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

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L-Selectin, α4β1, and α4β7 Integrins Participate in CD4+ T Cell Recruitment to Chronically Inflamed Small Intestine

Jesús Rivera-Nieves, Timothy Olson, Giorgios Bamias, Anthony Bruce, Michael Solga, Robert F. Knight, Sharon Hoang, Fabio Cominelli, and Klaus Ley

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/β7+ population and increased mucosal adressin cell adhesion molecule-1 (MadCAM-1) expression were observed within the intestinal lamina propria with disease progression. However, Ab blockade of the β7 integrin, the α4β7 heterodimer, MadCAM-1, or L-selectin did not attenuate inflammation. Blockade of two pathways (L-selectin and MadCAM-1 or α4 integrins) was required to improve ileitis. Further analyses showed that 55 ± 7% of the mesenteric lymph node α4β7+ 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 α4β1 and α4β7 integrins, markedly decreasing local production of TNF-α and IFN-γ. Thus, pathogenic CD4+ T cells not only use the physiologic α4β7/MadCAM-1 pathway, but alternatively engage α4β1 and L-selectin to recirculate to the cha

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C rohn’s disease (CD),1 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 specifically 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 wk 4 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 CD45RBhigh cells, predominantly induce ileitis, not colitis, demonstrating their inherent capacity to preferentially recirculate to the small intestine (11–14).

It is well established that β7 integrins play a critical role in lymphocyte gut homing (5, 15, 16). Integrin α4β7 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 αβ7/MadCAM-1 pathway as a disease-relevant homing mechanism is well established in colitic models (6–9), but not in models of ileitis.

Although αβ7-expressing cells migrate preferentially to gut, lymphocytes expressing the αβ7 integrin (VLA-4) preferentially traffic to nonintestinal sites (5, 16, 17). Integrin αβ7 binds to VCAM-1/VCAM-1, 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 αβ7+ cells tend to be αβ7negative or low and vice versa (5, 16, 20). The role of the αβ7/VCAM-1/fibronectin pathway in IBD has received
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 \(\beta_2\) integrins (14). Results from recent clinical trials with a humanized mAb to \(\alpha_4\beta_7\) integrins have suggested that \(\alpha_4\) 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 most of the naïve 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 \(\alpha_\beta_2/MAdCAM-1\) in the intestine or \(\alpha_\beta_2/VCAM-1\)/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 \(\alpha_\beta_4/MAdCAM-1\)-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 SAMP1/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 \(\alpha_4\) integrin moiety in \(\alpha_\beta_2\) and \(\alpha_4\beta_7\) or L-selectin and MAdCAM-1.

Materials and Methods

**Mice**

SAMP1/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). Fetal samples from SAMP1/YitFc mice were consistently negative for Helicobacter hepaticus, Helicobacter bilis, and other murine Helicobacter species, as well as for protozoa and helminthes. C3SnSmn.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 SAMP1/YitFc CD4\(^{+}\) T cell adoptive transfer and thereafter. Integri \(\beta_2\)\(^{-}\) mice were obtained 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 corksboard. Tissues were then fixed in 10% buffered Formalin or Bouin’s, embedded in paraffin, and cut into 3- to 5-\(\mu\)m sections. Resulting sections were stained with H&E. This tissue collection protocol reduced the histological score variability within experimental groups, compared with the mouse 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 \(\mu\)m) were cut on a cryostat (Micron 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-\mu\m cell strainer. Spleen RBCs were lysed by 15-min incubation in 1x 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 Turbostrat.

**CD4\(^{+}\) T cell adoptive transfer**

SAMP1/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\( \times \)10\(^7\) 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 \(\alpha_4\) (R1-2), \(\alpha_\beta_2\) (M293), \(\beta_2\) (HA2/5), and \(\alpha_\beta_2\) integrins (DATK-32) were used as well as anti-L-selectin (MEL-14) were incubated with leukocytes in suspension, including anti-CD4 (OKT4A) 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\(^5\) 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 \(\mu\)g/ml; BD Pharmingen). Supernatants were collected after 48 h and stored at −70°C.

**Cytometric bead array (CBA)**

TNF-\(\alpha\) and IFN-\(\gamma\) 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 \(\mu\)g each) against: 1) a combinatorial epitope on \(\alpha_\beta_2\) (DATK-32, rat IgG2a); 2) \(\beta_2\) 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 β7, β1, and α4 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 ± 2% vs 21 ± 4%, p < 0.01) (Fig. 1B, left). Over 95% of CD4 expressed β7 integrin by 4 wk (Fig. 1B, middle), and of those, the β7high population (mean fluorescence intensity (MFI) > 103) increased from 14 ± 3% to 21 ± 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 ± 3% CD44high, 85 ± 3% CD69+, 70 ± 8% CD45RBlow; 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 ± 12% vs 40 ± 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 β7 integrin (Fig. 1C, middle). CD4+ T cells from β7 integrin-deficient mice (β7−/−), which served as controls, showed MFI < 102 (Fig. 1C, shaded overlaid histogram). Consistent, but small increases in the β7high 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 ± 5% vs 28 ± 7%, p < 0.01) from 4 to 40 wk of age (Fig. 1D, left). However, the overall integrin β7 expression (~95% at 4 wk) decreased by 40 wk of age (51.5% ± 5), concomitant with the appearance of a β7negative (MFI < 10) population (Fig. 1D, middle). No significant differences were noted in noninflamed age-matched AKR mice (Fig. 1D, left). To further characterize the new β7negative splenocyte population, we determined whether they expressed β7 and α4 integrins, compared with age-matched AKR mice (Fig. 1, E and F). Marked expansion of the β7high and α4high (MFI > 103) population was observed in SAMP1/YitFc mice (Fig. 1, E and F, red boxes) from 4 to 40 wk of age. β7 integrin may associate with at least 10 other α7 integrin subunits (i.e., α7-1,−3, α7-11,−13) (33); however, α4 integrin dimerizes only with the β7 or β1 subunits. An increase in α4 in the setting of a decrease in β7/CD4+ splenocytes (Fig. 1D, middle) denotes an increase in α4β7 expression (VLA-4). Further analysis of both β7high and α4high populations (Fig. 1, E and F, offsets) confirmed that the majority of the expanded α4-positive cells (red overlaid histogram) were found within the increased β7high population (Fig. 1E, red box). The reverse analysis also localized the majority of the β7-expressing cells (red overlaid histogram) within the α4high population (Fig. 1F, red box). Analyses of α4 expression from splenocytes gated on intensity of β7 expression showed that cells within all three gates (L, M, and H) expressed integrin α4 (Fig. 1G). Two peaks of expression (high and low) were seen on the β7low cells (β7L), whereas the β7int (β7M) were predominantly α4high. Those that were β7high had variable intensity of α4 expression, but were predominantly α4low (β7H). The presence of two distinct peaks of α4 expression is consistent with a population expressing only one of the two α4 integrins (α4low), while the α4high most likely coexpress both α4β7 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 α4β7 ligand VCAM-1 was previously observed in SCID mice intestinal microvessels after adoptive transfer of SAMP1/YitFc CD4+ T cells (14).

Blockade of the α4β7/MAdCAM-1 pathway did not attenuate chronic ileitis

Expansion of the β7 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 β7 integrin subunit (FIB-504), α4β7 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...
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 μg/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 α4β7/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...
MA
cAM-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 α4β7, 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 ± 7% vs 30 ± 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 ± 7% vs 30 ± 8%, p < 0.01) (Fig. 4B). L-selectin was preferentially expressed by a subpopulation of MLN and splenic CD4+ T cells that were β7low (Fig. 4B) and coexpressed α4 integrin (Fig. 4C), but not αE integrin (Fig. 4D). To explore whether MLN L-selectin+ cells were strictly of naive phenotype, we determined the expression of surface markers consistent with an activated state. Within the CD4/L-selectin+ population, 10 ± 3% expressed CD69, 9 ± 2% were CD45RBlow, 12 ± 3% were CD44high, and 9 ± 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+ or L-selectin- subpopulations and cultured for 48 h under anti-CD3 stimulation. IFN-γ was produced predominantly by L-selectin- 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+ 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+ (L+) or CD4+/L-selectin- (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 unfractonated 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/MA
cAM-1 blockade, we characterized the LP mononuclear cells obtained from mice treated with isotype or combination anti-MA
cAM/L-selectin Abs using flow cytometry and cytokine

**FIGURE 2.** MLN and ileal LP MA
cAM-1 expression in SCID mice before and after CD4+ T cell transfer. Anti-MA
cAM-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. MA
cAM-1 expression increased in both MLN HEV (B) (×20) and intestinal LP vessels (arrowheads) (D) (×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.

**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 (L, n = 19), anti-β7 integrin (β7, n = 7), anti-α4β7 (α4β7, n = 7), anti-MA
cAM-1 (M, n = 16), anti-L-selectin (L, n = 12), or both anti-MA
cAM-1 and anti-L-selectin Abs (M + L) (n = 18), as described in Materials and Methods (mean ± SEM from six independent experiments). C and D, Effect of indicated treatment on intestinal architecture (representative micrographs, H&E, ×10).
FIGURE 4. L-selectin expression and integrin coexpression of CD4⁺ T cells from MLN or spleen of SAMP1/YitFc mice with or without ileitis. A–D, L-selectin, β7, α4, and αE integrin expression was analyzed in CD4 from mice with ileitis (40 wk old) compared with young (4 wk old) control SAMP1/YitFc mice, without ileitis. Cells gated on FSC, SSC, and CD4 were analyzed by flow cytometry. Representative data obtained from three to four SAMP1/YitFc mice at each time point run in duplicate. E, Activation marker expression by L-selectin<sup>+</sup> cells. F, IFN-γ and TNF-α production of unfraccionated CD4 (CD4), L-selectin<sup>+</sup> (L<sup>+</sup>), or L-selectin<sup>+</sup> (L<sup>-</sup>) CD4⁺ T cells. Cells were sorted by FACS and cultured, as described. Cytokine concentrations (mean ± SEM) were determined from SAMP1/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 ± SEM, n = 4/group).

CBA. LP mononuclear cell yields from mice treated with combination therapy (MEL-14 + MECA-367) were decreased ~7-fold (1.5 × 10⁶ vs 10 × 10⁶ cells/mouse) when compared with those from isotype-treated Abs (data not shown). Fluorescence intensity for β7 integrin in LP CD4⁺ T cells showed a bimodal distribution with a peak at low intensity (>10³ MFI < 10⁴) (β<sub>7</sub><sup>low</sup>) and a high peak (MFI > 10⁴) (β<sub>7</sub><sup>high</sup>) (Fig. 5, A and B). The β<sub>7</sub><sup>-</sup> population was defined as those with MFI < 10³, which corresponds to the MFI of β<sub>7</sub><sup>-</sup> 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<sub>7</sub><sup>low</sup>), whereas the β<sub>7</sub><sup>high</sup> population was unaffected (Fig. 5A, left). CD4 cells expressing high levels of α<sub>4</sub> also decreased, whereas those expressing integrin α<sub>E</sub> were unaffected in treated mice compared with controls (Fig. 5A, middle and right). To establish whether the decreased β<sub>7</sub><sup>low</sup> population coexpressed α<sub>4</sub> or α<sub>E</sub> integrin, α<sub>4</sub> integrin expression was determined in the β<sub>7</sub><sup>low</sup> and β<sub>7</sub><sup>high</sup> populations (Fig. 5B). Integrin α<sub>4</sub> was expressed predominantly by the β<sub>7</sub><sup>high</sup> population (unaffected by combined therapy). As β<sub>7</sub> dimerizes only with α<sub>4</sub> or α<sub>E</sub> integrins, our results demonstrate that the effect of the combined therapy affected the β<sub>7</sub><sup>low</sup> (α<sub>4</sub>β<sub>7</sub><sup>-</sup>) population, without detectable effect on α<sub>E</sub>β<sub>7</sub><sup>-</sup> 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 (C), compared with those treated with isotype control Ab (■). Therefore, most of the cells responsible for IFN-γ and TNF-α production were within the α<sub>4</sub>β<sub>7</sub><sup>-</sup> population, depleted by combined therapy.

Pathogenic T cells coexpress α<sub>4</sub> integrins

Lymphocytes use L-selectin to roll along the endothelium (5, 16), subsequently engaging integrins that mediate arrest (33, 45, 46). Activated integrin α<sub>4</sub>β<sub>7</sub> 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 α<sub>4</sub>β<sub>7</sub>, when it is functionally blocked (mAb) or genetically absent (β<sub>7</sub><sup>-</sup> mice). We then investigated whether other integrins were coexpressed by α<sub>4</sub>β<sub>7</sub> T cells in SCID mice with chronic ileitis. Thirty five ± 8% of the CD4⁺ T cells from noninflamed AKR mice coexpressed β<sub>7</sub> and β<sub>E</sub> integrins, whereas 80 ± 5% of those from mice with chronic ileitis were double positives (Fig. 6A). To further clarify whether the α<sub>4</sub>β<sub>7</sub>-positive population (β<sub>E</sub><sup>low</sup>) coexpressed β<sub>1</sub> integrin, cells were reacted with a mAb that recognizes a combinatorial epitope on α<sub>4</sub>β<sub>7</sub> integrin (clone DATK32). A total of 88 ± 4% of CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub> positives coexpressed integrin β<sub>1</sub> (Fig. 6A, right). Expression of β<sub>1</sub> integrin was not limited to the α<sub>4</sub>β<sub>7</sub>-positive T cells, as 92 ± 2% of those expressing integrin α<sub>E</sub> also expressed β<sub>1</sub> integrin (data not shown). Integrin polarization, believed to determine intestinal or peripheral homing, based on the reciprocal expression of α<sub>4</sub> integrins (α<sub>4</sub>β<sub>7</sub>; intestine/α<sub>E</sub>β<sub>7</sub>; 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 α<sub>4</sub>β<sub>7</sub> or alternatively α<sub>E</sub>β<sub>1</sub> 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<sub>4</sub> integrin moiety and therefore blocks both α<sub>4</sub>β<sub>7</sub> and α<sub>E</sub>β<sub>1</sub> 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<sub>E</sub> blockade on the active index (neutrophilic infiltrates) is in keeping with its widespread expression. Additional L-selectin blockade...
Discussion

The pre- eminent role of the $\alpha_4\beta_7$-MAAdCAM-1 pathway in physiologic 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 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 $\alpha_4\beta_7^+$ 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 $\beta_7$-expressing cells in spleen and for $\beta_7$-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 $\beta_7^{high}$ population in SAMP1/YitFc mice coexpressed integrin $\alpha_4$. Recent data support a regulatory role for $\alpha_4\beta_7^{high}$ 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 $\beta_7^{high}$ regulatory population (i.e., $\alpha_4\beta_7^{high}$) within the LP may be in part responsible for stabilization of disease severity after 40 wk (12). Increased $\alpha_4\beta_7^{high}$ 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 $\alpha_4\beta_7$-MAAdCAM-1 pathway, using mAb against the $\beta_7$ integrin subunit, the $\alpha_4\beta_7$ heterodimer or MAAdCAM-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 αβ7/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 ~25% of control by blocking the α4 subunit (PS2), the αβ7 integrin (DATK-32), or the β1 subunit (FIB-504). Similar results were observed in inflammatory recruitment to the large intestine using colitic mouse models. CD45RBhigh-induced colitis was equally attenuated by treatment with either FIB-504 or MECA-367 alone (6). Likewise, αβ7 integrin or MAdCAM-1 blockade attenuated dextran sulfate sodium-induced colitis (7, 50). Blockade of α4 integrins attenuated spontaneous colitis in nonhuman primates (9). Decreased lymphocyte adhesion was also observed in intravital microscopy studies of CD45RBhigh-transferred mice after treatment with MECA-367 (51).

In our studies, targeting any single component of the αβ7/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 naïve T cell recruitment into mucosal inductive sites (i.e., MLN, PP), as only combined deficiency of both β1 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, αβ7, and αβ1 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-selectinlow with disease progression, 50–60% of those within the MLN continued to express L-selectin and >90% of these coexpressed β1 integrin. Persistent L-selectin expression by CD4 T cells supports potential continued use in intestinal recruitment. Expression of integrin β1 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 naïve T cells, but ~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 express αβ7 integrin (5, 16), whereas those that are gut specific express αβ1 (5, 16, 17, 46). Prior reports have shown that under physiologic conditions, expression of αβ1 and αβ7 integrins is reciprocal (5, 16, 20, 47). However, in our chronically inflamed model, CD4+ T cells coexpress αβ7 and αβ1 integrins, allowing CD4+ T cell recruitment via the physiologic αβ7/MAdCAM-1 pathway or alternatively through αβ1. Activated αβ7 integrin can mediate arrest in the absence of L-selectin (45). In contrast, Gilbin et al. (61) have shown that L-selectin engagement acts as a trigger for αβ1 integrin-dependent T cell adhesion. Thus, CD4+ T cells that coexpress both α4 integrins continue to roll and adhere through L-selectin/αβ1 after Ab blockade of the αβ1/MAdCAM-1 pathway (Fig. 7). Consistent with this hypothesis, simultaneous blockade of αβ1 and αβ7 through interference with the shared α4 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-αβ7/MAdCAM-1 strategy stems from its additional interference with the αβ1 pathway. This is in keeping with the counterintuitive results from clinical trials in CD in which the less specific anti-human α4 integrin reagent (natalizumab; blocks both αβ7 and αβ1 integrins) resulted in clinical response, whereas specific blockade of the gut-homing integrin αβ7 (MLN-O2β) 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 (αβ1/αβ7 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 α4β7 integrin as part of the alternate adhesion pathways that continue to support CD44 T-cell recruitment to the chronically inflamed small bowel, after α4β7/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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