in the normal intestine and in the acute injury DSS colitis model. Furthermore, we observed that Foxp3+ T-lymphocytes, like B-lymphocytes, were dependent on $\alpha 4\beta 7$ function to localize to lymphoid aggregates. In contrast to the above observations, T-lymphocyte trafficking in T-lymphocyte-mediated colitis model was dependent on $\alpha 4\beta 7$ function. Anti- $\alpha 4\beta 7$ blockade did not alter intestinal T-lymphocyte populations or ameliorate acute injury DSS-induced colitis, but did improve chronic T-lymphocyte-mediated colitis as evidenced by decreased T-lymphocyte infiltration into the intestine and decreased production of inflammatory cytokines. These observations demonstrate differential dependence on $\alpha 4\beta 7$ by intestinal lymphocyte subpopulations and suggest that

$\alpha 4\beta 7$ blockade may be a more targeted therapy than previously appreciated by selectively blocking T-lymphocyte trafficking to the intestine during chronic T-lymphocyte-mediated inflammation.

MATERIALS AND METHODS

Animals

BALB/c mice, C57BL/6 mice, $\beta 7^{-/-}$ mice on the C57BL/6 background, and RAG $^{-/-}$ mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a specific pathogen-free facility and fed routine chow diet. Animals were 8 to 16 weeks of age at the time of analysis. Animal procedures and protocols were carried out in accordance with the Institutional Review Board at Washington University School of Medicine (St. Louis, MO).

Induction of DSS Colitis

The 7–8-week-old Balb/c mice were given 5% DSS (MW 36,000–50,000, MP Biomedicals, Solon, OH) in drinking water for 7 days and then remained on regular water for another 2 weeks. The mice were monitored daily and weight loss was recorded. At the time of sacrifice a piece of distal colon close to the rectum was fixed in 10% neutral formalin buffer (Fisher Scientific, Fair Lawn, NJ) and subjected to hematoxylin and eosin H&E staining for histological scoring as described.²¹ A second piece of distal colon was snap frozen at -80°C and subjected to myelo-peroxidase (MPO) activity analysis.²¹ The number of lymphoid aggregates was evaluated by B220 whole-mount staining and counted using a dissecting microscope at a magnification of 25 $\times$ or greater as previously described.²²

To observe if $\alpha 4\beta 7$ blockade plays a role in DSS colitis, mice were injected intraperitoneally (i.p.) with 200 μg of rat antimouse $\alpha 4\beta 7$ (BioExpress, West Lebanon, NH) or rat IgG (Southern Biotechnology Associates, Birmingham, AL) every other day. Treatment was initiated at the time of DSS exposure and continued for 1 week following DSS removal. The mice were sacrificed at the end of treatment and B220 clusters in the colon and DSS-colitis were compared between each group as described above.

Flow Cytometric Analysis

Single cell suspensions from lamina propria from small intestine and colon obtained as described previously were used for flow cytometric analysis. Antibodies used for analysis were antimouse CD62L (BD Pharmingen, San Diego, CA), antimouse CD44 (eBioscience, San Diego, CA), antimouse CD69 (eBioscience), antimouse CD3, anti-mouse CD19 (eBioscience), antimouse CD4 (eBioscience), antimouse CD25 (eBioscience), antimouse Foxp-3 (eBioscience), and appropriate isotype control antibodies (all from eBioscience). Data acquisition was performed on a FACScan cytometer (BD Bioscience, San Jose, CA) retro-fitted with a second laser using Cellquest (BD Bioscience) and Rainbow (Cytex, Fremont, CA) software. Data analysis was performed on a Macintosh G4 computer running FlowJo software (Tree Star, Ashland, OR) or CellQuest software (BD Bioscience). Dead cells were excluded based on forward and side light scatter and 7-AAD staining. Gates for positive staining was defined such that 1% of the analyzed population stained positive with the appropriate isotype control antibodies.

Immunohistochemistry

Intestines were opened and 1.5-cm sections were snap-frozen in OCT medium (Sakura Finetek, Torrance, CA). Seven μm sections were cut perpendicular to the axis of the villi (horizontal sections). Endogenous peroxidase activity was quenched with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 minutes at room temperature, endogenous biotin was

blocked with the Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA). Sections were washed in PBS 3 times, blocked with PBS plus 1% bovine serum albumin (BSA) for 30 minutes at room temperature, and incubated with the primary antibody for 1 hour at room temperature. Sections were washed in PBS 3 times. Sections incubated with unconjugated primary antibodies were subsequently incubated with biotinylated secondary antibodies for 1 hour at room temperature and washed 3 times in PBS. Detection utilized streptavidin conjugated Cy2 or streptavidin conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA). In experiments using multiple fluorophores, sections were treated with the Avidin/Biotin blocking kit and the above protocol was repeated using a second fluorophore for detection. Sections were counter-stained with Hoechst dye (Sigma-Aldrich, St. Louis, MO) to visualize nuclei. Images were acquired using Axiovision software (Carl Zeiss Microimaging, Thornwood, NY) and the number of positively staining cells per mm² surface area was obtained.

Splenocyte Transfer

In all, 3×10^5 CD4⁺ CD25⁻ splenocytes from C57BL/6 or $\beta 7^{-/-}$ mice were isolated by flow cytometric cell sorting and injected i.p. into gender-matched Rag^{-/-} mice. Mice were monitored with weekly weight loss, and 5 weeks after transfer the mice receiving wildtype splenocytes were given 200 μ g anti- $\alpha 4\beta 7$ or rat IgG i.p. every other day for 2 weeks. The intestinal inflammation was evaluated as described above.

Real-time Polymerase Chain Reaction (PCR) Analysis

Total RNA was isolated from the distal colon using Trizol (Invitrogen, Carlsbad, CA) and treated with DNase I (Ambion, Austin, TX) to remove contaminating DNA, and cDNA was synthesized using Superscript II RNase H-reverse transcriptase (Invitrogen) in the presence of random hexamer primers (Invitrogen). Expression of targets was detected by real-time PCR using ABI prism 7700 sequence detection system and SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA). The following primers were used for detection of the targets, forward primers are listed first followed by reverse primers: 18S 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAAT TACCGCGGCT-3', IFN- γ 5'-ATGAACGCTACACACTG CATC-3' and 5'-CCATCCTTTTGCCAGTTCCTC-3', TNF- α 5'-CCCTCACACTCAGATCATCTTCT-3' and 5'-GCTAC GACGTGGGCTACAG-3', IL-17 5'-TTTAACTCCCTT GGCGCAA-3' and 5'-CTTCCCTCCGCATT GACAC-3', MPO 5'-AGTTGTGCTGAGCTGTATGGA-3' and 5'-CGG CTGCTTGAAGTAAAAACAGG-3', IL-6 5'-CCAGAAA CCGCTATGAAGTTCCT-3' and 5'-CACCAG CATCAGT CCCAAGA-3', CD3e 5'-ATGCGGTGGAACACT TTCTGG-3' and 5'-GCACGTCAACTCTACACT GGT-3'

Samples were measured in triplicate. Relative quantitation of target expression using 18s as a housekeeping gene was determined using the comparative CT method as described in the ABI prism 7700 sequence detection system user bulletin.

Myeloperoxidase Activity

Colonic MPO activity was determined as described previously.²¹ Briefly, distal colon was homogenized in homogenate buffer containing 0.5% hexadecyltrimethylammoniumbromide (HTAB) and centrifuged at 13,000 rpm for 10 minutes. MPO activity of the supernatant was determined by measuring the H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine and expressed as units per gram of tissue.

Statistical Analysis

Data analysis using Student's *t*-test was performed using GraphPad Prism (San Diego, CA). A value of $P < 0.05$ was used as a cutoff for statistical significance.

RESULTS

Differential Dependence on $\alpha 4\beta 7$ by Intestinal Lymphocyte Subsets

While it is widely accepted that $\alpha 4\beta 7$ /MadCAM-1 interactions play roles in the localization of lymphocytes to the intestine, the essential nature of these interactions and the differential usage of this pathway by lymphocyte subsets and lymphoid compartments has not been previously addressed. To address this issue, lamina propria (LP) cellular populations were isolated from wildtype and $\beta 7^{-/-}$ mice and evaluated with flow cytometry. Consistent with prior observations, the CD19+ B-lymphocyte populations in the diffuse LP were decreased in the small intestine and colon of $\beta 7^{-/-}$ mice (Fig. 1A–D). Conversely, CD3+ T-lymphocytes populations were present in the LP of the small intestine and colon and were not affected by the absence of $\beta 7$ (Fig. 1A–D). Further analysis of the T-lymphocyte populations revealed that CD4 and CD8 T-lymphocyte populations were present in normal ratios and that there was no difference in the expression of cell surface markers associated with activation in the absence of $\beta 7$ (Fig. 1A–D). We observed no differences in the population of Foxp3+ CD4+ T-lymphocytes in the small intestine LP between wildtype and $\beta 7^{-/-}$ mice (Fig. 1A,B). Foxp3+ T-lymphocytes were rare in the colonic LP of mice of either genotype and subsequently differences in the presence of this lymphocyte population in the absence of $\beta 7$ could not be evaluated.

Prior studies have shown that $\alpha 4\beta 7$ plays a critical role in localizing B-lymphocytes to lymphoid aggregates in the uninflamed intestine, and accordingly plays a critical role in the development of B-lymphocyte containing lymphoid aggregates.⁶ To evaluate a similar role for $\alpha 4\beta 7$ in localizing T-lymphocyte to lymphoid aggregates we evaluated the presence of T-lymphocytes in the lymphoid aggregates in wildtype mice, $\beta 7^{-/-}$ mice, and mice receiving anti- $\alpha 4\beta 7$ or rat IgG for 2 weeks using immunohistochemistry. In contrast to our previous observations regarding B-lymphocytes, the density of CD3+ T-lymphocytes in lymphoid aggregates of both small intestine and colon was not reduced by the absence of $\beta 7$ or in the presence of $\alpha 4\beta 7$ blockade (Fig. 2A,B). Conversely we noted an increase in the density of CD3+ T-lymphocytes within the colonic aggregates of $\beta 7^{-/-}$ mice (Fig. 2B). While this could reflect an increased density of CD3+ T-lymphocytes due to the absence of B-lymphocytes within these aggregates, a similar increase was not seen in mice given anti- $\alpha 4\beta 7$ therapy, which also lack B-lymphocytes within aggregates. Alternatively, this could reflect a low level of inflammation in the intestine of mice with diminished B-lymphocyte populations. Lymphoid aggregates in the uninflamed intestine contain a population of Foxp3+ T-lymphocytes. To examine whether this T-lymphocyte subset is affected in the absence of $\alpha 4\beta 7$ function, we evaluated the presence of Foxp3+ T-lymphocytes in the lymphoid aggregates in wildtype mice and $\beta 7^{-/-}$ mice by immunohistochemistry. The density of Foxp3+ T-lymphocytes in lymphoid aggregates in the small intestine of $\beta 7^{-/-}$ mice was significantly lower when compared to C57BL/6 mice (Fig. 2C), indicating that in contrast to Foxp3⁻ T-lymphocytes, or Foxp3+ T-lymphocytes in the diffuse LP, Foxp3+ T-lymphocyte trafficking into lymphoid aggregates is impaired in the absence of $\alpha 4\beta 7$ function. We could not detect a Foxp3+ T-lymphocyte population in the lymphoid aggregates or the diffuse LP in the colon of wildtype or $\beta 7^{-/-}$ mice.

$\alpha 4\beta 7$ Blockade Inhibits Colonic Lymphoid Aggregate Formation, But Does Not Affect Disease Course or the Presence of Intestinal T-lymphocytes During Acute Injury

The above observations demonstrate that a major effect of $\alpha 4\beta 7$ blockade on intestinal lymphocytes in the uninflamed intestine are related to the loss of lymphocytes (B-lymphocytes and Foxp3+ T-lymphocytes) within lymphoid aggregates. Lymphoid aggregates develop in an $\alpha 4\beta 7$ -dependent manner in the uninflamed intestine.⁶ To evaluate the effects of $\alpha 4\beta 7$ blockade on lymphoid aggregate development during acute injury, we first evaluated the kinetics of lymphoid aggregate development in the acute injury DSS colitis model. We observed that the formation of lymphoid aggregates correlated with the peak of inflammation as demonstrated by weight loss, histological score, and MPO activity (Fig. 3A–D). Interestingly, lymphoid aggregates persisted after inflammation resolved (Fig. 3D), suggesting that these lymphoid aggregates were less likely to contribute to disease processes in this acute injury model. To evaluate the effect of $\alpha 4\beta 7$ blockade during acute injury, mice were given blocking antibodies to $\alpha 4\beta 7$ or control Ig and DSS in drinking water. We observed that $\alpha 4\beta 7$ blockade had no effect on the course of DSS colitis as evidenced by histologic score, MPO activity, or weight loss (Fig. 4A–C). However, $\alpha 4\beta 7$ blockade was effective at reducing the number of lymphoid aggregates formed during acute injury (Fig. 4D–F), demonstrating that like the uninflamed intestine, lymphoid aggregate development during acute injury is also dependent on $\alpha 4\beta 7$. Related to the inhibition of lymphoid aggregate development, we observed that blockade or loss of $\alpha 4\beta 7$ function during acute injury diminished the colonic LP B-lymphocyte population (Fig. 5A–D). Similar to our findings in the uninflamed intestine, we observed that during the presence of acute injury, $\beta 7$ deficiency or $\alpha 4\beta 7$ blockade did not affect the presence or activation status of intestinal T-lymphocyte populations (Fig. 5A–D). Similar to our findings above, Foxp3+ T-lymphocytes were rare in the colonic LP in the setting of acute injury, and therefore we were not able to evaluate changes in this T-lymphocyte population.

Loss of $\alpha 4\beta 7$ Function Reduces Intestinal T-lymphocyte Populations and Inflammatory Mediator Production in a Chronic Model of T-lymphocyte Dependent Intestinal Inflammation

$\alpha 4\beta 7$ blockade has shown promise as a therapy for IBD.^{23,24} While our understanding of the pathogenesis of IBD is incomplete, it is generally believed to be mediated by uncontrolled T-lymphocyte responses that are driven by the luminal microbiota. In order to evaluate the effects of $\alpha 4\beta 7$ blockade in an animal model more closely aligned with our understanding of the pathogenesis of IBD, we evaluated the effects of the loss of $\alpha 4\beta 7$ in the splenocyte transfer model of intestinal inflammation. RAG^{-/-} mice were adoptively transferred CD4+ CD25⁻, Treg depleted, splenocytes from $\beta 7$ ^{-/-} donors or wildtype donors. Recipients of wildtype splenocytes received control Ig or anti- $\alpha 4\beta 7$ blockade starting 5 weeks after splenocyte transfer. We observed that all groups of animals developed weight loss to similar degrees (Fig. 6A). There was a trend toward improved histologic score in recipients of $\beta 7$ ^{-/-} splenocytes, and a significant decrease in MPO expression in recipients of $\beta 7$ ^{-/-} splenocytes and recipients of wildtype splenocytes receiving $\alpha 4\beta 7$ blockade (Fig. 6A). We observed significantly decreased expression of inflammatory mediators IFN- γ , IL-6, IL-17, and TNF- α in animals with disrupted $\alpha 4\beta 7$ function (Fig. 6B). In contrast to our above observations in the uninflamed intestine or in the setting of acute injury, we observed that $\alpha 4\beta 7$ blockade resulted in a decrease in the population of intestinal T-lymphocytes and a decrease in the number of T-lymphocytes within the lymphoid aggregates (Fig. 6C). Congruent with our above observations, we found that loss of $\beta 7$ function resulted in a decrease in the number of Foxp3+ T-lymphocytes in the lymphoid aggregates in this T-lymphocyte-dependent chronic inflammatory model (Fig. 6C).

DISCUSSION

Biologic therapies targeting adhesion molecules have demonstrated efficacy in autoimmune and chronic inflammatory diseases. Currently, the most established of these therapies is an antibody directed against $\alpha 4$ integrins, natalizumab. $\alpha 4$ pairs with either $\beta 7$ or $\beta 1$ and therefore this strategy targets $\alpha 4\beta 7$, $\alpha 4\beta 1$, and their respective binding partners MadCAM-1 and VCAM-1. Natalizumab therapy effectively blocks lymphocyte recruitment into tissues and has shown promise for the treatment of multiple sclerosis and Crohn's disease.^{25–28} However, this therapy is complicated by a rare but significant incidence of progressive multifocal leukoencephalopathy in the treatment of both of these disorders.^{29,30} MadCAM-1 expression is relatively restricted to the mucosa, while VCAM-1 is more widely expressed, and this differential expression of the $\alpha 4$ integrin binding partners has raised promise that therapies selectively blocking $\alpha 4\beta 7$ /MadCAM-1 interactions may avoid some of the untoward effects of the more global $\alpha 4$ blocking strategies. Accordingly, $\alpha 4\beta 7$ blocking strategies have demonstrated promise as therapies for IBD.^{23,24} While it is widely accepted that $\alpha 4\beta 7$ /MadCAM-1 interactions play a role in lymphocyte trafficking to the intestine and that lymphocyte trafficking can affect diverse aspects of mucosal immunity, it is less well understood which aspects of mucosal immunity are entirely dependent on $\alpha 4\beta 7$ /MadCAM-1 interactions, and therefore would be affected by $\alpha 4\beta 7$ blockade. Furthermore, recent investigations revealed a critical role for $\alpha 4\beta 7$ in the development of intestinal lymphoid tissues in the uninflamed adult intestine,⁶ suggesting other potential effects of $\alpha 4\beta 7$ blockade as a therapeutic approach. To better understand the potential effects of $\alpha 4\beta 7$ blockade on the intestinal immune system we evaluated the loss of $\alpha 4\beta 7$ function on intestinal lymphocyte populations and lymphoid tissues in the uninflamed and inflamed intestine.

Early studies demonstrated a role for $\alpha 4\beta 7$ in the localization of lymphocytes to the intestine in the basal condition.^{2,10} These studies were performed prior to the appreciation of the diversity of lymphocyte populations, and therefore did not address the dependence of specific lymphocyte subtypes on $\alpha 4\beta 7$ for localization to the intestine. In addition, further observations suggested that the dependence of T-lymphocytes on $\alpha 4\beta 7$ may not be universal, and that during inflammation T-lymphocytes can use alternative homing mechanisms.³ In contrast to these studies, we observed that intestinal T-lymphocyte populations in the uninflamed intestine were largely unaffected by the loss of $\alpha 4\beta 7$ function. T-lymphocytes can play important roles in mucosal defense and repair during acute injury, and accordingly we evaluated intestinal T-lymphocyte populations during acute injury in the absence of $\alpha 4\beta 7$ function. We also observed that intestinal T-lymphocyte populations were largely unaffected by the loss of $\alpha 4\beta 7$ function during acute injury and, related to this, $\alpha 4\beta 7$ blockade had little effect on the disease course in the acute injury DSS colitis model. One exception we observed related to the presence of Foxp3+ T-lymphocytes within intestinal lymphoid tissues. Foxp3+ T-regulatory cells are instrumental in controlling immune responses and maintaining tolerance in the intestine.³¹ Recent investigations demonstrate that human Foxp3+ T-regulatory cells express $\alpha 4\beta 7$ early in life but lose $\alpha 4\beta 7$ expression in adulthood,³² suggesting that this population may be less affected by $\alpha 4\beta 7$ blockade. Related to this, we did not observe a decrease in Foxp3+ T-lymphocytes in the diffuse LP in the absence of $\alpha 4\beta 7$ function; however, we did observe that the localization of Foxp3+ T-lymphocytes, but not Foxp3– T-lymphocytes, to intestinal lymphoid tissues was affected by the loss of $\alpha 4\beta 7$ function. Therefore, in the uninflamed intestine and during acute injury $\alpha 4\beta 7$ blockade had little effect on intestinal T-lymphocyte populations with the exception of inhibiting the localization of Foxp3+ T-lymphocytes to the intestinal lymphoid tissues.

The pathogenesis of IBD is incompletely understood, but largely regarded as being mediated by chronic inappropriate T-lymphocyte responses driven by luminal microbiota. To examine

the effect of loss of $\alpha 4\beta 7$ function in a chronic T-lymphocyte-dependent model, we evaluated intestinal T-lymphocytes in the splenocyte transfer model of chronic intestinal inflammation. We observed that loss of $\alpha 4\beta 7$ function improved aspects of this disease model, including the infiltration of T-lymphocytes into the intestine and the production of inflammatory mediators. This correlates well with findings demonstrating efficacy of $\alpha 4\beta 7$ blockade in the treatment of IBD,^{23,24} and with prior studies demonstrating that adoptively transferred splenic T-lymphocytes use MadCAM-1-dependent interactions to adhere to the microvasculature of the chronically inflamed murine colon.¹¹ A minority of Foxp3+ T-lymphocytes are CD25-, and therefore despite transferring T-regulatory cell depleted, CD4+ CD25-, splenocytes, a few Foxp3+ cells will be present in the recipients. Related to our above observations in the uninflamed and acutely injured intestine, we also observed that loss of $\alpha 4\beta 7$ function reduced the population of Foxp3+ T-lymphocytes within the lymphoid aggregates in this chronic inflammation model.

The adult intestine has a unique ability to develop lymphoid tissues via a canonical lymphoid tissue inducer (LTi) cell-dependent pathway in response to physiologic inflammatory stimuli.³³ These lymphoid tissues, termed isolated lymphoid follicles (ILFs) in the murine small intestine, resemble single-domed Peyer's patch and can act as inductive sites of the intestinal immune system by promoting homeostatic immune response in unchallenged animals.³⁴ In the human intestine and murine colon these lymphoid tissues are generally referred to as lymphoid aggregates. How these structures develop and function during acute injury and chronic inflammation is largely unknown. Here we observed that, like the development of ILFs in the uninflamed murine small intestine, lymphoid aggregate formation in the murine colon at baseline and during acute injury is also dependent on $\alpha 4\beta 7$, suggesting that the developmental requirements for ILFs or lymphoid aggregates are shared throughout the intestinal tract and during acute injury. This dependence closely correlates with the dependence of B-lymphocytes on $\alpha 4\beta 7$ for their localization into the intestine. This may reflect the selectivity of intestinal B-lymphocytes for intestinal lymphoid tissues as opposed to the diffuse LP, or a requirement for B-lymphocyte priming within mucosal lymphoid tissues prior to their migration to the LP. Of note, Foxp3+ T-lymphocytes, but not Foxp3- T-lymphocytes, partially share this dependence, as in the absence of $\alpha 4\beta 7$ function this population was reduced in the lymphoid aggregates, yet the overall Foxp3+ T-lymphocyte population in the diffuse LP was unaffected. This difference could be a result of differential homing properties of two Foxp3+ T-lymphocyte populations that localize to different compartments within the intestine, or alternatively could reflect a dependence on B-lymphocytes, or other cell types dependent on $\alpha 4\beta 7$, within the lymphoid tissues for the localization or generation of Foxp3+ T-lymphocytes.

Lymphoid neogenesis is a feature of many chronic inflammatory conditions including infections and autoimmune diseases.³⁵⁻³⁷ The lymphoid tissues formed in these conditions could form via an LTi cell independent, noncanonical pathway, and are referred to as tertiary lymphoid tissues. These structures can be well organized, resembling lymph nodes, containing germinal centers, lymphatics, and high endothelial venules and can closely resemble the lymphoid aggregates present in the uninflamed intestine. There is also evidence suggesting that tertiary lymphoid tissues can support primary lymphocyte responses including oligoclonal B-cell expansion, somatic hypermutation, and terminal differentiation to plasma cells.³⁸⁻⁴⁰ Related to the current study, increased numbers of lymphoid aggregates are seen in inflammatory conditions in the human intestine including IBD,¹⁵⁻¹⁸ and several observations suggest that these are the sites containing the earliest manifestations of IBD.^{19,20} Because of the nature of the splenocyte transfer model, we are unable to definitively address whether $\alpha 4\beta 7$ blockade will block the development of all or some of the lymphoid aggregates during chronic inflammation. However, based on the observations with the acute injury model, it seems likely that $\alpha 4\beta 7$ blockade will inhibit

lymphoid tissue development during chronic inflammation, and this could contribute to the efficacy of this therapy.

$\alpha 4\beta 7$ plays diverse roles in the intestinal immune systems, including lymphocyte homing and the development and maintenance of lymphoid tissues. Because of the selective expression of MadCAM-1, the binding partner of $\alpha 4\beta 7$, blockade of this pathway has the promise to inhibit lymphocyte trafficking to the intestine while avoiding the untoward effects of more global antiadhesion molecule approaches. The findings presented here further expand this promise by demonstrating that intestinal T-lymphocyte populations are relatively unaffected by $\alpha 4\beta 7$ blockade in the uninflamed intestine and during acute injury. Conversely, $\alpha 4\beta 7$ blockade was effective at limiting the pathogenic T-lymphocyte population in the intestine during chronic T-lymphocyte dependent inflammation. Thus, $\alpha 4\beta 7$ blockade may offer both a tissue specific and lymphocyte subset specific therapy during chronic T-lymphocyte-dependent intestinal inflammation.

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References

- Berlin C, Berg EL, Briskin MJ, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell*. 1993; 74:185–195. [PubMed: 7687523]
- Hamann A, Andrew DP, Jablonski-Westrich D, et al. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol*. 1994; 152:3282–3293. [PubMed: 7511642]
- Rivera-Nieves J, Olson T, Bamias G, et al. L-selectin, alpha 4 beta 1, and alpha 4 beta 7 integrins participate in CD4+ T cell recruitment to chronically inflamed small intestine. *J Immunol*. 2005; 174:2343–2352. [PubMed: 15699171]
- Wagner N, Muller W. Functions of alpha 4- and beta 7-integrins in hematopoiesis, lymphocyte trafficking and organ development. *Curr Top Microbiol Immunol*. 1998; 231:23–32. [PubMed: 9479858]
- Mebius RE, Schadee-Eestermans IL, Weissman IL. MAdCAM-1 dependent colonization of developing lymph nodes involves a unique subset of CD4+CD3- hematolymphoid cells. *Cell Adhes Commun*. 1998; 6:97–103. [PubMed: 9823459]
- Wang C, McDonough JS, McDonald KG, et al. Alpha4beta7/MAd-CAM-1 interactions play an essential role in transitioning crypto-patches into isolated lymphoid follicles and a nonessential role in cryptopatch formation. *J Immunol*. 2008; 181:4052–4061. [PubMed: 18768861]
- Perez-Villar JJ, Zapata JM, Melero I, et al. Expression and function of alpha 4/beta 7 integrin on human natural killer cells. *Immunology*. 1996; 89:96–104. [PubMed: 8911146]
- Yang Y, Harrison JE, Print CG, et al. Interaction of monocytoïd cells with the mucosal addressin MAdCAM-1 via the integrins VLA-4 and LPAM-1. *Immunol Cell Biol*. 1996; 74:383–393. [PubMed: 8912000]
- Erle DJ, Briskin MJ, Butcher EC, et al. Expression and function of the MAdCAM-1 receptor, integrin alpha 4 beta 7, on human leukocytes. *J Immunol*. 1994; 153:517–528. [PubMed: 7517418]
- Wagner N, Lohler J, Kunkel EJ, et al. Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. *Nature*. 1996; 382:366–370. [PubMed: 8684468]

11. Shigematsu T, Specian RD, Wolf RE, et al. MAdCAM mediates lymphocyte-endothelial cell adhesion in a murine model of chronic colitis. *Am J Physiol Gastrointest Liver Physiol*. 2001; 281:G1309–1315. [PubMed: 11668040]
12. Picarella D, Hurlbut P, Rottman J, et al. Monoclonal antibodies specific for beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RBhigh CD4+ T cells. *J Immunol*. 1997; 158:2099–2106. [PubMed: 9036954]
13. Soriano A, Salas A, Sans M, et al. VCAM-1, but not ICAM-1 or MAdCAM-1, immunoblockade ameliorates DSS-induced colitis in mice. *Lab Invest*. 2000; 80:1541–1551. [PubMed: 11045571]
14. Matsuzaki K, Tsuzuki Y, Matsunaga H, et al. In vivo demonstration of T lymphocyte migration and amelioration of ileitis in intestinal mucosa of SAMP1/Yit mice by the inhibition of MAdCAM-1. *Clin Exp Immunol*. 2005; 140:22–31. [PubMed: 15762871]
15. Weninger W, Carlsen HS, Goodarzi M, et al. Naive T cell recruitment to nonlymphoid tissues: a role for endothelium-expressed CC chemokine ligand 21 in autoimmune disease and lymphoid neogenesis. *J Immunol*. 2003; 170:4638–4648. [PubMed: 12707342]
16. Surawicz CM, Belic L. Rectal biopsy helps to distinguish acute self-limited colitis from idiopathic inflammatory bowel disease. *Gastroenterology*. 1984; 86:104–113. [PubMed: 6689653]
17. Kaiserling E. Newly-formed lymph nodes in the submucosa in chronic inflammatory bowel disease. *Lymphology*. 2001; 34:22–29. [PubMed: 11307662]
18. Carlsen HS, Baekkevold ES, Johansen FE, et al. B cell attracting chemokine 1 (CXCL13) and its receptor CXCR5 are expressed in normal and aberrant gut associated lymphoid tissue. *Gut*. 2002; 51:364–371. [PubMed: 12171958]
19. Fujimura Y, Kamoi R, Iida M. Pathogenesis of aphthoid ulcers in Crohn's disease: correlative findings by magnifying colonoscopy, electron microscopy, and immunohistochemistry. *Gut*. 1996; 38:724–732. [PubMed: 8707119]
20. Gullberg E, Soderholm JD. Peyer's patches and M cells as potential sites of the inflammatory onset in Crohn's disease. *Ann N Y Acad Sci*. 2006; 1072:218–232. [PubMed: 17057202]
21. Stopfer P, Obermeier F, Dunger N, et al. Blocking lymphotoxin-beta receptor activation diminishes inflammation via reduced mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression and leucocyte margination in chronic DSS-induced colitis. *Clin Exp Immunol*. 2004; 136:21–29. [PubMed: 15030510]
22. Lorenz RG, Chaplin DD, McDonald KG, et al. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J Immunol*. 2003; 170:5475–5482. [PubMed: 12759424]
23. Feagan BG, Greenberg GR, Wild G, et al. Treatment of active Crohn's disease with MLN0002, a humanized antibody to the alpha4-beta7 integrin. *Clin Gastroenterol Hepatol*. 2008; 6:1370–1377. [PubMed: 18829392]
24. Feagan BG, Greenberg GR, Wild G, et al. Treatment of ulcerative colitis with a humanized antibody to the alpha4beta7 integrin. *N Engl J Med*. 2005; 352:2499–2507. [PubMed: 15958805]
25. Rudick RA, Stuart WH, Calabresi PA, et al. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N Engl J Med*. 2006; 354:911–923. [PubMed: 16510745]
26. Polman CH, O'Connor PW, Havrdova E, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med*. 2006; 354:899–910. [PubMed: 16510744]
27. Sandborn WJ, Colombel JF, Enns R, et al. Natalizumab induction and maintenance therapy for Crohn's disease. *N Engl J Med*. 2005; 353:1912–1925. [PubMed: 16267322]
28. Targan SR, Feagan BG, Fedorak RN, et al. Natalizumab for the treatment of active Crohn's disease: results of the ENCORE Trial. *Gastroenterology*. 2007; 132:1672–1683. [PubMed: 17484865]
29. Van Assche G, Van Ranst M, Sciot R, et al. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N Engl J Med*. 2005; 353:362–368. [PubMed: 15947080]
30. Yousry TA, Major EO, Ryschkewitsch C, et al. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. *N Engl J Med*. 2006; 354:924–933. [PubMed: 16510746]

31. Barnes MJ, Powrie F. Regulatory T cells reinforce intestinal homeostasis. *Immunity*. 2009; 31:401–411. [PubMed: 19766083]
32. Grindebacke H, Stenstad H, Quiding-Jarbrink M, et al. Dynamic development of homing receptor expression and memory cell differentiation of infant CD4⁺CD25^{high} regulatory T cells. *J Immunol*. 2009; 183:4360–4370. [PubMed: 19734224]
33. Eberl G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat Rev Immunol*. 2005; 5:413–420. [PubMed: 15841100]
34. Lorenz RG, Newberry RD. Isolated lymphoid follicles can function as sites for induction of mucosal immune responses. *Ann N Y Acad Sci*. 2004; 1029:44–57. [PubMed: 15681742]
35. Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. *Nat Rev Immunol*. 2006; 6:205–217. [PubMed: 16498451]
36. Drayton DL, Liao S, Mounzer RH, et al. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol*. 2006; 7:344–353. [PubMed: 16550197]
37. Hjelmstrom P. Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. *J Leukoc Biol*. 2001; 69:331–339. [PubMed: 11261778]
38. Schroder AE, Greiner A, Seyfert C, et al. Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. *Proc Natl Acad Sci U S A*. 1996; 93:221–225. [PubMed: 8552609]
39. Sims GP, Shiono H, Willcox N, et al. Somatic hypermutation and selection of B cells in thymic germinal centers responding to acetylcholine receptor in myasthenia gravis. *J Immunol*. 2001; 167:1935–1944. [PubMed: 11489973]
40. Stott DI, Hiepe F, Hummel M, et al. Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjogren's syndrome. *J Clin Invest*. 1998; 102:938–946. [PubMed: 9727062]

FIGURE 1.

T-lymphocyte trafficking into the diffuse LP is independent of $\beta 7$ function in the uninfamed murine intestine. LP lymphocyte populations in the small intestine (A,B) and colon (C,D) from wildtype and $\beta 7^{-/-}$ mice were evaluated using flow cytometry. The CD19+ B-lymphocyte population was significantly decreased in the small intestine and colon in the absence of $\beta 7$, while the CD3+ T-lymphocyte population was unaffected (A–D). In the absence of $\beta 7$ the small intestine and colonic LP CD3+ T-lymphocyte population displayed a normal ratio of CD4+ to CD8+ T-lymphocytes and the expression of cell surface markers associated with activation (CD44, CD69, and CD62L) was unchanged (A–D). In addition, we observed no difference in the global population of Foxp-3+ CD4+ T-lymphocytes in the small intestinal LP in the absence of $\beta 7$ (A). $n = 3$ for each group. * $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

Foxp3+ T-lymphocytes, but not Foxp3- T-lymphocytes, are reduced in lymphoid aggregates in the absence of $\alpha 4\beta 7$. The presence of T-lymphocytes in the lymphoid aggregates was evaluated in wildtype mice, $\beta 7^{-/-}$ mice, and mice receiving anti- $\alpha 4\beta 7$ or rat IgG for 2 weeks using immunohistochemistry. The density of CD3+ T-lymphocytes in lymphoid aggregates of both small intestine and colon was not affected by the absence of $\beta 7$ or anti- $\alpha 4\beta 7$ blockade (A,B). However, the density of Foxp3+ T-lymphocytes in lymphoid aggregates in the small intestine of $\beta 7^{-/-}$ mice was significantly lower when compared to C57BL/6 mice (C). * $P < 0.05$. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Colonic lymphoid aggregates form at the peak of inflammation and persist after colitis resolution. To evaluate the timing of lymphoid aggregate development during acute injury and correlate this with disease progression, Balb/c mice were given 5% DSS for 7 days and followed for the development of weight loss, the formation of lymphoid aggregates, and colonic inflammation. DSS-induced colitis peaked at day 7 after DSS treatment demonstrated by maximal weight loss (A), histological score (B), and MPO activity (C). Inflammation began to regress at day 7 after removal of DSS. Coincident with the peak of inflammation, the numbers of lymphoid aggregates peaked at 7 days after DSS treatment and remained elevated after the removal of DSS despite the regression of clinical inflammation (D). $n = 3$ for each group.

FIGURE 4.

$\alpha 4\beta 7$ blockade prevents colonic lymphoid aggregate formation, but is ineffective at blocking inflammation in DSS-induced colitis. To evaluate the ability of $\alpha 4\beta 7$ blockade to alter the course of acute injury and alter the development of lymphoid aggregates during acute injury, Balb/c mice were given 5% DSS in drinking water for 7 days and 200 μ g anti- $\alpha 4\beta 7$ antibody or rat IgG by i.p. every other day for 14 days and evaluated for colonic inflammation and the number of lymphoid aggregates throughout the course of disease. Anti- $\alpha 4\beta 7$ blockade did not show an effect on DSS-induced colitis as demonstrated by no difference in weight loss (A), histological score (B,E,F), and MPO activity (C); however, anti- $\alpha 4\beta 7$ blockade dramatically decreased the number lymphoid aggregates and prevented new lymphoid aggregate formation during colitis (D). * $P < 0.05$. Scale bar = 200 μ m. $n = 3$ or more mice in each group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Colonic T-lymphocyte populations are unaffected by $\alpha 4\beta 7$ blockade during acute injury. To evaluate the effect of loss of $\alpha 4\beta 7$ function on intestinal lymphocyte populations during an acute injury, C57BL/6 mice and $\beta 7^{-/-}$ mice (A,B) and Balb/c mice given control antibodies or anti- $\alpha 4\beta 7$ blockade (C,D) were given 5% DSS in drinking water as described in Materials and Methods and evaluated for changes in the colonic LP populations by flow cytometry. In both instances we observed a decrease in the colonic CD19+ B-lymphocyte population (B,D), consistent with the observations of the loss of $\beta 7$ function in the uninflamed intestine. In both situations in which $\alpha 4\beta 7$ function was lost, CD3+ T-lymphocyte populations were unaltered and displayed no significant changes in the ratios of CD4+ to CD8+ T-lymphocytes or the expression of cell surface markers associated with activation (A–D). * $P < 0.05$, $n = 3$ for each group. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

FIGURE 6.

α 4 β 7 blockade reduces intestinal T-lymphocyte populations and decreases the production of inflammatory cyto-kines in a chronic model of T-cell-mediated colitis. To evaluate the effect of α 4 β 7 blockade in T-cell-mediated intestinal inflammation, Rag^{-/-} mice were injected with 3×10^5 CD4⁺ CD25⁻ splenocytes from C57BL/6 or β 7^{-/-} donors. Recipients of wildtype splenocytes were given anti- α 4 β 7 or rat IgG for 2 weeks starting 5 weeks after transfer as described in Materials and Methods. The colitis activity was determined by weight loss, histologic score, and MPO expression (A), the expression of proinflammatory cytokines (B), and the presence of intestinal T-lymphocyte populations (C) were measured 7 weeks after transfer. There was no statistically significant difference in weight loss or histology score between the groups, but recipients receiving α - α 4 β 7 antibodies or β 7^{-/-} splenocytes had significantly reduced expression of MPO (A). Recipients receiving α - α 4 β 7 antibodies or β 7^{-/-} splenocytes had significantly reduced expression of the proinflammatory cytokines TNF- α , IL-17, IFN- γ , and IL-6 (C). Unlike the observations in the intestine without inflammation or during acute injury, we observed a significant decrease in the population of intestinal T-lymphocytes in the diffuse LP, as determined by the expression of CD3, and in the lymphoid aggregates, as determined by immunohistochemistry (C). Consistent with the observations in the uninfamed intestine, we observed a decrease in the population of Fop3⁺ cells within the lymphoid aggregates of recipients of β 7^{-/-} lymphocytes (C). * $P < 0.05$. $n = 4$ for each group.