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Neutrophil Migration across Intestinal Epithelium: Evidence for a Role of CD44 in Regulating Detachment of Migrating Cells from the Luminal Surface

Jennifer C. Brazil,* Winston Y. Lee,† Keli N. Kolegraff,† Asma Nusrat,† Charles A. Parkos,‡ and Nancy A. Louis*‡

The migration of polymorphonuclear leukocytes (PMNs) across the intestinal epithelium is a histopathological hallmark of many mucosal inflammatory diseases including inflammatory bowel disease. The terminal transmigration step is the detachment of PMNs from the apical surface of the epithelium and their subsequent release into the intestinal lumen. The current study sought to identify epithelial proteins involved in the regulation of PMN migration across intestinal epithelium at the stage at which PMNs reach the apical epithelial surface. A panel of Abs reactive with IFN-γ-stimulated T84 intestinal epithelial cells was generated. Screening efforts identified one mAb, GM35, that prevented PMN detachment from the apical epithelial surface. Microsequencing studies identified the GM35 Ag as human CD44. Transfection studies confirmed this result by demonstrating the loss of the functional activity of the GM35 mAb following attenuation of epithelial CD44 protein expression. Immunoblotting and immunofluorescence revealed the GM35 Ag to be an apically expressed v6 variant exon-containing form of human CD44 (CD44v6). ELISA analysis demonstrated the release of soluble CD44v6 by T84 cells during PMN transepithelial migration. In addition, the observed release of CD44v6 was blocked by GM35 treatment, supporting a connection between CD44v6 release and PMN detachment. Increased expression of CD44v6 and the GM35 Ag was detected in inflamed ulcerative colitis tissue. This study demonstrates that epithelial-expressed CD44v6 plays a role in PMN clearance during inflammatory episodes through regulation of the terminal detachment of PMNs from the apical epithelial surface into the lumen of the intestine. The Journal of Immunology, 2010, 185: 7026–7036.

Polymorphonuclear leukocytes (PMNs) play a key role in host defense through phagocytosis and destruction of invading microorganisms and as such are important effectors of the acute inflammatory response (1). A key event in these processes is the migration of PMNs out of the circulation and across both endothelial and epithelial tissue barriers in response to chemotactic stimuli. Although the steps involved in PMN migration across vascular endothelium have been extensively characterized (2–4), much less is known about the sequential cell–cell interactions that define PMN transepithelial migration. Previous studies have demonstrated stimulus-specific PMN epithelial migratory interactions, with N-formyl-methionyl-leucyl-phenylalanine (fMLP) mediating its effects through binding of leukocyte-specific β2 integrins to epithelial counterligands (5), but with other chemoattractants, such as IL-8 and C5a, acting independently of β2 integrins (6). Subsequent migration by PMN into the intraepithelial space is reported to be regulated through epithelial glycoprotein CD47 interaction with PMN-expressed signal regulatory protein α (7–9). In addition, neutrophil expressed junctional adhesion-like protein binding to epithelial coxsackie and adenovirus receptor protein has been shown to regulate the passage of PMNs through epithelial tight junctions (10).

As well as expressing ligands facilitating initial PMN binding to the basal surface and subsequent intracellular passage, the intestinal epithelium also expresses proteins mediating PMN detachment and clearance from the luminal surface. Although these epithelial ligands have not been extensively characterized, it has been previously reported that epithelial ICAM-1 is expressed apically and acts as a PMN retention ligand under inflammatory conditions (11). In addition, PMN FcR interactions with apical epithelial proteins have also been implicated in PMN-epithelial retention (12). More recently, it has been reported that decay-accelerating factor functions as an antiadhesive epithelial glycoprotein that regulates PMN detachment from the epithelium (13). An increased understanding of the processes governing the terminal release of PMNs into the intestinal lumen has important clinical implications, as inflammatory infiltrates characterized by accumulations of PMNs within mucosal tissues are pathognomonic of both acute and chronic inflammatory conditions. Specifically, PMN accumulation and abscess formation within intestinal crypts at the apical epithelial surface are pathological features of multiple inflammatory disease processes of the intestine including ulcerative colitis (14), infectious colitis (15), and necrotizing enterocolitis (16).

In the current study, we aimed to identify epithelial ligands important in the terminal stages of PMN transmigration, including...
detachment into the intestinal lumen. To this end, a panel of mAbs was generated against IFN-γ-treated T84 epithelial plasma membranes. This screening process identified one mAb, designated GM35, that inhibited the detachment/release of PMN from the apical surface of polarized monolayers of epithelial cells following fMLF-stimulated PMN migration. Extensions of these observations revealed the GM35 Ag to be an apically expressed, 67 kDa variant containing form of human CD44 (CD44v6). The current study also demonstrated that T84 epithelial cells release soluble CD44v6 during PMN transmigration. The release of epithelial CD44v6 during transmigration was blocked by apical treatment of epithelia with GM35, suggesting a connection between CD44v6 protein shedding and PMN detachment. Our results indicate that CD44v6 acts as a mediator in PMN clearance from the apical surface of the intestinal epithelium. Increased understanding of the role of CD44v6 in PMN epithelial clearance has the potential to allow for the design of specific targeting mechanisms to better facilitate intestinal inflammatory resolution.

Materials and Methods

Cell culture

Cultures of T84 cells (passage 68–72) (17), HT29 cells (passage 128–138) (18), Caco2 cells (passage 28–38) (5), SK-CO15 cells (passage 8–11) (19), and HeLa cells (passage 63–68) (20) were grown as described previously. Human dermal microvascular endothelial cells (HDMECs) (passage 4–9) were purchased from Promocell (Heidelberg, Germany) and grown according to the manufacturer’s instructions.

Abs and reagents

Monoclonal anti-CD44v3, anti-CD44v5, and anti-CD44v6 Abs were purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-CD44v7 and anti-CD44v10 Abs were purchased from Abcam (Cambridge, MA). Monoclonal anti-CD16 F(ab′)2 and anti-CD32 (Fab′)2 Abs were purchased from Fitzgerald Industries International (Concord, MA). Monoclonal anti-desmin or mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). J10.4, an isotype-matched binding control IgG1 for GM35, the anti-CD11b/CD18 mAb CBRM1/29, and CBRM1/30 were purchased from Promocell (Heidelberg, Germany) and grown according to the manufacturer’s instructions.

PMN isolation

PMNs were isolated from whole blood obtained from normal human volunteers, with approval from the Emory University Institutional Review Board, from human subjects, by using a previously described procedure (24). PMNs were resuspended in HBSS with 10 mM HEPES (pH 7.4) and without Ca2+ or Mg2+ at a concentration of 5 × 107 cells/ml. PMNs isolated in this way were 97% pure and >95% viable and were used for transmigration within 2 h of isolation.

PMN transmigration and cell adhesion assays

For transmigration experiments, cells were grown on collagen-coated, permeable 0.33-μm polycarbonate filters (5 μm pore size; Costar, Cambridge, MA) as described previously (7, 17, 25). All epithelial migration experiments were performed in the presence of a chemotactic gradient of 100 nM fMLF and in the physiologically relevant basolateral-to-apical direction (i.e., inverted monolayers) unless otherwise indicated, and all HDMEC transmigration studies were performed in the apical-to-basolateral direction with a 10 nM fMLF gradient as described previously (26). For migration experiments, 1 × 105 PMNs were added to the upper chambers of transwell inserts and migration levels assessed at 37°C following indicated intervals. Transmigrated PMNs were quantified by colorimetric enzyme activity assay specific for the PMN azurophilic marker myeloperoxidase (MPO) as described previously (27). Briefly, migrated PMNs were lysed by the addition of Triton X-100 to a final concentration of 0.5% and acidified by the addition of citrate buffer (100 mM [pH 4.2]). PMN standards in the range of 0.05 × 106–1.0 × 106 PMNs were prepared and similarly lysed. Standard and experimental sample aliquots (in triplicate) were added to equal volumes of MTS solution (1 mM ABTS, 0.03% H2O2, and 100 mM sodium citrate buffer [pH 4.2]) in a 96-well plate, and resulting color was quantitated on a plate reader at 405 nm. A standard curve with numbers of PMNs versus MPO activity was constructed and used to determine the number of migrated PMNs in experimental samples. PMNs remaining adherent to T84 monolayers after basolateral-to-apical migration were quantified using a previously described monolayer washing procedure (12). Briefly, following completion of transmigration, T84 monolayers were removed and transferred to new tissue culture plates containing 1 ml HBSS+well plates. Cells were spun for 5 min (50 × g, 4°C) and detached PMNs quantified by MPO assay as above.

For IFN-γ studies, T84 epithelial monolayers grown to 70–80% confluence in 24-well tissue-culture plates were treated for 24 h with 100 U/ml IFN-γ immediately prior to measurement of PMN adhesion. PMN adhesion to confluent T84 and HT29 epithelial cells was measured directly using modifications of previous protocols (13, 28, 29). Briefly, epithelial cells were washed free of media and treated with 10 μg/ml GM35 or isotype control binding Ab in the presence of 100 nM MIFL for 10 min at 37°C. 2′,7′-bis(carboxyethyl)-5-(and)-6-carboxyfluorescein (BCECF)-labeled PMNs (5 × 105) preincubated with relevant Abs were added to epithelial monolayers, and plates were centrifuged at 50 × g for 5 min to uniformly settle PMN before adhesion was allowed to proceed for 10 min at 37°C. Monolayers were gently washed with HBSS+, and fluorescence intensity (excitation, 485 nm; emission, 530 nm) was measured on a fluorescence plate reader. Adherent PMN numbers were determined from standard curves generated by serial dilution of known numbers of BCECF-acetoxyethyl ester-labeled cells.

Immunoblotting and immunofluorescence

Cell lysates for Western blotting were prepared with the following lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% TX-100, 1 mM Na3VO4, and 1 mM PMSF) supplemented with 10% mammalian tissue protease inhibitor mixture (Sigma-Aldrich). For immunoprecipitation experiments, precleared cell lysates were incubated with 2 μg relevant mAb for 4 h at 4°C followed by incubation with protein G-Sepharose beads overnight at 4°C. Washed immunoprecipitates and regular cell lysates were boiled in SDS-PAGE sample buffer under reducing conditions and subjected to SDS-PAGE followed by transfer to polyvinylidene fluoride under standard conditions. Membranes were blocked with 0.5% milk and incubated with 1 μg/ml GM35 or anti-CD44 variant Abs. Primary Abs were detected using HRP-linked secondary Abs (Jackson Immuno-
Research Laboratories, West Grove, PA). All blocking, Ab incubations, and intervening washes with TBS-Tween 20 were carried out using the SNAP i.d. protein detection system (Millipore, Billerica, MA).

Immunofluorescent labeling of T84 epithelial cells was achieved as follows. Nonpermeabilized T84 monolayers were fixed using 10% formalin (20°C, 20 min) and subsequently blocked with 2% BSA in PBS. Monolayers were then incubated with 10 μg/ml GM35 labeled with Zenon Alexa Fluor 488 mouse IgG1, 5 μg/ml anti-CD44 Ab labeled with Zenon Alexa Fluor 568 mouse IgG1 or Alexa Fluor 568 mouse IgG2a, or 10 μg/ml anti-CD55 Ab labeled with Zenon Alexa Fluor 568 Mouse IgG2a for 1 h at room temperature. After three washes with PBS, monolayers were mounted in ProLong anti-fade embedding solution (Invitrogen). Images shown were representative of at least three experiments with multiple images taken per monolayer.

For human tissue staining, frozen sections (6 μm) of discarded resection specimen colonic mucosa from patients with ulcerative colitis were obtained. Inflamed and noninflamed sections of discarded tissue were characterized based on observed disease extent and activity. Tissue was fixed in absolute ethanol, nonspecific protein binding was blocked with 3% BSA, and tissue sections were incubated with primary Abs, washed in HBSS+, and subsequently labeled with appropriate secondary Abs. All procedures on discarded human tissue were carried out under Emory Institutional Review Board approval. All images were captured using an LSM 510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) with pan-Neofluar 40×/1.3 oil objective using software supplied by the vendor.

shRNA and DNA transfections

For CD44 knockdown studies, one of four HuSh 29-mer shRNA constructs against CD44 (p313, p314, p315, p316) or an Scr construct was transfected into HT29 cells. Transfection complexes consisting of 1 μg plasmid DNA, 3 μl Lipofectamine 2000, and 100 μl Opti-MEM I (Invitrogen) were incubated for 30 min at room temperature before addition to HT29 cells grown in six-well culture plates. Extent of protein knockdown was assessed after 72 h by immunoblotting for CD44 as described above. The functional effect of CD44 knockdown on mAb GM35 activity was measured by transfecting HT29 cells with the shRNA constructs 72 h before examining the effect of GM35 on PMN–HT29 adhesion as described above.

ELISA detection of soluble CD44

PMNs were isolated and prompted to migrate across confluent T84 monolayers in the physiologically relevant basolateral to apical direction in the presence or absence of apically applied GM35 (10 μg/ml) as described above. Samples from the apical reservoir were removed at 0, 5, 15, 30, 45, and 60 min and assessed for levels of soluble CD44 standard (sCD44std) and soluble CD44v6 (sCD44v6) using CD44std and CD44v6 ELISA kits, respectively, according to manufacturer’s instructions. A standard curve was prepared from six standard dilutions of sCD44std or sCD44v6 and levels of sCD44std and sCD44v6 in experimental samples, and standards were measured at 450 nm.

Data analysis

Data were analyzed by two-factor ANOVA using Prism 5 for Mac OSX version 5.0.1a 1992–1998 (GraphPad, San Diego, CA). Values are expressed as the mean ± SE from a minimum of at least three independent experiments.

Results

To identify epithelial mediators of PMN clearance upregulated by inflammatory stimuli, T84 cells were treated with the proinflammatory cytokine IFN-γ, a key mediator of intestinal inflammation (30) and barrier function (31). Cytokine-treated T84 epithelial cell membrane preparations were then used to generate mouse mAbs against epithelial epitopes. The resulting Abs were tested using T84 monolayers, as a model intestinal epithelial cell line, to determine their influence on PMN transepithelial migration (TEM) in response to fMLF. Upon initial investigation, one subclone IgG1 Ab, designated GM35, was found to significantly reduce the level of PMN transmigration when applied to the apical surface of T84 monolayers and was chosen for further investigation.

GM35 inhibits detachment of transmigrated PMNs from the apical epithelial surface

Initial observations revealed that GM35 reduced the levels of PMN migration across T84 epithelial monolayers in a dose-dependent manner in the physiologically relevant basolateral-to-apical direction. At a concentration of 10 μg/ml, GM35 reduced detectable PMN numbers in the apical chamber by ≥80% (p < 0.001) compared with either a no mAb control (Fig. 1A) or an isotype-matched noninhibitory binding control mAb (Fig. 1C). GM35 had no effect on apical to basolateral TEM of PMNs (Fig. 1E), suggesting that GM35 influences PMN transmigration in a polarized fashion. Further, GM35 had no effect on transendothelial migration (Fig. 1F), implying that the effects of GM35 on PMN transmigration are restricted to interactions between PMN and the epithelium. This epithelial specificity of GM35 is further supported by flow cytometric analyses, which indicate that GM35 does not bind PMNs (data not shown).

To determine if the GM35-mediated reduction in PMN TEM was due to interference with the process of PMN detachment from the apical epithelial surface, we measured the numbers of PMNs that had reached but not detached from the apical epithelial surface. Fig. 1B demonstrates that GM35 increases the level of PMN adherence to the apical surface of the epithelium with 10 μg/ml increasing the number of adherent PMNs by ≥80% (p < 0.001) in a dose-dependent manner. Taken together, these data demonstrate that mAb GM35 exerts its effects on PMN transmigration through promoting PMN adherence to the apical surface of the epithelium.

Previous studies have demonstrated that interactions between epithelium-bound Ab and PMN FcRs influence PMN transmigration. Specifically, Reaves et al. (12) report an FcR CD32A epitope-mediated decrease in PMN detachment from the epithelium during PMN transmigration. To rule out the involvement of PMN FcRs in GM35 activity, transmigration assays in the presence of anti-FcR Abs were carried out. Fig. 1C demonstrates that the GM35-induced attenuation of PMN transmigration is independent of PMN FcRs in that anti-CD16 F(ab′)2 and anti-CD32 F(ab′)2 Abs, which bound PMN by FACS (data not shown), had no effect on the GM35-mediated increase in PMN adhesiveness. This result was further supported by the demonstration that GM35 F(ab′)2 fragments, which were determined to be 98% pure by gel electrophoresis and densitometry (data not shown), resulted in the same increase in PMN adherence/decrease in PMN detachment as intact GM35 mAb (Fig. 1C).

Kinetic analysis of the GM35 effect on PMN TEM revealed a maximal effect detectable at 60 min (p < 0.001), with significant effects on detachment of transmigrated PMN also detectable at 2 h (p < 0.01) and 3 h (p < 0.001) (Fig. 1D).

To confirm the specific effect of GM35 on PMN-epithelial adhesion/attachment interactions, the effect of this mAb on PMN adhesion to epithelial cells was measured directly. GM35 (10 μg/ml) significantly increased the level of PMN adhesion to T84 cells compared with either a no Ab control (p < 0.01) or an isotype-matched noninhibitory binding control Ab (p < 0.01) (Fig. 2A). Further, as was observed during transmigration, GM35 exerted its effects independently of PMN FcRs in that anti-CD16 and anti-CD32 F(ab′)2 Abs had no effect on the GM35-mediated increase in PMN adhesion. In addition, whereas the anti-CD11b/CD18 Ab CBRM1/29 effectively blocked PMN epithelial adhesive interactions, it had no effect on the ability of GM35 to alter PMN adhesion, suggesting that GM35 acts independently of the β2 integrin Mac-1. A similarly significant increase (p < 0.01) in PMN adhesion induced by 10 μg/ml GM35 was also observed for HT29 cells (Fig. 2B).
FIGURE 1. GM35 inhibits detachment of transmigrated PMNs from the apical epithelial surface. Confluent T84 monolayers were pretreated apically with indicated concentrations of GM35 mAb (GM35) before $1 \times 10^6$ PMNs were added to the basolateral surface. PMNs were allowed to migrate in the physiologically relevant basolateral to apical direction for 1 h in response to a 100 nM gradient of fMLF. The number of migrated PMNs (A) and the number of PMNs that were adherent to the apical epithelial surface (B) were quantified by MPO assay. Data are means ± SE ($n = 5$). Transmigration assays were also performed in the presence of apically applied GM35, GM35 F(ab')2, or isotype-matched binding control IgG1, with PMN exposed to 5 μg/ml functionally inhibitory F(ab')2 anti-FCR CD32 and CD16 mAbs (C). D, Confluent T84 monolayers were treated apically with 10 μg/ml GM35 (open squares) or vehicle (closed squares) before the addition of $1 \times 10^6$ PMNs. PMN transmigration was then measured over a 3-h time course. E, A total of $1 \times 10^6$ PMNs was added to confluent T84 monolayers pretreated apically with 10 μg/ml GM35, or isotype-matched binding control IgG1 before migration in the apical-to-basolateral direction was measured by MPO assay. F, Confluent HDMEC monolayers were pretreated apically with 10 μg/ml GM35 or isotype control IgG1 before the apical addition of $1 \times 10^6$ PMNs. PMNs were allowed to migrate for 1 h in response to a 10 nM gradient of fMLF. The number of migrated PMNs was quantified by MPO assay. Data are mean ± SE ($n = 3$). Significance was defined at $p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 

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PMN transmigration across Caco2 monolayers (Fig. 3A). Functional effects on T84 and HT29 cells, GM35 had no effect on HeLa, PMNs, or HDMECs (Fig. 3B). A significant increase in the GM35-mediated increase in PMN adhesion to T84 cells following treatment with IFN-\(\gamma\) was observed in T84 and HT29 cells but not in Caco2, SK-CO15, the GM35 ligand. Expression of the protein recognized by GM35 was observed in T84 and HT29 cells but not in Caco2, SK-CO15, HeLa, PMNs, or HDMECs (Fig. 3A). Also, in contrast to observed functional effects on T84 and HT29 cells, GM35 had no effect on PMN transmigration across Caco2 monolayers (Fig. 3B).

Identification of the GM35 protein Ag as CD44

Further experiments were performed to identify the Ag recognized by mAb GM35. A total of 500 cm\(^2\) confluent T84 cells were used to generate sufficient GM35 Ag, as detailed in Materials and Methods. Although protein identification was limited by the highly glycosylated nature of the GM35 Ag, four tryptic peptides resulting from mass spectrometry showed direct sequence homology with human CD44 (Fig. 4A). The polymorphic, though monogenic, CD44 family of proteins is composed of 19 exons, 10 of which (exons 1–5 and 15–19) are nonvariant and included in the ubiquitously expressed 80–95 kDa standard form of CD44, termed CD44s (32). The remaining variant exons can be differentially inserted into the mature mRNA via alternative splicing. Giving rise to tissue-specific variant CD44 isoforms, the expression of which has been reported in endothelial cells, epithelial cells, activated lymphocytes, and some tumor cells (33–36).

To demonstrate a direct requirement for CD44 binding in GM35 activity, HT29 cells were transfected with one of four gene-silencing shRNA plasmids specific for human CD44 (p313, p314, p315, p316) or with a control Scr plasmid. HT29 cells were chosen for transfection studies over T84 cells due to the difficulties of transfecting T84 epithelial cells by conventional methods. Knockdown of CD44 protein expression was verified by Western blot (Fig. 4B). Data show that transfection of HT29 cells with either of the CD44 gene-silencing plasmids p315 or p316 markedly reduced the expression of CD44v6 (a large glycosylated isoform of CD44 previously reported to be expressed in both T84 and HT29 cells [37]). In contrast, the Scr plasmid and two of the shRNA plasmids p313 and p314 failed to reduce the level of CD44v6 protein expression. Importantly, the same two shRNA plasmids that reduced expression levels of CD44v6 also resulted in a significant reduction in the expression of the GM35 Ag.

Having demonstrated a decrease in GM35 Ag expression upon CD44 knockdown, we next determined the functional effect of CD44 knockdown on the GM35-mediated increase in PMN adhesion. GM35 at 10 \(\mu\)g/ml significantly increased PMN adhesion compared with no Ab control in nontransfected cells (\(p < 0.001\)) and in HT29 cells transfected with a Scr plasmid (\(p < 0.01\)) (Fig. 4C). Transfection of HT29 cells with plasmids p313 or p314 (which had failed to significantly alter the expression of CD44 or the GM35 Ag; Fig. 4B) had no effect on the GM35-induced increase in PMN adhesion (data not shown).
In contrast, transfection of HT29 cells with plasmids that successfully knocked down expression of CD44 (p315 or p316) resulted in loss of the GM35-mediated increase in PMN adhesion.

This loss of the functional activity of the GM35 mAb following attenuation of epithelial CD44 expression is consistent with our observation that GM35 mediates its effects on PMN transmigration through interaction with epithelial expressed CD44.

Identification of the GM35 Ag as CD44v6

To determine the specific CD44 splice variant recognized by GM35, T84 and HT29 cell lysates were probed with Abs specifically raised against different variants of CD44. Immunoblotting with a panel of variant-specific CD44 Abs revealed that only an anti-CD44v6 Ab bound to a glycoprotein with a m.w. similar to that recognized by mAb GM35 (Fig. 5A). Conversely, anti-CD44v3, anti-CD44v4,5, anti-CD44v7, and anti-CD44v10 Abs recognized smaller nonglycosylated proteins, suggesting that the GM35 Ag represents an epithelial CD44v6. The binding of GM35 to CD44v6 was further explored through coimmunoprecipitation experiments. CD44v6 immunoprecipitates contained readily detectable amounts of the heavily glycosylated ~195 kDa GM35 ligand (Fig. 5B). By contrast, immunoprecipitates of CD44v4,5 (an isotype-matched variant CD44 mAb) did not contain the large glycosylated protein recognized by GM35. However, several smaller nonglycosylated proteins (between 75 kDa and 100 kDa) recognized by anti-CD44v4,5 mAbs were also recognized by GM35. These bands may represent nonspecific binding or may represent smaller proteins, different from the large 195-kDa CD44v6-containing proteins that are also recognized by GM35.

GM35 colocalizes apically with CD44v6

Examination of the subcellular colocalization of specific CD44 variant proteins with the GM35 Ag in nonpermeabilized T84 monolayers revealed a pattern of apical staining (Fig. 6A). In addition, the only variant form of CD44 found to colocalize apically with GM35 was CD44v6 (Fig. 6B). Analysis of protein localization in permeabilized T84 cells also revealed apical colocalization of CD44v6 and the GM35 Ag as well as basolateral colocalization of GM35 with CD44v6, CD44v3, and CD44v4,5 (data not shown). In addition, analysis of colocalization of GM35 and CD55 (an apically expressed protein previously implicated in PMN release from the apical surface of the epithelium) revealed only intermittent, patchy areas of colocalization, distinct from the almost complete colocalization observed between the GM35 Ag and CD44v6.

This apical colocalization of CD44v6 and the GM35 Ag is consistent with the interaction of GM35 and CD44v6, disrupting
PMN detachment from the apical epithelial surface. Examination of the expression of CD44v6 in uninflamed versus inflamed ulcerative colitis tissue demonstrated an increase in the expression of this variant form of CD44 in the inflamed tissue (Fig. 6C). This is in keeping both with the findings of others (38) and with the recognition of CD44v6 by GM35, as a similar pattern of tissue staining was also observed with mAb GM35 (Fig. 3C). Taken together, these data suggest that GM35 binding to apically expressed epithelial CD44v6 interferes with the process of PMN detachment from the luminal surface of the epithelium.

GM35 binding blocks PMN-dependent release of sCD44v6
Recent studies have identified a role for extracellular domain shedding in the functioning of CD44 variant proteins (39). Therefore, we sought to examine whether the release of sCD44v6 fragments occurs during PMN migration across epithelium. Analysis of the presence of CD44 proteins released by T84 epithelial cells during PMN transmigration (Fig. 7A, 7B) revealed significant levels of both CD44std and CD44v6 released between 30 and 60 min of PMN migration. Furthermore, levels of detectable protein were similar for both CD44s and CD44v6, suggesting rec-
ognition of a single v6 domain containing CD44 protein recognized by both capture Abs. To ascertain if apical exposure of T84 cells to GM35 affected the release of sCD44 protein fragments, assays in the presence of GM35 (10 μg/ml) were carried out. These data revealed that apical treatment of T84 cells with GM35 prevents the release of sCD44std and sCD44v6 (Fig. 7C, 7D). In contrast to the effects seen when GM35 is present for the duration of the migration assay, addition of mAb GM35 following migration of PMNs did not prevent the detection of sCD44s or sCD44v6 (data not shown). These data therefore demonstrated that GM35 did not prevent binding of released CD44 proteins to the capture or detection Abs of the ELISA, but rather that GM35 interfered with the release of sCD44 proteins. Taken together, these data suggest that binding of GM35 to CD44v6 prevents PMN-TEM–dependent release of CD44v6 and that this failure to release CD44v6 potentially contributes to the retention of PMN at the apical epithelial surface.

Discussion
Mucosal recruitment of PMNs involves sequential migration across endothelial, lamina propria, and epithelial barriers (40). Subsequent adhesion of PMNs to apical epithelial membranes can result in activated PMNs persisting in crypt abscesses, an event that has implications for disease states including inflammatory bowel disease, periodontitis, cystitis, and infectious enterocolitis (41). As epithelial proteins involved in the retention/release of PMNs have not been comprehensively characterized, the current study devised a monoclonal strategy to screen for apically expressed epithelial ligands that modulate the late stages of PMN migration. This strategy identified one mAb, GM35, that was found to regulate PMN detachment from the apical surface of epithelial cell monolayers. Specifically, treatment of confluent epithelial monolayers with GM35 significantly inhibited the terminal release of PMN from the apical epithelial surface.

Expansion of these observations identified the GM35 Ag as a member of the highly polymorphic, though monogenic, CD44 protein family. CD44 class I transmembrane glycoproteins have previously been implicated in cell–cell adhesion and cytoskeletal rearrangements, as well as in cell signaling, cell survival, and malignant transformation processes (42–44). This multifunctional capacity is facilitated by the expression of different CD44 isoforms generated both from alternative splicing and posttrans-
ational modifications of the extracellular domain of CD44 proteins.

The large m.w. and highly glycosylated nature of the GM35 Ag in the current study was highly suggestive of binding to a variant exon-containing form of CD44 rather than binding to the ubiquitously expressed 80–95 kDa nonvariant form of CD44 (CD44s). Further analysis revealed binding of GM35 to CD44v6. The expression of CD44v6 in epithelial cells reported in the current work is supported by previous studies that report that alternative splicing of CD44 is mediated by tissue-specific factors with certain variant exon products being differentially expressed (or at least predominantly found) on defined tissues, such as epithelium (45–48). Further, the expression of the GM35 ligand in T84 and HT29 cells but not in other epithelial cell lines is in keeping with its binding to CD44v6, as it has been reported that this large, heavily glycosylated protein is expressed in these epithelial cell lines but not in Caco2 colon cells (37). This cell-specific expression pattern of the glycoprotein recognized by GM35 in the current study is supported by other previously reported cell-specific differences in protein glycosylation (49, 50). Further, glycosylation and more specifically the oligosaccharides that decorate cell surface O- and N-linked glycoproteins have previously been shown to play an important role in the regulation of leukocyte trafficking (51).

Our results also indicate that expression of the GM35 ligand is increased by treatment of T84 cells with IFN-γ. It has previously been reported that the expression of CD44v6 in epithelial cells increases during the inflammatory response associated with inflammatory bowel disease (52) and more specifically during the inflammatory response associated with ulcerative colitis (38). One specific feature of CD44 variant proteins (including CD44v6) that may be related to the mechanism of GM35 described in the current study is the extracellular proteolytic cleavage of CD44 proteins in response to stimuli. Such proteolytic cleavage of CD44 has recently emerged as a key mechanism underlying its functional regulation, but the biological effects of this event and the proteases involved have not been comprehensively characterized (39). In the current study, we demonstrate the release of sCD44v6 protein during PMN transmigration and further that GM35 prevents this release of sCD44v6. It is therefore plausible that the release of the CD44v6 extracellular domain from the apical surface of the epithelium can facilitate PMN detachment and that the GM35-mediated blockade of CD44v6 release results in the observed PMN accumulation at the apical epithelial surface.

Although it has been previously suggested that the expression of specific isoforms of CD44 plays a role in the regulation of the immune response, as well as in the development of autoimmune disorders (33, 53, 54), the mechanisms whereby CD44 variants govern leukocyte recruitment have not been fully characterized. It has been previously detailed that CD44v3 expressed basolaterally on epithelial cells regulates transmigration via binding to PMN CD11b/CD18 (5). However, the basolateral expression and m.w. of this CD44v3 isoform show it to be a different CD44 variant than the one recognized by GM35 in the current study. In addition,
The authors have no financial conflicts of interest.

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Disclosures

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