

L-Selectin, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ Integrins Participate in CD4⁺ T Cell Recruitment to Chronically Inflamed Small Intestine¹

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CD4⁺ T cells are essential for development and perpetuation of Crohn's disease, a chronic immune-mediated condition that affects primarily the small intestine. Using novel models of Crohn's disease-like ileitis (i.e., SAMP1/YitFc and CD4⁺ T cell transfer models), we have begun to understand the adhesive pathways that mediate lymphocyte trafficking to the chronically inflamed small bowel. Expansion of the CD4/ β_7 ⁺ population and increased mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression were observed within the intestinal lamina propria with disease progression. However, Ab blockade of the β_7 integrin, the $\alpha_4\beta_7$ heterodimer, MAdCAM-1, or L-selectin did not attenuate inflammation. Blockade of two pathways (L-selectin and MAdCAM-1 or α_4 integrins) was required to improve ileitis. Further analyses showed that $55 \pm 7\%$ of the mesenteric lymph node $\alpha_4\beta_7$ ⁺ CD4 expressed L-selectin. These L-selectin⁺ T cells were the main producers of TNF- α and the predominant ileitis-inducing subpopulation. Mechanistically, combined blockade of L-selectin and MAdCAM-1 depleted the intestinal lamina propria of CD4⁺ T cells that aberrantly coexpressed $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins, markedly decreasing local production of TNF- α and IFN- γ . Thus, pathogenic CD4⁺ T cells not only use the physiologic $\alpha_4\beta_7$ /MAdCAM-1 pathway, but alternatively engage $\alpha_4\beta_1$ and L-selectin to recirculate to the chronically inflamed small intestine. *The Journal of Immunology*, 2005, 174: 2343–2352.

Crohn's disease (CD),³ one of the chronic inflammatory bowel diseases (IBD), affects primarily the small intestine, particularly the terminal ileum, whereas ulcerative colitis (UC) affects exclusively the large intestine (1, 2). Like many chronic immune-mediated diseases, lymphocytes that produce Th1 cytokines (i.e., IFN- γ , TNF- α) play a crucial role (1–4). Pathogenic T cells acquire immunologic memory and recirculate, being therefore essential for the maintenance of chronic inflammatory processes. To recirculate, lymphocytes use a defined repertoire of adhesion molecules and chemokine receptors that recognize counterreceptors and chemokines on endothelial cells (5). Their regulated expression helps to initiate and terminate physiologic inflammatory responses. However, in immune-mediated diseases, their inappropriate expression contributes to dysregulated responses, perpetuation of chronic inflammation, and tissue injury.

Although prior studies have described expression and blockade of various adhesion molecules and chemokines in the inflamed colon (6–9), the mechanisms of CD4⁺ T cell trafficking specifi-

cally to the chronically inflamed small intestine are poorly understood. Recently, a spontaneous murine model of chronic ileitis (i.e., SAMP1/Yit) has been described (10). As in the human disease, these mice develop discontinuous, transmural chronic inflammation predominantly in the small intestine (10, 11). Immunologically, the ileitis is characterized by early overproduction of Th1 cytokines, with high levels of IFN- γ and TNF- α by 4 wk of age (11, 12). The disease is in part mediated by lymphocytes that intensely infiltrate the lamina propria (LP), display an activated phenotype, and have the ability to adoptively transfer disease to SCID mice (11–14). However, SAMP1/YitFc CD4⁺ T cells, different from CD45RB^{high} cells, predominantly induce ileitis, not colitis, demonstrating their inherent capacity to preferentially recirculate to the small intestine (11–14).

It is well established that β_7 integrins play a critical role in lymphocyte gut homing (5, 15, 16). Integrin $\alpha_4\beta_7$ binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), a member of the Ig superfamily of adhesion molecules (5, 15–17). MAdCAM-1 acts as a key intestinal addressin or address code molecule for intestinal homing (5, 15–18). In the adult human or mouse, MAdCAM-1 is constitutively expressed in postcapillary venules of intestinal LP, mesenteric lymph nodes (MLN), and Peyer patches (PP) high endothelial venules (HEV) (5, 16–18). MAdCAM-1 is also aberrantly expressed in the inflamed pancreas and up-regulated in chronically inflamed small and large intestines of patients with UC and CD (18, 19). The role of $\alpha_4\beta_7$ /MAdCAM-1 pathway as a disease-relevant homing mechanism is well established in colitic models (6–9), but not in models of ileitis.

Although $\alpha_4\beta_7$ -expressing cells migrate preferentially to gut, lymphocytes expressing the $\alpha_4\beta_1$ integrin (VLA-4) preferentially traffic to nonintestinal sites (5, 16, 17). Integrin $\alpha_4\beta_1$ binds to VCAM-1/fibronectin, expressed on endothelial and other cells at sites of inflammation (5, 9, 16). Expression of these integrins under physiologic conditions is reciprocal, and gut-homing $\alpha_4\beta_7$ ⁺ cells tend to be $\alpha_4\beta_1$ ^{negative} or low and vice versa (5, 16, 20). The role of the $\alpha_4\beta_1$ /VCAM-1/fibronectin pathway in IBD has received

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³ Abbreviations used in this paper: CD, Crohn's disease; CBA, cytometric bead array; CII, chronic inflammatory index; FSC, forward light scatter; HEV, high endothelial venule; IBD, inflammatory bowel disease; LP, lamina propria; LPL, LP lymphocyte; MAdCAM, mucosal addressin cell adhesion molecule; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; PP, Peyer patch; SSC, side light scatter; UC, ulcerative colitis.

only limited attention. VCAM-1 blockade significantly reduced inflammation in the adoptive transfer model of ileitis, only when combined with an Ab to ICAM-1, an endothelial adhesion molecule that serves as a ligand for β_2 integrins (14). Results from recent clinical trials with a humanized mAb to α_4 integrins have suggested that α_4 integrins may be relevant therapeutic targets in CD (21–23).

L-selectin is a C-type lectin that binds to carbohydrate moieties presented on glycoprotein scaffolds such as P-selectin glycoprotein ligand-1, CD34, or glycosylation-dependent cell adhesion molecule-1 (24–26). MAdCAM-1 can also serve as a ligand for L-selectin when properly glycosylated (27). L-selectin mediates much of the naive lymphocyte rolling seen in PP HEV of noninflamed mice (28) and is involved in lymphocyte recruitment to MLN and Peyer patches (16, 29). L-selectin engagement may facilitate subsequent binding through $\alpha_4\beta_7$ /MAdCAM-1 in the intestine or $\alpha_4\beta_1$ /VCAM-1/fibronectin elsewhere (5, 16). In chronic ileitis, the role of L-selectin has not been explored.

In the current study, we addressed the hypothesis that in chronic ileitis, pathogenic CD4⁺ T cells may be recruited through an $\alpha_4\beta_7$ /MAdCAM-1-independent pathway, not used in physiologic recruitment. To dissect these alternate pathways, we chose a three-pronged approach. First, we determined adhesion molecule expression of CD4⁺ T cells in SAMPI/YitFc mice before and after the onset of disease. Next, we asked which subsets produced relevant proinflammatory cytokines and induce ileitis. Based on these findings, we developed therapeutic strategies that greatly ameliorated ileitis by targeting the shared α_4 integrin moiety in $\alpha_4\beta_1$ and $\alpha_4\beta_7$ or L-selectin and MAdCAM-1.

Materials and Methods

Mice

SAMPI/YitFc mice were generated by brother-sister mating for over 30 generations from two breeding pairs provided by S. Matsumoto (Yakult Institute for Microbiological Research, Tokyo, Japan) and kept under specific pathogen-free conditions at the University of Virginia (11, 12). As most identifiable genes were AKR derived, age-matched AKR/J mice were used as controls (30). Fecal samples from SAMPI/YitFc mice were consistently negative for *Helicobacter hepaticus*, *Helicobacter bilis*, and other murine *Helicobacter* species, as well as for protozoa and helminths.

C3SnSmm.CB17-Prkdc^{scid}/J SCID mice (6–8 wk old) (The Jackson Laboratory) were purchased and housed at our vivarium in specific pathogen-free conditions for 1 wk before SAMPI/YitFc CD4⁺ T cell adoptive transfer and thereafter. Integrin $\beta_7^{-/-}$ mice were provided by N. Wagner (University of Cologne, Cologne, Germany) (15). All animal-handling procedures were approved by the institutional committee for animal use.

Tissue collection and histological analyses

Mice were anesthetized and euthanized at the times required by the experimental design. The MLN was identified at the confluence of the mesenteric vasculature and harvested. The distal ilea (10 cm) were resected, opened, rinsed of debris, oriented from distal to proximal, and pinned longitudinally in corkboard. Tissues were then fixed in 10% buffered Formalin or Bouin's, embedded in paraffin, and cut into 3- to 5- μ m sections. Resulting sections were stained with H&E. This tissue collection protocol reduced the histological score variability within experimental groups, compared with the swiss roll tissue configuration used in prior studies (14). Histological assessment of ileal inflammation was performed by a single pathologist in a blinded fashion, using a standardized semiquantitative scoring system, as described previously (14). Briefly, scores ranging from 0 (normal histology) to 3 (maximum severity of histologic changes) were used to evaluate histologic severity of: 1) active inflammation (infiltration with neutrophils), or 2) chronic inflammation (lymphocytes, plasma cells, and monocytes in the mucosa and submucosa). The final histologic index is determined by multiplying the histologic grade by a numerical assessment of involvement, which represents the percentage of diseased cross-sectional area (14).

Immunohistochemistry

SCID mice (before or 6 wk postadoptive T cell transfer) were injected with primary mAb (MECA-367 against MAdCAM-1) i.p. or i.v. MLN and ilea were harvested 30 min after mAb injection and snap frozen, and sections (5 μ m) were cut on a cryostat (Microm HM505N). Secondary staining was conducted with rabbit anti-rat Ab HRP (Vector Laboratories). Nonspecific binding was reduced using normal mouse serum (Sigma-Aldrich). Tissues from SCID mice after CD4⁺ T cell transfer, injected with rat IgG2a isotype Ab, served as controls.

Lymphocyte isolation

MLN and spleens were aseptically removed at the time of necropsy. Single cell suspensions were obtained by gently pressing the MLN or spleen against a 100- μ m cell strainer. Spleen RBCs were lysed by 15-min incubation in 1 \times ammonium chloride lysing reagent (BD Pharm Lyse; BD Pharmingen). LP lymphocyte (LPL) isolation was performed, as previously described (11), with the following modifications. Briefly, freshly resected terminal ilea were washed with cold PBS and cut into 2- to 5-mm pieces. Tissues were incubated in HBSS solution containing 15 mM HEPES and 1 mM EDTA at room temperature three times, until debris and epithelial cells were removed and the supernatant solution appeared clear. Subsequently, tissues were digested in RPMI 1640 with 10% FBS, 15 mM HEPES, 1% penicillin/streptomycin, and 300 U/ml collagenase VIII (Sigma-Aldrich), at 37°C for 60 min. For cell culture purposes, lymphocyte-enriched populations were isolated at the 40/70% interface of a discontinuous Percoll gradient.

T cell enrichment and separation

Enriched T cell fractions were obtained by incubation with anti-CD4-bound magnetic beads and sorted into discrete populations, using a magnetic cell-sorting system (Miltenyi Biotec), following manufacturer's instructions. For FACS sorting, cells were incubated with PE-labeled anti-L-selectin Abs (MEL-14) and separated using a FACSVantage SE Turbosort.

CD4⁺ T cell adoptive transfer

SAMPI/YitFc (30- to 40-wk-old) mice MLN were harvested and rendered into a single cell suspension, as above, followed by positive or negative selection for CD4, using magnetic beads. CD4⁺ lymphocytes (1×10^5 /mouse) were injected i.p. or i.v. into 8-wk-old MHC-matched SCID mice (The Jackson Laboratory). The 10^5 CD4⁺ T cell/mouse dose resulted in decreased mortality, compared with the 10^6 CD4⁺ T cell/mouse dose used on prior studies (14). Neither the selection protocols (positive or negative) nor the route of injection (i.p. or i.v.) altered the severity or time course of disease. Mice were housed in a barrier facility and fed irradiated, standard chow. After 5 wk, the adoptively transferred mice showed ileitis with moderate to severe LP leukocyte infiltration and architectural changes (villus and crypt distortion, goblet cell hyperplasia, and hypertrophy of the muscularis propria).

Flow cytometry

Fluorescently tagged mAbs reactive with α_4 (R1-2), α_E (M290), β_7 (M293), β_1 (HA2/5), and $\alpha_4\beta_7$ (DATK-32) integrins as well as against L-selectin (MEL-14) were incubated with lymphocytes in suspension, including anti-CD4 (GK1.5) for gating. Cells were fixed with 1% paraformaldehyde, and three- to four-color analyses were performed using the FACSCalibur system (BD Immunocytometry Systems). Further analysis was performed using FLOWJo software (Tree Star).

Cell culture

For cytokine secretion studies, lymphocytes were cultured in 96-well round-bottom plates at 10^6 cells/ml in complete medium (RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin) with or without anti-CD3e stimulation (10 μ g/ml; BD Pharmingen). Supernatants were collected after 48 h and stored at -70°C .

Cytometric bead array (CBA)

TNF- α and IFN- γ protein contents from cell culture supernatants were determined using the mouse Th1/Th2 CBA (BD Biosciences), following the manufacturer's instructions. Samples were analyzed by multicolor analysis on FACSCalibur (BD Biosciences), using the BD CBA software.

Therapeutic interventions

mAbs (200 μ g each) against: 1) a combinatorial epitope on $\alpha_4\beta_7$ (DATK-32, rat IgG2a); 2) β_7 integrin (FIB-504, rat IgG2a); 3) MAdCAM-1 (MECA-367, rat IgG2a); 4) L-selectin (MEL-14, rat IgG2a) or irrelevant

isotype control (rat IgG2a) were injected i.p. every other day (Q.O.D.) for 3 days, 5 wk after adoptive transfer. Mice were sacrificed 16–18 h after the last dose. Hybridomas were obtained from American Type Culture Collection. Abs were produced and purified from hybridoma supernatants at the University of Virginia Biomolecular Core Facility.

Statistics

Statistical analyses for flow cytometry, CBA, and inflammatory indices were performed using the two-tailed unpaired Student's *t* test or one-way ANOVA for multiple comparisons using the Student-Newman-Keuls method (SigmaStat; SPSS). Data were expressed as mean and SEM. Statistical significance was set at $p < 0.05$.

Results

Expansion of integrin-expressing CD4⁺ T cell populations with progression of disease

SAMP1/YitFc mice, like patients with CD, spontaneously develop chronic inflammation in their terminal ilea (chronic ileitis), a location distinct from all previously described models of IBD, which develop colitis (31, 32). The disease progresses from 4 to 40 wk of age, with no histologic evidence of disease by 4 wk and maximum severity by 40 wk (Fig. 1A).

Detailed analysis of adhesion molecule expression within the ileal LP, MLN, and spleen in function of disease progression may shed light on the adhesive pathways used by lymphocytes while trafficking to these compartments. Expression of integrins β_7 , β_1 , and α_4 was analyzed by flow cytometry in SAMP1/YitFc mice before (4 wk) and after the development of chronic ileitis (40 wk), compared with noninflamed age-matched AKR controls. Within the ileal LP, the inflammatory infiltrate increased markedly from 4 to 40 wk of age. The villous and crypt architecture were almost completely replaced by a dense inflammatory infiltrate composed mainly of lymphocytes and granulocytes (Fig. 1A). Lymphocytic infiltration, as reflected by chronic inflammatory indices (CII), increased from 4 wk (CII = 0.5 ± 0.2) to 40 wk (CII = 3.5 ± 0.7) (data not shown). Accordingly, the percentage of CD4⁺ T cells within the ileal LP increased 2-fold by 40 wk ($9 \pm 2\%$ vs $21 \pm 4\%$, $p < 0.01$) (Fig. 1B, left). Over 95% of CD4 expressed β_7 integrin by 4 wk (Fig. 1B, middle), and of those, the β_7^{high} population (mean fluorescence intensity (MFI) $> 10^2$) increased from $14 \pm 3\%$ to $21 \pm 5\%$ ($p < 0.05$) from 4 to 40 wk in SAMP1/YitFc mice (Fig. 1B, middle), but not in age-matched noninflamed AKR controls (Fig. 1B, right). Most LP CD4 from 40-wk-old SAMP1/YitFc mice had an activated phenotype ($89 \pm 3\%$ CD44^{high}, $85 \pm 3\%$ CD69⁺, $70 \pm 8\%$ CD45Rb^{low}; data not shown).

The cellularity of the MLN (a relevant trafficking compartment in physiologic and chronic inflammation) increased ~10-fold (5 ± 3 to 50 ± 10 million cells/mouse, $p < 0.01$) from 4 to 40 wk of age (data not shown) (12). Although the CD4⁺ T cell percentage did not increase from 4 to 40 wk of age ($50 \pm 12\%$ vs $40 \pm 8\%$, NS) (Fig. 1C, left), once corrected for MLN cellularity, there was a ~8-fold increase in total CD4⁺ T cells (2.5 ± 0.4 to 20 ± 0.8 million cells, $p < 0.01$) (data not shown), of which $>95\%$ expressed β_7 integrin (Fig. 1C, middle). CD4⁺ T cells from β_7 integrin-deficient mice ($\beta_7^{-/-}$), which served as controls, showed MFI $< 10^1$ (Fig. 1C, shaded overlaid histogram). Consistent, but small increases in the β_7^{high} cells were observed within the MLN, which contains cells originating from intestine and other sites (Fig. 1C, middle). No age-related changes were seen in noninflamed AKR control mice (Fig. 1C, right).

To evaluate whether the peripheral compartments were affected by disease progression, a similar analysis was performed on splenocytes (Fig. 1, D–G). Splenic cellularity increased from 42.8 ± 9 to 102 ± 7 million cells (data not shown) with concomitant increased CD4⁺ T cell fraction ($17 \pm 5\%$ vs $28 \pm 7\%$, $p <$

0.01) from 4 to 40 wk of age (Fig. 1D, left). However, the overall integrin β_7 expression ($\sim 95\%$ at 4 wk) decreased by 40 wk of age ($51.5\% \pm 5$), concomitant with the appearance of a $\beta_7^{\text{negative}}$ (MFI $< 10^1$) population (Fig. 1D, middle). No significant differences were noted in noninflamed age-matched AKR mice (Fig. 1D, left). To further characterize the new $\beta_7^{\text{negative}}$ splenocyte population, we determined whether they expressed β_1 and α_4 integrins, compared with age-matched AKR mice (Fig. 1, E and F). Marked expansion of the β_1^{high} and α_4^{high} (MFI $> 10^2$) population was observed in SAMP1/YitFc mice (Fig. 1, E and F, red boxes) from 4 to 40 wk of age. β_1 integrin may associate with at least 10 other α integrin subunits (i.e., α_{1-3} , α_{5-11} , α_v) (33); however, α_4 integrin dimerizes only with the β_7 or β_1 subunits. An increase in α_4 in the setting of a decrease in $\beta_7^+/CD4^+$ splenocytes (Fig. 1D, middle) denotes an increase in $\alpha_4\beta_1$ expression (VLA-4). Further analysis of both β_1^{high} and α_4^{high} populations (Fig. 1, E and F, offsets) confirmed that the majority of the expanded α_4 -positive cells (red overlaid histogram) were found within the increased β_1^{high} population (Fig. 1E, red box). The reverse analysis also localized the majority of the β_1 -expressing cells (red overlaid histogram) within the α_4^{high} population (Fig. 1F, red box). Analyses of α_4 expression from splenocytes gated on intensity of β_7 expression showed that cells within all three gates (L, M, and H) expressed integrin α_4 (Fig. 1G). Two peaks of expression (high and low) were seen on the β_7^{low} cells (β_7L), whereas the β_7^{int} (β_7M) were predominantly α_4^{high} . Those that were β_7^{high} had variable intensity of α_4 expression, but were predominantly α_4^{low} (β_7H). The presence of two distinct peaks of α_4 expression is consistent with a population expressing only one of the two α_4 integrins (α_4^{low}), while the α_4^{high} most likely coexpresses both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins.

Increased MAdCAM-1 expression in chronic ileitis

SAMP1/YitFc CD4⁺ T cells compared with cells from AKR mice produce significantly higher levels of TNF- α and IFN- γ and adoptively transfer disease to MHC-matched SCID recipients (11–13). MAdCAM-1 is induced by TNF- α (34), and in SAMP1/YitFc mice MAdCAM-1 expression increased with worsening disease from 4 to 40 wk of age (our unpublished results). To determine whether SAMP1/YitFc CD4⁺ T cells induce MAdCAM-1 expression over constitutive levels in SCID endothelium, immunohistochemical studies were performed before and after T cell transfer (Fig. 2). MAdCAM-1 expression increased in MLN HEV in SCID mice after T cell transfer (Fig. 2B), compared with mice before transfer (Fig. 2A). Within the intestinal LP, numerous microvessels (red arrowhead) showed MAdCAM-1 expression after T cell transfer (Fig. 2D), compared with basal expression in mice before adoptive transfer (Fig. 2C). Similarly, increased expression of the $\alpha_4\beta_1$ ligand VCAM-1 was previously observed in SCID mice intestinal microvessels after adoptive transfer of SAMP1/YitFc CD4⁺ T cells (14).

Blockade of the $\alpha_4\beta_7$ /MAdCAM-1 pathway did not attenuate chronic ileitis

Expansion of the β_7 integrin-expressing CD4⁺ T cell population and of MAdCAM-1 after T cell transfer supports a role for these molecules in the pathogenesis of chronic ileitis. To specifically address this hypothesis, SCID mice with established chronic ileitis were treated with anti-isotype Abs (I) or with neutralizing mAbs against: the β_7 integrin subunit (FIB-504), $\alpha_4\beta_7$ integrin (DATK-32), MAdCAM-1 (M) (MECA-367), or L-selectin (L) (MEL-14). Intriguingly, these interventions were ineffective in reducing the severity of acute or chronic infiltrates, compared with isotype Ab-treated controls (I) (Fig. 3, A and B). Saturation of binding sites at

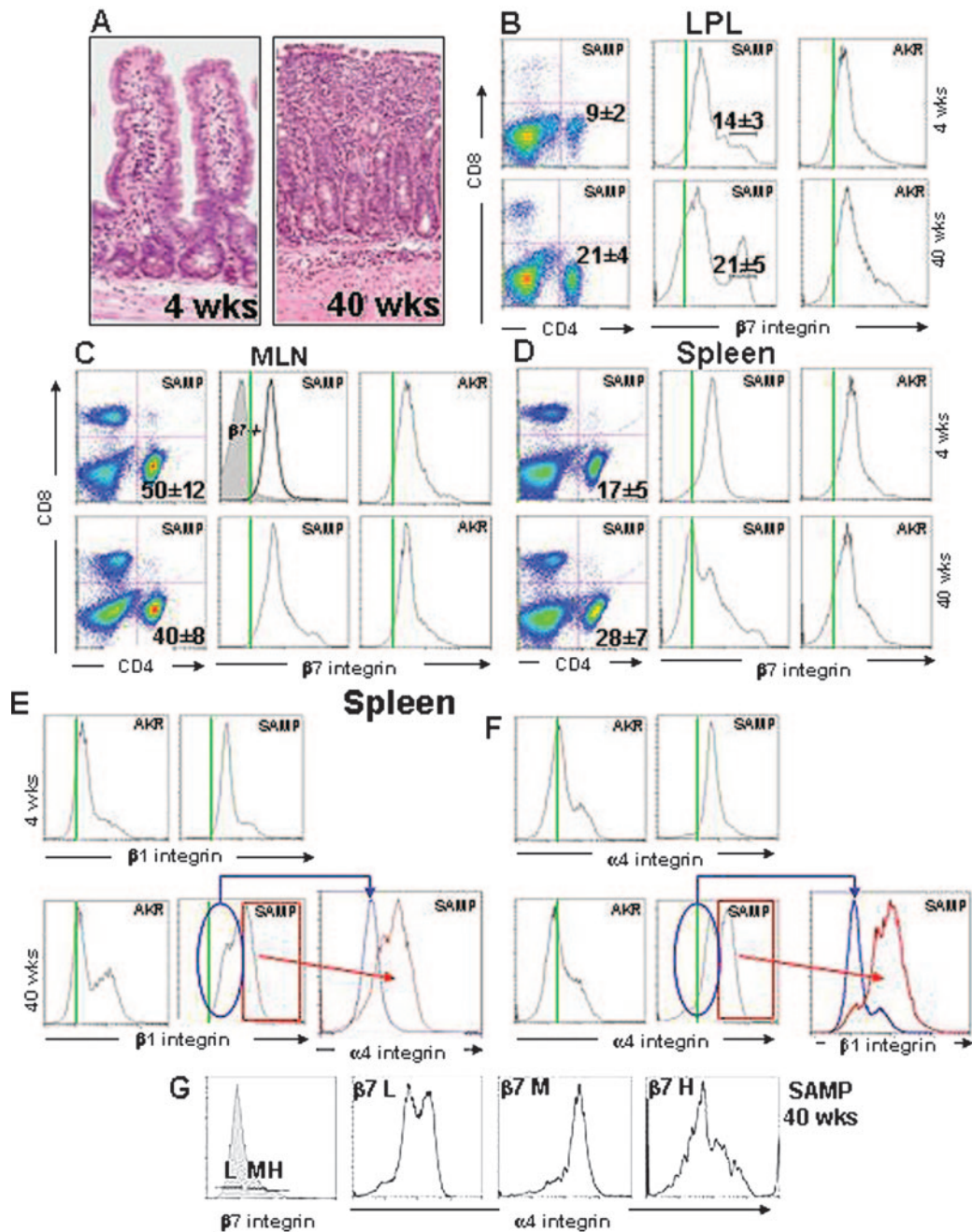


FIGURE 1. Integrin expression in SAMPI/YitFc (SAMP) $CD4^+$ T cells within lymphoid compartments before and after the development of chronic ileitis compared with uninflamed age-matched AKR controls (AKR). Integrin expression was analyzed by flow cytometry. *B–D, Left panels*, Gated on forward light scatter (FSC) and side light scatter (SSC). Histograms were additionally gated on CD4. The green vertical line throughout represents 95% of isotype MFI. *A*, Representative micrographs of disease progression from 4 to 40 wk of age (H&E, $\times 20$). *B–D, Left panels*, CD4/CD8 T cell distribution from indicated compartments before and after the development of chronic ileitis. *B–G, Middle and right panels*, $CD4^+$ T cell expression of β_7 , β_1 , and α_4 integrins within the indicated compartments in SAMPI/YitFc and AKR mice, as indicated. *E and F, Offsets*, Coexpression of α_4 integrin within the β_1^{low} (blue oval and histogram) and β_1^{high} (red square and histogram) cells (*E*), and of β_1 integrins within the α_4^{low} and α_4^{high} $CD4^+$ splenocytes of SAMPI/YitFc mice (*F*). *G*, Splenocyte α_4 integrin expression gated on intensity of β_7 expression. Representative data obtained from three to four mice at each time point run in duplicate or triplicate.

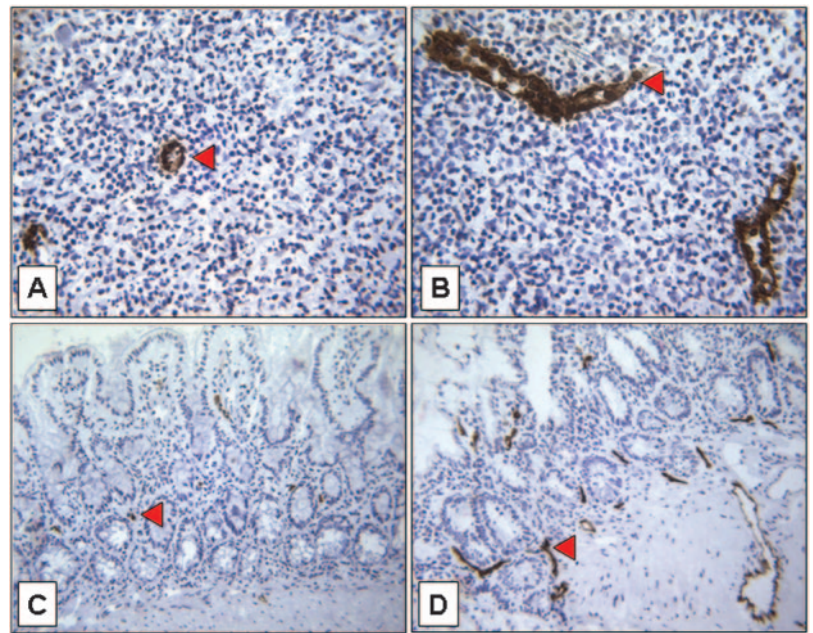
our dosing interval was confirmed by flow cytometry performed on MLN cells obtained from mice treated with DATK-32 or FIB-504, 48 h before sampling (data not shown). MAdCAM-1 blockade was tested at increasing doses (200–1000 $\mu\text{g}/\text{mouse}$), as well as per different treatment schedules (Q.O.D. at week 5 posttransfer or biweekly starting at the time of transfer for 6 wk) (data not shown). MAdCAM-1 blockade failed to attenuate ileitis at all doses and treatment schedules tested. The failure of these therapeutic interventions was puzzling, as previous studies had shown that these

Abs attenuated colonic inflammation in animal models (6–9). It was therefore likely that $\alpha_4\beta_7/\text{MAdCAM-1}$ -independent pathways were involved in trafficking to the small intestine.

Combined blockade of L-selectin and MAdCAM-1 attenuated chronic ileitis

L-selectin ligands have been identified in multiple chronic inflammatory animal models and human diseases, including CD and UC (25, 35–41). We therefore simultaneously targeted L-selectin and

FIGURE 2. MLN and ileal LP MAdCAM-1 expression in SCID mice before and after CD4⁺ T cell transfer. Anti-MAdCAM-1 Ab was administered before (A and C) and 6 wk after (B and D) transfer. Immunohistochemistry was performed on frozen sections, as per *Materials and Methods*. MAdCAM-1 expression increased in both MLN HEV (B) ($\times 20$) and intestinal LP vessels (arrowheads) (D) ($\times 10$) with development of chronic ileitis compared with SCID tissues before adoptive transfer (A and C). Representative micrographs from tissues obtained from three mice before and after transfer.



MAdCAM-1, using neutralizing Abs MEL-14 and MECA-367 combined (M + L). Significant amelioration of acute and chronic inflammatory infiltrates was observed (Fig. 3, A and B), along with partial restoration of the villous and crypt architectures (Fig. 3, C and D).

Populations of L-selectin-expressing CD4⁺ T cells coexpress $\alpha_4\beta_7$, produce TNF- α , and are predominantly responsible for induction of ileitis

In an attempt to understand the role of L-selectin in inflammatory recruitment to the small intestine, further analyses of relevant populations were performed in the context of disease progression in MLN compared with spleen. Splenic L-selectin-high cells decreased from 4 to 40 wk ($85 \pm 7\%$ vs $30 \pm 8\%$) (Fig. 4, A and B,

bottom). In contrast, a significantly higher percentage of MLN CD4 continued to express L-selectin by 40 wk, compared with spleen ($55 \pm 7\%$ vs $30 \pm 8\%$, $p < 0.01$) (Fig. 4B). L-selectin was preferentially expressed by a subpopulation of MLN and splenic CD4⁺ T cells that were β_7^{low} (Fig. 4B) and coexpressed α_4 integrin (Fig. 4C), but not α_E integrin (Fig. 4D). To explore whether MLN L-selectin^{positive} cells were strictly of naive phenotype, we determined the expression of surface markers consistent with an activated state. Within the CD4/L-selectin^{positive} population, $10 \pm 3\%$ expressed CD69, $9 \pm 2\%$ were CD45RB^{low}, $12 \pm 3\%$ were CD44^{high}, and $9 \pm 3\%$ expressed CD25 (Fig. 4E). Thus, L-selectin expression does not preclude an activated state.

To determine the contribution of L-selectin-expressing CD4⁺ T cells to the overall cytokine profile in this model, CD4⁺ T cells were sorted based on their L-selectin expression into L-selectin^{positive} or L-selectin^{negative} subpopulations and cultured for 48 h under anti-CD3 stimulation. IFN- γ was produced predominantly by L-selectin^{negative} CD4⁺ T cells, whereas TNF- α (a pivotal cytokine in human and murine chronic ileitis) (42–44) was produced predominantly by L-selectin-expressing CD4⁺ T cells (Fig. 4F). L-selectin^{positive} CD4 did not produce IL-4 or IL-5 (data not shown). To probe the functional role of L-selectin-expressing cells on the induction of chronic ileitis, sorted cells (i.e., CD4⁺, CD4⁺/L-selectin^{positive} (L⁺) or CD4⁺/L-selectin^{negative} (L⁻)) were adoptively transferred into SCID mice, and the severity of ileitis was induced by subpopulations evaluated, as described (14). Active inflammatory indices were 6 ± 0.8 , 4.5 ± 0.9 , and 2.5 ± 0.8 , whereas chronic inflammatory indices were 5.6 ± 0.4 , 5.5 ± 0.5 , and 2.25 ± 0.4 , respectively. The severity of the disease induced by unfractionated CD4 and that induced by L-selectin-expressing cells was not significantly different, whereas indices were significantly decreased in mice transferred with cells that did not express L-selectin ($p < 0.05$) (Fig. 4G). These data suggest that the disease-inducing population is found within the CD4 cells that express L-selectin.

To probe the mechanisms of action of combined L-selectin/MAdCAM-1 blockade, we characterized the LP mononuclear cells obtained from mice treated with isotype or combination anti-MAdCAM/L-selectin Abs using flow cytometry and cytokine

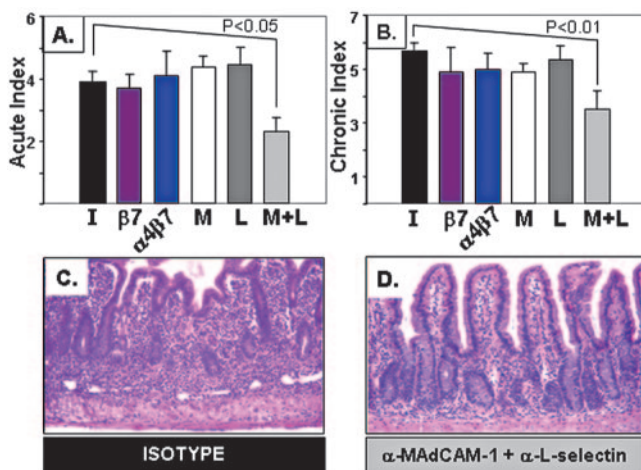
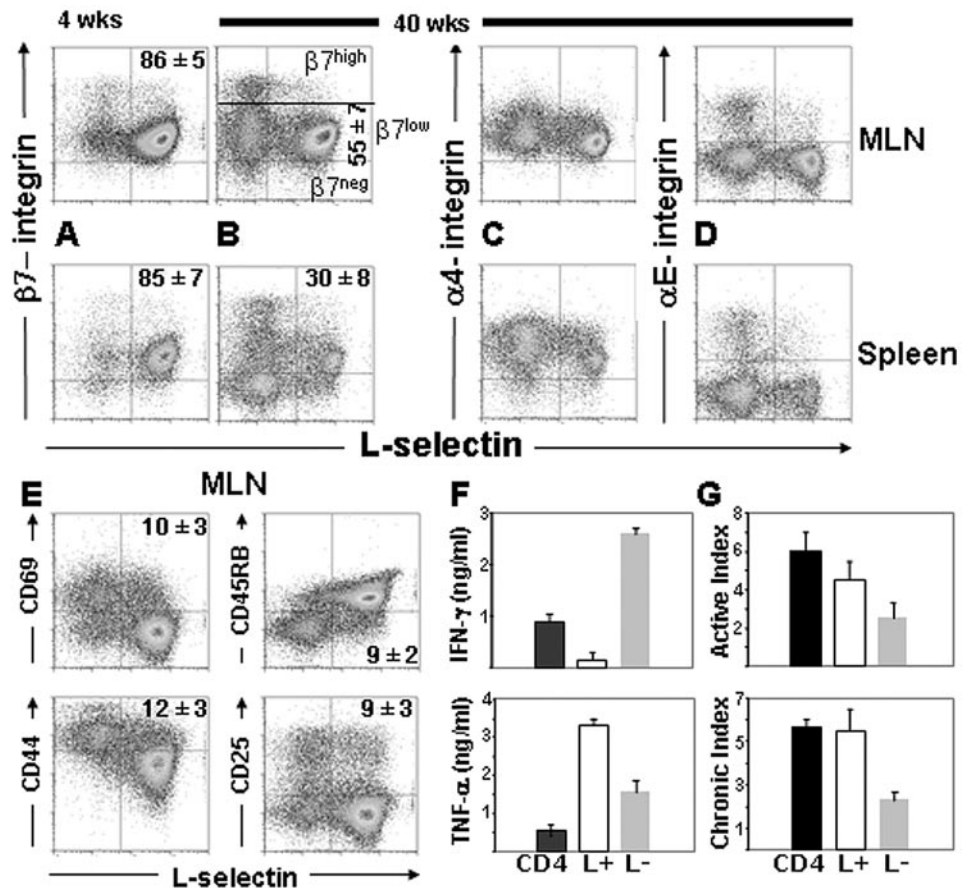


FIGURE 3. Effect of Ab blockade on inflammatory indices and histological features in mice with chronic ileitis. A and B, Inflamed SCID mice were injected with isotype mAb (I, $n = 19$), anti- β_7 integrin (β_7 , $n = 7$), anti- $\alpha_4\beta_7$ ($\alpha_4\beta_7$, $n = 7$), anti-MAdCAM-1 (M, $n = 16$), anti-L-selectin (L, $n = 12$), or both anti-MAdCAM-1 and anti-L-selectin Abs (M + L) ($n = 18$), as described in *Materials and Methods* (mean \pm SEM from six independent experiments). C and D, Effect of indicated treatment on intestinal architecture (representative micrographs, H&E, $\times 10$).

FIGURE 4. L-selectin expression and integrin coexpression of CD4⁺ T cells from MLN or spleen of SAMPI/YitFc mice with or without ileitis. A–D, L-selectin, β_7 , α_4 , and α_E integrin expression was analyzed in CD4 from mice with ileitis (40 wk old) compared with young (4 wk old) control SAMPI/YitFc mice, without ileitis. Cells gated on FSC, SSC, and CD4 were analyzed by flow cytometry. Representative data obtained from three to four SAMPI/YitFc mice at each time point run in duplicate. E, Activation marker expression by L-selectin^{positive} cells. F, IFN- γ and TNF- α production of unfractionated CD4 (CD4), L-selectin^{positive} (L⁺), or L-selectin^{negative} (L⁻) CD4⁺ T cells. Cells were sorted by FACS and cultured, as described. Cytokine concentrations (mean \pm SEM) were determined from SAMPI/YitFc-pooled cells ($n = 6$) run in triplicate, as per *Materials and Methods*. G, Sorted populations were adoptively transferred to SCID recipients, and the severity of ileitis was evaluated, as described (mean \pm SEM, $n = 4$ /group).



CBA. LP mononuclear cell yields from mice treated with combination therapy (MEL-14 + MECA-367) were decreased \sim 7-fold (1.5×10^6 vs 10×10^6 cells/mouse) when compared with those from isotype-treated Abs (data not shown). Fluorescence intensity for β_7 integrin in LP CD4⁺ T cells showed a bimodal distribution with a peak at low intensity ($>10^1$ MFI $< 10^2$) (β_7^{low}) and a high peak (MFI $> 10^2$) (β_7^{high}) (Fig. 5, A and B). The $\beta_7^{\text{negative}}$ population was defined as those with MFI $< 10^1$, which corresponds to the MFI of $\beta_7^{-/-}$ cells (Fig. 1C, shaded histogram) and the PE-labeled isotype Ab control (rat IgG2a). Simultaneous MAdCAM-1/L-selectin blockade (dotted line) reduced only the first peak of expression (β_7^{low}), whereas the β_7^{high} population was unaffected (Fig. 5A, left). CD4 cells expressing high levels of α_4 also decreased, whereas those expressing integrin α_E were unaffected in treated mice compared with controls (Fig. 5A, middle and right). To establish whether the decreased β_7^{low} population coexpressed α_4 or α_E integrin, α_E integrin expression was determined in the β_7^{low} and β_7^{high} populations (Fig. 5B). Integrin α_E was expressed predominantly by the β_7^{high} population (unaffected by combined therapy). As β_7 dimerizes only with α_4 or α_E integrins, our results demonstrate that the effect of the combined therapy affected the β_7^{low} ($\alpha_4\beta_7^+$) population, without detectable effect on $\alpha_E\beta_7^+$ population (Fig. 5A). Furthermore, IFN- γ and TNF- α production were markedly reduced in MLN (Fig. 5C) and intestinal LPL (Fig. 5D) from treated mice (\square), compared with those treated with isotype control Ab (\blacksquare). Therefore, most of the cells responsible for IFN- γ and TNF- α production were within the $\alpha_4\beta_7^+$ population, depleted by combined therapy.

Pathogenic T cells coexpress α_4 integrins

Lymphocytes use L-selectin to roll along the endothelium (5, 16), subsequently engaging integrins that mediate arrest (33, 45, 46).

Activated integrin $\alpha_4\beta_7$ supports lymphocyte arrest in the absence of L-selectin (45), but as the bonds established by selectins are too short-lived to arrest cells at the vessel wall, other integrin(s) must compensate for $\alpha_4\beta_7$, when it is functionally blocked (mAb) or genetically absent ($\beta_7^{-/-}$ mice). We then investigated whether other integrins were coexpressed by $\alpha_4\beta_7^+$ T cells in SCID mice with chronic ileitis. Thirty five \pm 8% of the CD4⁺ T cells from noninflamed AKR mice coexpressed β_1 and β_7 integrins, whereas 80 \pm 5% of those from mice with chronic ileitis were double positives (Fig. 6A). To further clarify whether the $\alpha_4\beta_7$ -positive population (β_7^{low}) coexpressed β_1 integrin, cells were reacted with a mAb that recognizes a combinatorial epitope on $\alpha_4\beta_7$ integrin (clone DATK32). A total of 88 \pm 4% of CD4/ $\alpha_4\beta_7$ positives coexpressed integrin β_1 (Fig. 6A, right). Expression of β_1 integrin was not limited to the $\alpha_4\beta_7$ -positive T cells, as 92 \pm 2% of those expressing integrin α_E also expressed β_1 integrin (data not shown). Integrin polarization, believed to determine intestinal or peripheral homing, based on the reciprocal expression of α_4 integrins ($\alpha_4\beta_7$: intestine/ $\alpha_4\beta_1$: periphery), was not observed in the chronically inflamed ileal LP (5, 16, 20, 47). We therefore hypothesized that lymphocytes may roll through L-selectin, subsequently engaging $\alpha_4\beta_7$ or alternatively $\alpha_4\beta_1$ integrins, which in the chronically inflamed state were coexpressed. We then targeted both pathways using mAb clone PS-2, an Ab that blocks the shared α_4 integrin moiety and therefore blocks both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins. Treatment of inflamed mice with PS-2 resulted in significant reduction of acute and chronic inflammatory indices (Fig. 6B), compared with mice treated with isotype Abs. The more pronounced effect of α_4 blockade on the active index (neutrophilic infiltrates) is in keeping with its widespread expression. Additional L-selectin blockade

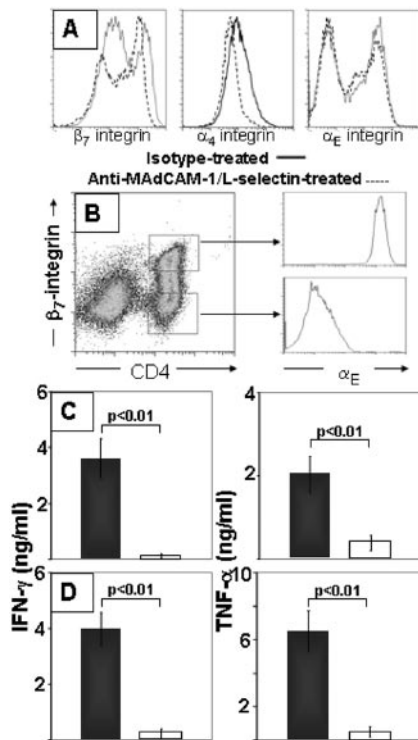


FIGURE 5. Effect of L-selectin + MAdCAM-1 blockade on LP β_7^+ , α_4^+ , α_E^+ CD4 T cell populations and on MLN/LPL cytokine production. LPL were obtained from mice treated with combination (dotted line) ($n = 9$) or isotype mAb (solid line) ($n = 10$); gated on FSC, SSC, and CD4; and analyzed by flow cytometry. **A**, CD4⁺/integrin β_7^{low} and α_4^{high} decreased, whereas neither the β_7^{high} nor the $\alpha_E^{\text{positive}}$ was affected. **B**, Analysis of α_E^+ integrin expression within β_7^{low} or β_7^{high} populations demonstrated that most $\alpha_E^{\text{positive}}$ cells were found within the β_7^{high} population. Representative histograms and dot plots of pooled cells from three independent experiments. **C** and **D**, Effect of combined L-selectin and MAdCAM-1 blockade on Th1 cytokine production by MLN and LPL. IFN- γ and TNF- α production in MLN (**C**) and LPL (**D**) from mice receiving anti-MAdCAM-1/L-selectin Abs (□), compared with mice treated with isotype Abs (■). Cells were harvested and cultured, as described. Cytokine concentrations (mean \pm SEM) from experimental mice-pooled cells ($n = 6/\text{group}$), run in triplicate, were determined as per *Materials and Methods*.

did not further enhance the effect of single anti- α_4 treatment, consistent with the hypothesis that the added benefit of L-selectin blockade may be due to interference with the $\alpha_4\beta_1$ -dependent pathway (Fig. 6B).

Discussion

The pre-eminent role of the $\alpha_4\beta_7/\text{MAdCAM-1}$ pathway in physiological lymphocyte recruitment to the gut and in colitic models is well established in the literature (5–9, 15–17). However, the adhesive pathways that lead to induction and maintenance of chronic small intestinal inflammation are not well defined. SAMP1/YitFc mice, like patients with CD, develop dysregulated chronic inflammation, uniquely localized to the small intestine and induced by proinflammatory cytokine overproduction that begins by 4 wk of age and lasts throughout their life span (10–13). We hypothesized that dysregulated cytokine production induces increased expression of constitutive adhesion molecules, as well as aberrant expression of molecules not used physiologically for gut homing. Through flow cytometric analysis of lymphoid compartments and targeted blockade of molecules expressed by disease-inducing CD4⁺ T cells, we have begun to understand the adhesive pathways responsible for trafficking to the chronically inflamed

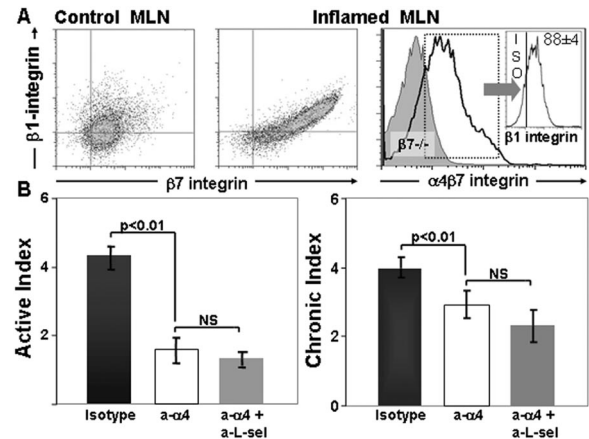


FIGURE 6. MLN CD4⁺ T cell integrin coexpression and blockade of α_4 integrins in chronic ileitis. **A**, CD4⁺ T cell integrin expression was analyzed in uninfamed AKR and SCID mice with chronic ileitis using flow cytometry. **B**, $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins were simultaneously blocked through their shared α_4 integrin moiety (clone PS-2) anti- α_4 (a- α_4) ($n = 8$), isotype mAb (isotype) ($n = 12$), or combined L-selectin/ α_4 blockade (a- α_4 + a-L-sel) ($n = 13$), as described. Severity of ileitis was evaluated, as per *Materials and Methods* (mean \pm SEM from two independent experiments).

small intestine. Our findings in ileitis, compared with prior reports in noninflamed mice and models of colitis (5, 6–9, 45), have begun to uncover differences that may be attributable to the chronic nature of SAMP1/YitFc ileitis, as well as to its distinct small intestinal localization.

Expansion of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ CD4⁺ T cell populations was observed within intestinal (LP and MLN) and peripheral compartments (spleen), with development of chronic ileitis, but not in age-matched control mice, consistent with induction by proinflammatory cytokines. Expansion of not only $\alpha_4\beta_7$ -expressing cells within intestinal effector sites, but of $\alpha_4\beta_1^+$ cells in the periphery suggests that immune dysregulation in these mice is not restricted to the intestine, but systemic in nature. In fact, SAMP1/YitFc mice develop systemic manifestations (e.g., cutaneous, ocular) reminiscent of the dermatologic manifestations (i.e., pyoderma gangrenosum, erythema nodosum) and uveitis associated with CD (12). The observed enrichment for β_1 -expressing cells in spleen and for β_7 -expressing cells in intestine was consistent with the hypothesis that $\alpha_4\beta_1$ integrin regulates peripheral T cell trafficking, whereas $\alpha_4\beta_7$ regulates gut homing. Our subsequent data demonstrate that the discrete skin:gut demarcations seen in physiologic lymphocyte recruitment are less defined in chronic inflammation.

Most of the expanded β_7^{high} population in SAMP1/YitFc mice coexpressed integrin α_E . Recent data support a regulatory role for $\alpha_E\beta_7^+$ CD4 T cells in intestinal inflammation (48). In SAMP1/YitFc mice, ileitis progressively worsens between 4 and 40 wk, but subsequently stabilizes or improves (12). It is possible that expansion of the β_7^{high} regulatory population (i.e., $\alpha_E\beta_7^+$) within the LP may be in part responsible for stabilization of disease severity after 40 wk (12). Increased $\alpha_E\beta_7^+$ regulatory cells may counteract the proinflammatory effect of pathogenic populations in the LP, allowing SAMP1/YitFc mice to live their full life span (60–70 wk).

Targeted blockade of the $\alpha_4\beta_7/\text{MAdCAM-1}$ pathway, using mAb against the β_7 integrin subunit, the $\alpha_4\beta_7$ heterodimer or MAdCAM-1, in increasing doses and with adequate saturation of binding sites, was ineffective in ameliorating ileitis. This was particularly intriguing given the strong body of evidence that supports

a pivotal role for the $\alpha_4\beta_7$ /MAdCAM-1 pathway in intestinal trafficking. Using short-term lymphocyte-homing assays in noninflamed mice, Hamann et al. (49) decreased recruitment into PP or intestine down to $\sim 25\%$ of control by blocking the α_4 subunit (PS2), the $\alpha_4\beta_7$ integrin (DATK-32), or the β_7 subunit (FIB-504). Similar results were observed in inflammatory recruitment to the large intestine using colitic mouse models. CD45RB^{high}-induced colitis was equally attenuated by treatment with either FIB-504 or MECA-367 alone (6). Likewise, $\alpha_1\beta_1$ integrin or MAdCAM-1 blockade attenuated dextran sulfate sodium-induced colitis (7, 50). Blockade of α_4 integrins attenuated spontaneous colitis in nonhuman primates (9). Decreased lymphocyte adhesion was also observed in intravital microscopy studies of CD45RB^{high}-transferred mice after treatment with MECA-367 (51).

In our studies, targeting any single component of the $\alpha_4\beta_7$ /MAdCAM-1 pathway was ineffective, whereas combined blockade of L-selectin and MAdCAM-1 significantly ameliorated ileitis. Mechanistically, the effect on disease severity could be due to decreased naive T cell recruitment into mucosal inductive sites (i.e., MLN, PP), as only combined deficiency of both β_7 and L-selectin depleted the cellularity of MLN (52). Alternatively, L-selectin could participate in effector T cell recruitment directly into the LP. This possibility is not often considered, as most lymphocytes within the intestinal LP lack L-selectin (53). Our analysis of the LP populations affected by the combined treatment supports the latter hypothesis. We propose that the disease-inducing population coexpresses L-selectin, $\alpha_4\beta_7$, and $\alpha_4\beta_1$ integrins. Upon its arrival and transmigration into the effector site (intestinal LP), L-selectin is shed. In fact, the intestinal LP is not the only effector site in which most lymphocytes lack L-selectin, as those within the lung in pneumonia, in allograft rejection, and sites of viral infection lack L-selectin as well (54–57). In this context, it is easier to understand the role of L-selectin ligands described at sites of chronic inflammation in multiple human diseases and animal models, where they support L-selectin-mediated recruitment of naive and subsets of pathogenic effector/memory T cells (25, 26, 35–41).

The natural history of human IBD suggests that trafficking to small and large intestines proceeds through distinct pathways. Although UC involves strictly the large intestine, CD involves predominantly the terminal ileum (1–3). To maintain these strict demarcations, the recirculating lymphocyte pool must possess a repertoire of adhesion molecules and chemokine receptors that allows distinction between small and large intestine. Our data suggest that L-selectin may be part of the address code for homing to chronically inflamed small bowel. Further expression studies showed that while the majority of SAMP1/YitFc peripheral CD4⁺ T cells become L-selectin^{low} with disease progression, 50–60% of those within the MLN continued to express L-selectin and >90% of these coexpressed β_7 integrin. Persistent L-selectin expression by CD4 T cells supports potential continued use in intestinal recruitment. Expression of integrin β_7 is often interpreted as evidence of memory acquisition and commitment to gut homing, whereas L-selectin expression is often seen as proof of antigenic inexperience and often as a sine qua non for the naive state. In our chronically inflamed model, L-selectin expression was not restricted to naive T cells, but $\sim 10\%$ of the L-selectin-expressing cells were activated. In keeping with our observations, others have shown that L-selectin is re-expressed after lymphocyte activation and subpopulations of memory and effector lymphocytes continue to express or re-express L-selectin (58–60).

Lymphocytes acquire the predilection to recirculate back to the site in which they first encountered their cognate Ag (5, 16, 46). The literature provides strong evidence that in physiologic recruitment, lymphocytes homing to extraintestinal tissues preferentially

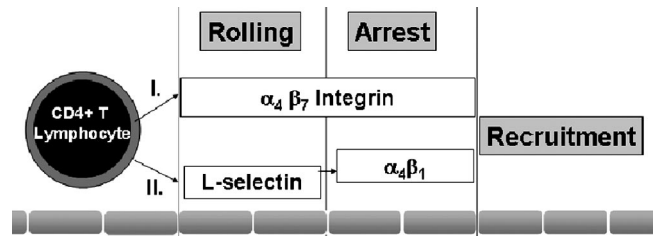


FIGURE 7. Alternate adhesive pathways in chronic ileitis. Dysregulated cytokine production in chronic ileitis induces effector CD4⁺ T cell coexpression of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins. Pathogenic CD4⁺ T cells therefore: *I*, roll and arrest on activated $\alpha_4\beta_7$ integrin, or *II*, alternatively engage L-selectin/ $\alpha_4\beta_1$ integrins, which continue to support pathologic recruitment into the chronically inflamed small intestine, after blockade of the $\alpha_4\beta_7$ /MAdCAM-1 pathway.

express $\alpha_4\beta_1$ integrin (5, 16), whereas those that are gut specific express $\alpha_4\beta_7$ (5, 16, 17, 46). Prior reports have shown that under physiologic conditions, expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins is reciprocal (5, 16, 20, 47). However, in our chronically inflamed model, CD4⁺ T cells coexpress $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins, allowing CD4⁺ T cell recruitment via the physiologic $\alpha_4\beta_7$ /MAdCAM-1 pathway or alternatively through $\alpha_4\beta_1$. Activated $\alpha_4\beta_7$ integrin can mediate arrest in the absence of L-selectin (45). In contrast, Gibling et al. (61) have shown that L-selectin engagement acts as a trigger for $\alpha_4\beta_1$ integrin-dependent T cell adhesion. Thus, CD4⁺ T cells that coexpress both α_4 integrins continue to roll and adhere through L-selectin/ $\alpha_4\beta_1$ after Ab blockade of the $\alpha_4\beta_7$ /MAdCAM-1 pathway (Fig. 7). Consistent with this hypothesis, simultaneous blockade of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ through interference with the shared α_4 integrin moiety (PS2) resulted in significant attenuation of ileitis. Additional L-selectin blockade was not necessary, suggesting that the added benefit of L-selectin blockade over the anti- $\alpha_4\beta_7$ /MAdCAM-1 strategy stems from its additional interference with the $\alpha_4\beta_1$ pathway. This is in keeping with the counterintuitive results from clinical trials in CD in which the less specific anti-human α_4 integrin reagent (natalizumab; blocks both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins) resulted in clinical response, whereas specific blockade of the gut-homing integrin $\alpha_4\beta_7$ (MLN-O₂) was not more efficacious than placebo (21, 62). None of the reported combined antiadhesion molecule strategies in mice (current studies) or humans (natalizumab) results in complete resolution of the inflammatory process; thus, pathogenic T cells may further engage other, not yet described, adhesive pathways. This study only begins to uncover the complexity of the migratory pathways responsible for the maintenance of the chronically inflamed state.

Physiologic leukocyte recruitment is a highly regulated process with a limited number of decision points along every step of the adhesion cascade (4, 16, 45). Expression of specific combinations of adhesion molecules on lymphocyte subpopulations determines their capacity to reach specific tissues, where appropriate endothelial ligands are often restrictively expressed (e.g., MAdCAM-1:gut vs VCAM-1:periphery). This results in orderly recruitment that begins and terminates physiologic inflammatory responses. The current study demonstrates that in chronic inflammation, continuous dysregulated production of proinflammatory cytokines results in inappropriately increased adhesion molecule expression (e.g., MAdCAM-1), as well as aberrant expression of molecules not normally expressed by specific lymphocyte subpopulations and tissues ($\alpha_4\beta_1$: $\alpha_4\beta_7$ coexpression), exponentially increasing the lymphocyte chances of finding appropriate endothelial ligands.

Therefore, when one adhesion molecule is blocked, others compensate for its deficiency. These redundant adhesive pathways favor adhesiveness over tissue specificity, resulting in failure of termination of the inflammatory response and perpetuation of the chronic inflammatory process.

Targeted blockade of specific adhesion molecules has rapidly evolved from model systems to human clinical trials for multiple sclerosis and CD (21–23). Unfortunately, our understanding of the basic mechanisms of action of these antiadhesion molecule strategies is limited (22). Our findings lead us to propose that due to redundancies in the lymphocyte adhesion cascade in chronic inflammation, targeting two or more adhesion pathways may result in improved clinical response, compared with the current single adhesion molecule blockade strategies. Moreover, our studies identify L-selectin and $\alpha_4\beta_1$ integrin as part of the alternate adhesive pathways that continue to support CD4⁺ T cell recruitment to the chronically inflamed small bowel, after $\alpha_4\beta_7$ /MAdCAM-1 blockade. Further dissection of redundant adhesive pathways in chronic ileitis may help us optimize therapies already in clinical use and potentially develop new strategies for the treatment of CD (63).

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