P-Selectin and P-Selectin Glycoprotein Ligand 1 Mediate Rolling of Activated CD8$^+$ T Cells in Inflamed Colonic Venules

Muhammad Asaduzzaman, PhD, Andrada Mihaescu, MD,* Yusheng Wang, PhD,* Tohru Sato, MD,† and Henrik Thorlacius, MD, PhD**

Background: Activated T cells regulate inflammatory diseases in the intestinal tract; however, the adhesive mechanisms governing CD8$^+$ T-cell recruitment in the colon are not known.

Methods: Herein, we used a graft-versus-host disease (GvHD) model to study CD8$^+$ T-cell rolling and adhesion in the large intestine by use of intravital fluorescence microscopy. Graft-versus-host disease was induced by transferring 50×10^6 allogeneic donor splenocytes from BDF1, B6, H-2b mice to recipient BDF1, H-2^{bxd} mice. After 8 days, rhodamine-labeled CD8$^+$ T cells (4×10^6) from healthy and GvHD mice were injected into both healthy and GvHD recipient mice, and CD8$^+$ T-cell–endothelium interactions were studied in the colon.

Results: Activated CD8$^+$ T cells from GvHD mice expressed higher levels of P-selectin ligand and decreased levels of L-selectin. Immunoneutralization of P-selectin and P-selectin glycoprotein ligand 1 reduced CD8$^+$ T-cell rolling and adhesion in inflamed colonic venules by more than 71%. Inhibition of E-selectin had no effect on GvHD-induced CD8$^+$ T-cell–endothelium interactions.

Conclusions: We conclude that P-selectin and P-selectin glycoprotein ligand 1 are dominating molecules in supporting adhesive interactions of CD8$^+$ T cells in inflamed colonic venules and may be useful targets to protect against pathological inflammation in the large bowel.

Key Words: adhesion, colon, inflammation, lymphocyte, rolling

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Recruitment of activated CD8$^+$ T cells is a critical component in tissue injury and inflammation. The recruitment process of lymphocytes is a multistep process, comprising initial rolling followed by firm adhesion and transendothelial migration.[1] In general, this rolling adhesive interaction is considered to be a precondition for subsequent firm adhesion and tissue accumulation of lymphocytes.[1] Lymphocyte rolling is mediated by the selectin family of adhesion molecules, including P-, E-, and L-selectins and their corresponding ligands and α4-integrins under certain circumstances.[1] The selectin ligands are complex glycoproteins and, for selectin specificity and function, poorly understood. Nonetheless, P-selectin glycoprotein ligand 1 (PSGL-1) is a well-accepted high-affinity ligand of P-selectin, although PSGL-1 also binds to E-selectin.[2,3] In addition, E-selectin ligand 1 has been shown to exhibit high affinity to E-selectin, although a functional role of E-selectin ligand 1 in vivo remains to be demonstrated. The L-selectin ligands are mainly expressed in lymphoid organs, although data support the presence of an inducible L-selectin ligand also on nonlymphoid endothelial cells.[2] Indeed, convincing data have documented that rolling and recruitment of naive lymphocytes are mediated by L-selectin in secondary lymphoid organs,[1,2] and E-selectin seem to play a key role in the accumulation of activated T cells in the skin.[2] However, the adhesive mechanisms regulating CD8$^+$ T-cell rolling and adhesion in the gastrointestinal tract remain elusive.

Thus, the purpose of this study was to determine the role of endothelial selectins and PSGL-1 in the recruitment of activated CD8$^+$ T cells in the inflamed colon. For this purpose, we used a graft-versus-host disease (GvHD) model and adoptive transfer of activated CD8$^+$ T cells and analyzed the molecular mechanisms supporting CD8$^+$ T-cell–endothelium interactions in the colonic microvasculature by use of inverted intravital fluorescence microscopy.

MATERIALS AND METHODS

Animals and GvHD

C57BL/6 (B6, H-2^b) and B6D2F1 (BDF1, H-2^{bxd}) male mice (21–26 g) were used as donors and recipients, respectively. All mice were kept on a 12:12-hour light-dark cycle with free access to food and tap water. Mice were anesthetized with 75-mg/kg ketamine hydrochloride and 25-mg/kg xylazine. All experiments were approved by the Regional Ethical Committee for Animal Experimentation at Lund University. Graft-versus-host disease was induced as previously described.[4] In brief, 50×10^6 allogeneic donor splenocytes were intravenously (IV) transferred into recipient mice. Previous experience has shown that a clear-cut graft-versus-host immune response is established after 8 days.[4]

Cell Isolation

For intravital microscopy experiments, spleen cells were harvested from both healthy and GvHD mice and stained with rhodamine (Sigma Chemical Co, St Louis, MO). Graft-versus-host disease splenocytes were isolated from mice killed 8 days after initial splenocyte transfer. CD8$^+$ T cells were purified by use of a magnetic bead isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

From the *Department of Surgery, Malmö University Hospital, Lund University, Malmö, Sweden; and †Department of Medicine, Stanford University School of Medicine, Stanford, CA.

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Reprints: Henrik Thorlacius, MD, PhD, Department of Surgery, Malmö University Hospital, Lund University, 205 02 Malmö, Sweden. E-mail: henrik.thorlacius@med.lu.se.

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The purity of $CD8^+$ T cells was analyzed by double staining cells with rat anti–mouse CD8 and hamster anti–mouse CD3 antibodies (Abs), which was found to be higher than 90% after bead isolation. The rhodamine-labeled $CD8^+$ T cells were kept on ice in the dark until use.

Flow Cytometry

Isolated spleen cells ($1–2 \times 10^6$) were stained with the following rat anti–mouse Abs (BD Pharmingen, San Diego, CA): CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD45.1 (clone A20), T-cell receptor β (clone H57-597), and L-selectin (clone Mel-14). A P-selectin–immunoglobulin M fusion protein was used to stain for P-selectin ligand as previously described.[5] Flow cytometry was performed using the FACSCalibur and LSR II (BD Biosciences, Stockholm, Sweden). Data were acquired and analyzed on CellQuest-Pro (BD Biosciences).

Intravital Fluorescence Microscopy

Rhodamine-labeled $CD8^+$ T cells (4×10^6) from healthy and GvHD mice were injected into the jugular vein of both healthy and GvHD recipient mice. Inverted intravital fluorescence microscopy of colonic microcirculation was used to quantify $CD8^+$ T-cell rolling and adhesion in postcapillary venules (15–35 μm) as described previously in detail.[6] Briefly, observations of the colonic microcirculation were made using an Olympus microscope (IX70; Olympus Optical Co GmbH, Hamburg, Germany) equipped with lenses (10/NA 0.25 and 40/NA 0.6). The microscopic image was televised using a charge-coupled device videocamera (FK 6990 Cohu; Pieper GmbH, Berlin, Germany) and recorded on videotape for subsequent off-line analysis. To determine the adhesive mechanisms behind $CD8^+$ T-cell rolling and adhesion, monoclonal Abs (40 μg per mouse) against PSGL-1 (2PH1), E-selectin (UZ4, kindly donated by Dr Rupert Hallmann), and P-selectin (RB40.34) and a control Ab (R3-34) were injected IV before transfer of rhodamine-labeled $CD8^+$ T cells. Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Microcirculatory analysis included determination of leukocyte rolling by counting the number of rolling leukocytes passing by a reference point in the venule per 30 seconds and expressed as cells per minute. Rolling ratio (%) of $CD8^+$ T cells was measured by counting the number of $CD8^+$ T-cell rolling by number of total flux in the colonic venule in each minute. Because rolling ratio is related to the total cell flux, potential changes in hemodynamics are compensated for herein. The number of firmly adherent $CD8^+$ T cells was measured by counting the number of $CD8^+$ T cells adhering to the venular endothelium (500- to 800-μm long segments) and those that remained stationary during the observation period of 30 seconds, and the number was expressed as cells per millimeter venule length.

Quantitative Real-Time Polymerase Chain Reaction

Total cellular RNA was extracted from both activated and unactivated colon tissues using an acid guanidinium thiocyanate–phenol–chloroform method (TRIzol Reagent; Gibco, Grand Island, NY) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants according to the manufacturer's protocol. RNA concentration and purity were determined by measuring absorbance spectrophotometrically at 260 and 280 nm. Then, total RNA was converted to complementary DNA using real-time polymerase chain reaction (RT-PCR) with Super-Script One-Step RT-PCR system (Gibco). Mouse β-actin served as an endogenous control gene for relative quantification using the comparative threshold cycle method. All reactions were performed in triplicate on an Mx3000P RT-PCR System (Stratagene, Stockholm, Sweden) using 2× SYBR Green (Stratagene). The primer sequences of P- and E-selectin and β-actin were as follows: P-selectin (forward) 5'-ACG AGC TGG ACG GAC CCG-3' and (reverse) 5'-GGC TGG CAC TCA AAT TTA CAG-3'; E-selectin (forward) 5'-GGT AGT TGC ACT TTC TGC GG-3' and (reverse) 5'-CCT TCT GTG GCA GCA TGT TC-3'; and β-actin (forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3' and (reverse) 5'-TCT CCA GGG AGG AAG AGG AT-3'.

Statistics

Statistical evaluations were performed using Kruskal-Wallis 1-way analysis of variance on ranks for unpaired samples (Dunn post hoc test). The results are presented as mean ± SEM; n represents the number of animals. Differences were considered to be significant at $P < 0.05$.

RESULTS

We first wanted to establish whether there are differences in the adhesive interactions between unactivated and activated (GvHD) $CD8^+$ T cells from healthy and GvHD mice, respectively, for rolling and adhesion in colonic venules of GvHD mice. It was found that the rolling ratio and the number of adherent $CD8^+$ T cells from healthy donors (unactivated cells) were 28.5 ± 1.8% and 5.0 ± 2.4 cells per millimeter, respectively. In contrast, the rolling ratio and the number of adherent $CD8^+$ T cells from GvHD mice (activated cells) were 72.3 ± 12.3% and 15.3 ± 5.3

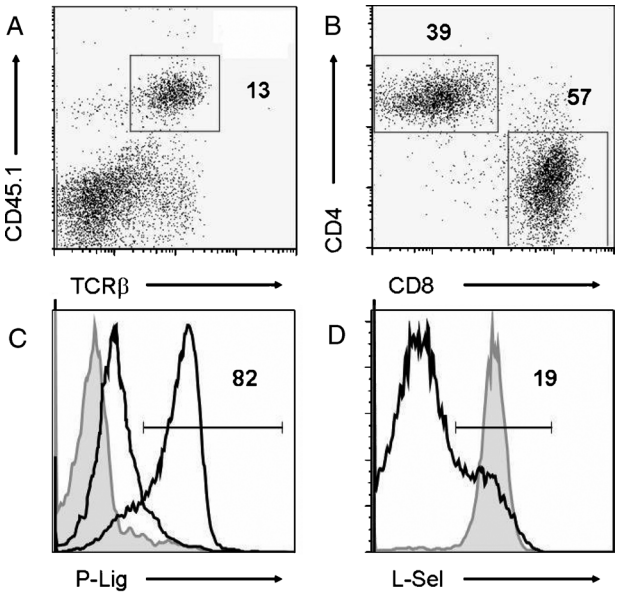

FIGURE 1. Graft-versus-host disease was induced by transferring 50×10^6 congenic splenocytes ($CD45.1^+$) into BDF1 mice. The mice were killed on day 8 and splenocytes harvested and stained for flow cytometry. Donor splenocytes (a) were analyzed for CD4 and CD8 populations (b). P-selectin ligand (P-Lig) expression on donor CD8 cells in normal (c; solid line left) and GvHD (c; solid line right) mice are shown. Percent positive cells based on EDTA control (c; gray line). L-selectin expression on donor CD8 cells in normal (d; gray line) and GvHD (d; solid line) mice are shown. Percent positive cells are indicated in the histograms. Representative plots shown (n = 3).

cells per millimeter, respectively ($P < 0.05$ vs. unactivated $CD8^+$ T cells). To select targets for inhibiting $CD8^+$ T-cell–endothelium interactions, we compared the starting phenotype of donor $CD8^+$ T cells from normal mice with the developed phenotype of activated donor $CD8^+$ T cells from GvHD mice. The activated donor $CD8^+$ T cells from GvHD mice exhibited an activated phenotype compared with the control $CD8^+$ T cells from healthy mice (Fig. 1). Moreover, we also examined the expression of tissue homing molecules on donor $CD8^+$ T cells, reasoning that those overexpressed might be involved in the recruitment process. Comparatively, activated $CD8^+$ T cells from GvHD mice expressed higher levels of P-selectin ligand and decreased levels of L-selectin (Fig. 1). Based on this phenotype, we assessed the importance of PSGL-1 and P- and E-selectins that PSGL-1 can bind to. Thus, monoclonal Abs against the endothelial selectins and PSGL-1 were injected into GvHD recipient before transfer of the rhodamine-labeled activated donor $CD8^+$ T cells. It was found that the rolling ratio of $CD8^+$ T cells was 8.1 ± 2.5% in negative control mice (no GvHD), which was significantly increased to 72.3 ± 12.3% in GvHD mice ($P < 0.05$, Fig. 2A, n = 4). Notably, GvHD increased the messenger RNA expression of E-selectin by more than 10-fold but had no effect on the gene expression of P-selectin in the colon (n = 3). We observed that immunoneutralization of both P-selectin and PSGL-1 markedly reduced the $CD8^+$ T-cell rolling ratio to 11.7 ± 6.2% and 21.2 ± 8.7% ($P < 0.05$, Fig. 2A, n = 4), corresponding to an 84% and 71% reduction in rolling, respectively, when compared with the control Ab treatment. However, immunoneutralization of E-selectin had no effect on the $CD8^+$ T-cell rolling ratio (50.6 ± 7.8%) compared with isotype-matched control Ab ($P > 0.05$, Fig. 2A). Moreover, the number of adherent $CD8^+$ T cells was 1.3 ± 0.7 cells per millimeter in the negative control group (no GvHD), which was significantly increased to 15.3 ± 5.3 cells per millimeter in GvHD mice ($P < 0.05$, Fig. 2B, n = 4). Moreover, we observed that administration of the Abs against P-selectin and PSGL-1 reduced the number of adherent $CD8^+$ T cells down to 1.0 ± 1.0 and 2.8 ± 1.6 cells per millimeter, respectively ($P < 0.05$). Thus, immunoneutralization of P-selectin and PSGL-1 decreased GvHD-induced adhesion of $CD8^+$ T cells in colonic venules by more than 82%. However, there was no difference in the adhesion of activated $CD8^+$ T cells in mice pretreated with the anti–E-selectin Ab when compared with the GvHD mice treated with the control Ab ($P > 0.05$, Fig. 2B).

DISCUSSION

The present study demonstrates a critical role of P-selectin and PSGL-1 in mediating rolling of activated $CD8^+$ T cells in inflamed venules in the colon. Moreover, inhibition of P-selectin and PSGL-1 abolished not only rolling interactions but also adhesion of activated $CD8^+$ T cells in colonic venules, suggesting that P-selectin/PSGL-1–dependent rolling is a precondition for the subsequent firm adhesion of $CD8^+$ T cells in the colon. In contrast, inhibition of E-selectin had no influence on $CD8^+$ T-cell rolling and adhesion in inflamed colonic venules. Thus, these findings indicate that P-selectin and PSGL-1 may be useful target to ameliorate pathological inflammation in the large bowel.

$CD8^+$ T cells play a prominent role in several inflammatory diseases in the colon, including GvHD and Crohn disease, although the adhesive mechanisms have remained elusive. Dynamic expression of specific adhesion molecules regulate T-cell homing to sites of inflammation.[1] We observed that activated $CD8^+$ T cells from GvHD mice down-regulated expression of L-selectin. Such a reduction in L-selectin on activated T cells is a well-known phenomenon, which prohibits T cells from recirculating to secondary lymphatics while they acquire adhesive capabilities to enter inflamed peripheral tissues. Nonfunctional PSGL-1 is constitutively expressed on almost all lymphocytes, but functional binding of PSGL-1 to P-selectin requires post-translational modifications of PSGL-1 by glycosyltransferases up-regulated in activated lymphocytes.[2] Herein, we studied functional P-selectin ligand expression by quantifying binding of P-selectin on $CD8^+$ T cells from the spleen of normal and GvHD mice. We observed that GvHD markedly increased P-selectin ligand expression on activated $CD8^+$ T cells from GvHD mice, suggesting that functional PSGL-1 is up-regulated on splenic $CD8^+$ T cells in GvHD animals. Thus, considered together, these data suggest that these activated $CD8^+$ T cells form GvHD mice have increased ability to accumulate at sites of inflammation. Indeed, we observed that the fraction of activated

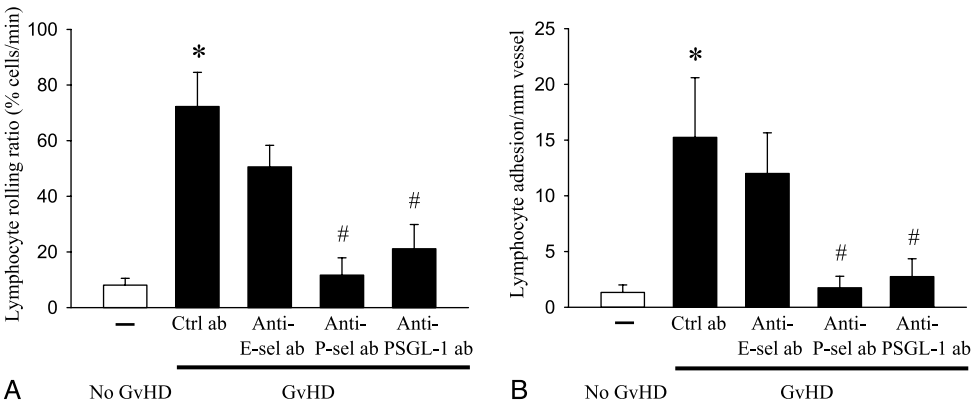

FIGURE 2. $CD8^+$ T-cell (A) rolling ratio and (B) firm adhesion in inflamed colonic venules of GvHD mice. Graft-versus-host disease was induced by transferring 50×10^6 congenic splenocytes ($CD45.1^+$) into BDF1 mice. Activated $CD8^+$ T cells (4×10^6) were isolated from GvHD mice at day 8, labeled with rhodamine, and transferred into either GvHD mice or healthy BDF1 mice (No GvHD). $CD8^+$ T cell–endothelium interactions were quantified by the use of inverted intravital fluorescence microscopy. Mice were pretreated IV with a control Ab (Ctrl Ab) or Abs against E-selectin (Anti–E-sel Ab), P-selectin (Anti–P-sel Ab), and PSGL-1 (Anti–PSGL-1 Ab). Data represent mean ± SEM, and n = 4. $^*P < 0.05$ versus No GvHD and $^\#P < 0.05$ versus control Ab.

$CD8^+$ T cells interacting with the microvascular endothelium in the inflamed colon was significantly higher compared with that of unactivated T cells from healthy mice.

Considering our finding that activated $CD8^+$ T cells from GvHD mice express higher levels of functional PSGL-1 and that PSGL-1 can bind to both P- and E-selectins,[2,3] we asked whether these molecules may regulate $CD8^+$ T-cell–endothelium interactions in the inflamed colon by use of a new inverted intravital fluorescence microscopical technique that allows detailed studies of leukocyte-endothelium interactions in the large bowel.[6] Herein, we show for the first time that P-selectin and PSGL-1 are critical in supporting rolling of activated $CD8^+$ T cells in inflamed colonic venules in a GvHD model. In fact, immunoneutralization of P-selectin and PSGL-1 reduced $CD8^+$ T-cell rolling by more than 71%. However, we observed that $CD8^+$ T-cell rolling was intact in GvHD mice pretreated with an Ab against E-selectin, although E-selectin expression in the colon was markedly increased in GvHD mice. In this context, it should be mentioned that some studies have suggested that P- and E-selectins are redundant and that inhibition of both P- and E-selectins is necessary to reduce leukocyte rolling.[6] However, our present data show that inhibition of P-selectin is sufficient to abolish $CD8^+$ T-cell rolling in the gut, indicating that P-selectin plays a nonredundant role in the intestine. Moreover, we found that inhibition of P-selectin and PSGL-1 not only abolished rolling but also markedly reduced firm adhesion of $CD8^+$ T cells in the colonic venules of GvHD mice, suggesting that P-selectin/PSGL-1–mediated rolling may be a precondition for subsequent adhesion of activated $CD8^+$ T cells in the inflamed colon. Thus, our results suggest that P-selectin and PSGL-1 are dominating and nonredundant adhesions molecules mediating initial interactions between activated $CD8^+$ T cells and endothelial cells in the colonic microcirculation. In this context, it is interesting to know that P-selectin has been shown to support neutrophil rolling in the colon in experimental models of ischemia/reperfusion and colitis.[7] Taken together, it may be proposed that an emerging body of data indicate that P-selectin and PSGL-1 may be key molecules in regulating early and critical interactions between most leukocyte subtypes and inflamed endothelial cells in the large bowel. In addition, it may also be mentioned that our data extend on 2 recent studies reporting that P-selectin and PSGL-1 are important for $CD4^+$ T-cell rolling in the small bowel.[8,9]

In conclusion, our novel data demonstrate that P-selectin and PSGL-1 mediate rolling of activated $CD8^+$ T cells in the inflamed colon. Moreover, inhibition of P-selectin and PSGL-1 attenuates adhesion of activated $CD8^+$ T cells, suggesting that P-selectin/PSGL-1–dependent rolling is a precondition for firm adhesion of activated $CD8^+$ T cells in the colon. Thus, targeting P-selectin and PSGL-1 may protect against $CD8^+$ T-cell–mediated tissue injury in conditions with pathological inflammation in the large bowel.

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