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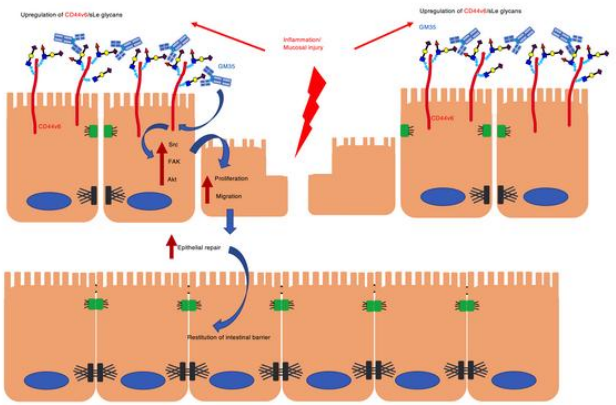
Graphical abstract



Targeting Epithelial Expressed Sialyl Lewis Glycans Improves Colonic Mucosal Wound Healing and Protects against Colitis.

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Abstract

Dysregulated healing of injured mucosa is a hallmark of many pathological conditions including inflammatory bowel disease. Mucosal injury and chronic intestinal inflammation are also associated with alterations in epithelial glycosylation. Previous studies have revealed the inflammation induced glycan sLe^a on epithelial CD44v6 acts as a ligand for transmigrating PMN. Here we report that robust sialylated Lewis glycan expression is induced in colonic mucosa from individuals with ulcerative colitis (UC) and Crohn's disease (CD) as well as in colonic epithelium of mice with DSS colitis. Targeting of sialylated epithelial Lewis glycans with mAb GM35 reduced disease activity and improved mucosal integrity during DSS induced colitis in mice. Wound healing studies revealed increased epithelial proliferation and migration responses as well as improved mucosal repair following ligation of epithelial sialyl Lewis glycans. Finally, we show GM35-mediated increases in epithelial proliferation and migration are mediated through activation of kinases that signal downstream of CD44v6 (Src, FAK, Akt). These findings suggest that sialylated Lewis glycans on CD44v6 represent epithelial targets for improved recovery of intestinal barrier function and restitution of mucosal homeostasis following inflammation or injury.

Introduction

The intestinal epithelium forms a critical barrier separating luminal antigens from underlying mucosal tissues. Epithelial wounds in the intestine resulting from mechanical damage or inflammation must be efficiently repaired in order to maintain gut homeostasis and ensure adequate absorption of nutrients. Mucosal wound closure in the gut is mediated by a complex spatiotemporal interplay between intestinal epithelial cells (IECs) and innate and adaptive immune cells that are recruited into damaged tissues. Re-epithelization of denuded intestinal mucosa is facilitated by migration of epithelial cells from wound-adjacent crypts over the wound bed accompanied by epithelial proliferation and differentiation to re-establish tissue architecture and barrier function (1-3).

Epithelial cell migration is a complicated process that is, in part, facilitated by binding interactions between migrating cells and underlying extracellular matrix. Motility of epithelial cells also requires activation of intracellular signaling cascades triggered by engagement of cell surface receptors. The CD44 family of cell adhesion molecules play a well described role in epithelial cell-cell and cell-matrix adhesive interactions (4). In particular the heavily glycosylated cancer associated CD44 family member CD44v6 is known to play an important role in epithelial migration and proliferation (5). In previous studies investigating mechanisms of intestinal inflammation and repair, we produced an anti-sialyl Lewis glycan mAb (GM35) that specifically recognizes O-glycan linked sialylated Lewis glycans expressed on CD44v6 in inflamed human intestinal mucosa. Further, in addition to marking inflamed colonic mucosa, GM35-targeting of epithelial sialyl Lewis A (sLe^a)/CD44v6 blocked transepithelial migration of human neutrophils. Importantly, these studies revealed that, in addition to binding the human glycan sLe^a, GM35 also recognizes an additional Lewis glycan family member (sialyl Lewis c [sLe^c]) which serves as a

precursor to sLe^a and is expressed by both humans and mice. Furthermore, targeting of sialyl Lewis glycans on CD44v6 with GM35 *in vivo* blocked PMN transmigration to the small intestine and improved epithelial barrier function (6, 7).

The importance of glycan mediated binding events for restoration of mucosal homeostasis following injury is supported by studies on Galectin 2 and Galectin 4 that have been shown to promote re-epithelization of intestinal wounds by enhancing binding interactions between epithelial cells and β-galactoside-containing glycans on cell surface and matrix glycoproteins (8). Similarly, other studies have demonstrated a role for cell surface N-glycans in organizing cell-cell junction dynamics and promoting epithelial cell motility (9). However, despite previous reports of glycan regulated epithelial mobility and intestinal wound repair, the potential of epithelial sialyl Lewis glycans as targets for amelioration of intestinal inflammation and promotion of mucosal wound healing have not been explored to date.

In this study, we demonstrate that antibody mediated targeting of sialyl Lewis glycan epitopes on epithelial CD44v6 protects against DSS-induced colitis and promotes repair of damaged intestinal mucosa *in vivo*. Data demonstrate that protective or pro-repair effects of GM35 are not mediated by reduced PMN mucosal trafficking but rather are a direct consequence of increased epithelial migration and proliferation responses triggered by sialylated Lewis glycan engagement. As delayed wound repair is a pathological hallmark of multiple conditions including inflammatory bowel disease (IBD) as well as anastomotic failure, targeting inflammation-induced carbohydrate signatures including Sialyl Lewis glycans to reduce inflammation and restore gut homeostasis may represent a promising new therapeutic opportunity.

Results

Increased expression and co-localization of sialyl Lewis glycans and CD44v6 in inflamed colonic mucosa

Previous studies have shown that the anti-glycan mAb GM35 binds to sLe^a on CD44v6 expressed by inflamed epithelium and reduces PMN TEpM (6, 7). However, the potential of sialylated Lewis glycans as targets to reduce intestinal inflammation *in vivo* has not been explored to date. Immunohistochemical analyses of normal intestinal mucosa (Fig. 1A) or non-involved/non-inflamed intestinal mucosa from patients with CD (Fig. 1C) revealed minimal basal expression of sialylated Lewis glycans. In contrast, significantly increased expression of sialylated Lewis glycans was detected by mAb GM35 in inflamed intestinal mucosa from patients with UC and CD (Fig. 1B,D,E **, $p<0.01$). Immunofluorescence analyses were performed to determine expression and localization of sialyl Lewis glycans/CD44v6 in murine colonic mucosa. In normal colonic epithelium, expression of sialyl Lewis glycans (green) and CD44v6 (red) were detected in crypt bases, consistent with previous reports identifying CD44v6 as a marker of crypt cells in normal murine intestinal mucosa (10). Low levels of expression of sialyl Lewis glycans/CD44v6 was also detected at the very apical or luminal aspect of the colonic intestinal epithelium (white arrows, Fig. 1F). Co-localization of sialyl Lewis glycans and CD44v6 was also observed (merged images, Fig. 1F). Importantly, in inflamed epithelium, detection of sialyl Lewis glycans and CD44v6 was increased, with expression observed along the entire length of colonic crypts. Furthermore, consistent with specific recognition of glycans on CD44v6 by GM35, co-localization of CD44v6 and sialyl Lewis glycans was observed along the length of inflamed colonic epithelial crypts *in vivo*. In contrast, there was no co-localization of sialyl Lewis glycans recognized by GM35 with the highly O-glycosylated mucin protein and goblet cell marker Muc-2 in normal or

inflamed intestinal mucosa (Fig. 1G). Furthermore, Muc-2 staining revealed the expected loss of goblet cells following DSS induced colitis (11).

Targeting sialyl Lewis glycans on CD44v6 protects against DSS-induced colitis

Given the observed inflammation induced increases in sialyl Lewis glycans on epithelial CD44v6, experiments were performed to probe the functional effect(s) of targeting these glycoepitopes *in vivo*. Disease progression in DSS colitis was assessed in mice treated with GM35 to ligate sialylated Lewis glycans. Systemic treatment with mAb GM35 (to target sialyl Lewis glycans on CD44v6) significantly decreased disease severity relative to mice treated with isotype control IgG mAb on days 5 to 8 in the DSS model of acute colitis (**, $p<0.01$, ***, $p<0.001$, Fig. 2A). Analysis of body weight revealed that GM35 treatment prior to and during 2.5% DSS administration significantly reduced weight loss compared to control IgG treated animals (*, $p<0.05$, Fig. 2C). In addition, histological analyses of colonic sections from mice treated with GM35 revealed that decreased disease activity and weight loss scores correlated with a significant reduction in mucosal injury (*, $p<0.05$, Fig. 2D, E). Importantly, GM35-treated mice showed less histological evidence of injury, highlighted by preservation of surface epithelium and significantly less mucosal ulceration. In contrast animals treated with isotype control mAb + 2.5% DSS had large areas of ulceration together with increased numbers of infiltrated immune cells (Fig. 2D, black arrows). Analysis of PMN migration into the proximal colon in response to a solution of luminally applied LTB_4 revealed no difference in transmigrated PMN mediated by GM35 (Fig. 2F,G), suggesting that protective effects observed during DSS colitis are not a result of reduced PMN trafficking.

In order to determine if GM35 targeting of epithelial sialyl Lewis glycans was protective against colitis without mAb pre-treatment, experiments were performed in which mice were

exposed to DSS followed by treatment with GM35 on days 1,3,6 and 8. As was observed in Fig. 2, GM35 targeting of sialylated epithelial Lewis glycans significantly reduced DSS induced weight loss and mucosal injury compared to IgG or PBS treated control mice (*, $p<0.05$ **, $p<0.01$, Suppl. Fig. 1). Taken together, these findings suggest that epithelial sialyl Lewis glycans on CD44V6 are upregulated during inflammation *in vivo* and can be selectively targeted to reduce disease severity during colitis.

Targeting sialyl Lewis glycan epitopes on CD44v6 improves healing of biopsy-induced mucosal wounds in vivo

We next examined direct effects of targeting sialyl Lewis glycans with mAb GM35 on colonic wound healing *in vivo* (schematic, Fig. 3A) using a well characterized biopsy wounding model (12). As can be seen in Fig. 3B, direct injection of GM35 in wound beds 24 hours post biopsy resulted in significantly increased rates of wound closure one day later (day 2) compared to injection with an IgG ctrl mAb (34% ± 1.2 vs 25% ± 1.7, **, $p<0.01$). A similar statistically significant increase in wound healing downstream of wound bed injection of GM35 was also observed 72 hours post biopsy/Day 3 (46% ± 3.7 vs 36% ± 1.9, ****, $p<0.0001$). Similarly, systemic treatment (intraperitoneal injection of GM35) resulted in significantly increased colonic mucosal wound healing relative to injection of an IgG control mAb on day 2 (42% ± 2.7 vs 28% ± 1.7, ****, $p<0.0001$) and day 3 (50% ± 3.3 vs 38% ± 2.2, ****, $p<0.0001$) post injury (Fig. 3C,D). These data demonstrate that sialyl Lewis glycans can be selectively targeted *in vivo* to significantly enhance mucosal wound closure. Furthermore, these findings suggest that inflammation induced intestinal glycans represent potential targets to improve mucosal repair in chronic conditions such as IBD.

Given that previous *in vitro* studies have shown inhibition of PMN migration across monolayers of human IEC downstream of targeting sLe^a on CD44v6 (7), we examined effect(s) of GM35 treatment on immune cell infiltration of mucosal wounds *in vivo*. Consistent with the lack of inhibition of PMN trafficking to the colon mediated by GM35 (Fig. 2 G, F), flow cytometric analyses of colonic wound infiltrating myeloid cells revealed no significant differences in recruitment of neutrophils, monocytes, macrophages or eosinophils to wounds following treatment with GM35 compared to an IgG control mAb (Suppl. Fig. 2). Importantly these findings highlight that GM35 mediated improvement in colonic mucosal wound healing are a result of epithelial rather than immune cell mediated effects. Indeed, no expression of sLe^a/sLe^c on neutrophils, monocytes, B cells, T cells, basophils or eosinophils was observed (Suppl. Fig. 3), demonstrating restricted epithelial expression of sialylated Lewis glycans on CD44v6 recognized by GM35 *in vivo*. In addition to improved mucosal repair, labeling of wounds with GM35 and anti-CD44v6 mAbs revealed increased expression of sialyl Lewis glycans/CD44v6 along the entire length of epithelial crypts directly adjacent to wound beds (Fig. 3E) consistent with the induction of expression of sialylated Lewis glycans observed during DSS-induced colitis (Fig. 1B). Furthermore, epithelial co-localization of sialyl Lewis glycans and CD44v6 was observed. Taken together these results highlight the increased nature of expression of sialylated Lewis glycans on epithelial CD44v6 following wounding or inflammation in the colon.

Given that coordinated epithelial cell proliferation and migration are necessary for mucosal wound healing, we examined effects of targeting sialyl Lewis glycans on IEC proliferation focusing analyses on crypts adjacent to biopsy induced mucosal wounds. As can be seen in Fig. 3F, intraperitoneal injection of GM35 resulted in a significant increase in the number of proliferating epithelial cells in wound-adjacent crypts compared to IgG injected controls (66% ±

5.3 vs 51% ± 7.02, ***, p > 0.001). These results suggest that increased epithelial migration and proliferation contributes to faster wound repair downstream of sialyl Lewis glycan targeting *in vivo*.

Targeting sialyl Lewis glycans epitopes on CD44v6 by GM35 increases migration and proliferation of human intestinal epithelial cells

Given the increased colonic wound healing observed downstream of targeting of sialyl Lewis glycans *in vivo*, we performed experiments directed at analysis of wound closure using monolayers of human epithelial cells. IEC monolayers were treated with GM35, a second commercially available anti-sLe^a mAb NS19-9, an anti-peptide CD44v6 mAb or an IgG control mAb. As can be seen in Fig. 4A, treatment of T84 IECs with GM35 or NS19-9 resulted in significantly enhanced wound closure 12-, 18- and 24-hours post wounding when compared to control IgG treated or anti-CD44v6 mAb treated epithelial cells (* $p<0.05$, ** $p<0.01$). In addition, staining of T84 IEC monolayers 24 hours after scratch wounding revealed expression and co-localization of GM35 binding sialylated Lewis glycans and CD44v6 at the wound edge (Fig. 4B). Similar to effects observed in T84 IECs, increased epithelial migration/wound closure was also observed in 2D monolayers of primary human colonoid derived epithelial cells (Fig. 4C) after targeting sialyl Lewis glycans with GM35 (77.2% ± 3.4 vs 59.8 ± 7.0, **, $p<0.01$).

Given the observations of sialyl Lewis glycan mediated increases in epithelial proliferation *in vivo* (Fig. 3F), we examined epithelial proliferation rates 18 hours after wounding of T84 IECs (Fig. 4D, E). Consistent with *in vivo* results, treatment of human epithelial cells with GM35 resulted in a significant increase in the percentage of proliferating cells near wound edges compared to IgG treated controls (38% ± 6.9 vs 27% ± 5.6, **, $p<0.01$). A similar increase in

proliferation was observed after treatment with another sLe^a binding mAb NS19-9 (39% ± 7.8 vs 27% ± 5.6, **, $p<0.01$). These findings demonstrate that targeting of sialylated Lewis glycans upregulated on wounded human epithelium results in increased rates of proliferation and migration thereby facilitating mucosal repair.

Targeting of sialyl Lewis glycans activates intracellular signaling in human IECs

CD44v6 is a known regulator of intracellular signaling events important for proliferation and migration of human epithelial cells (13). Therefore, we assessed the effect of targeting sialyl Lewis glycans on human epithelial intracellular signaling following injury. As shown in Fig. 5A, there was increased phosphorylation of Src at Tyr416 18 hours following wounding of human epithelial cells treated with GM35 compared to an IgG control. Similarly, there was increased activation of FAK (Tyr925) and Akt (Thr308 and Ser473) in IECs following ligation of sialyl Lewis glycans on CD44v6 by GM35. By contrast, no activation of p38 MAPK was observed downstream of sialyl Lewis glycan engagement highlighting the specificity of signaling events mediated by GM35. Collectively, these findings support a model shown in Fig. 5B whereby GM35 enhances wound closure by binding to sialyl Lewis glycans on intestinal epithelial CD44v6 to activate intracellular signaling pathways implicated in the regulation of epithelial proliferation and migration.

Discussion

Delayed intestinal epithelial wound repair is a pathological hallmark of ulcerative colitis (UC) and Crohn's disease (CD). Chronic intestinal inflammation is also associated with epithelial specific alterations in glycosylation (7). Using mAb GM35 (which is specific for O-glycan linked sialylated Lewis glycans on epithelial CD44v6) (6, 7) we show robust epithelial expression of immature sialylated glycans in inflamed colonic mucosa from individuals with UC and Crohn's disease. This is in contrast to the lack of sLe^a expression detected in normal non-inflamed colonic mucosa and is supported by previous studies demonstrating overexpression of immature glycans in individuals with active IBD (6, 7, 14). In keeping with these data, our previous studies have also demonstrated specific upregulation of epithelial CD44v6/sLe^a mediated by the proinflammatory cytokine IFNγ (6). Furthermore, previous studies have demonstrated increased sLe^a expression in inflamed pancreatic epithelial cells (15, 16) suggesting the importance of sLe^a as a marker of inflammation in multiple epithelial lined organs.

Previous work has shown that in addition to binding to the human glycan sLe^a, GM35 also recognizes a second glycan; the non-fucosylated biosynthetic precursor of sLe^a termed sialyl Lewis c (sLe^c) which is expressed in both humans and mice (17-19)]. We observed increased expression of sLe^c and CD44v6 in inflamed murine colonic mucosa, demonstrating that immature or less structurally complex sialylated glycans are indeed markers of epithelial inflammation in both humans and mice. In addition, as has been reported for inflamed human intestinal epithelium, co-localization of murine sialyl Lewis glycans and the epithelial specific CD44 variant CD44v6 was observed suggesting protein specific decoration with these neoglycans in inflamed intestinal mucosa *in vivo*. Similar co-localization of sLe^a and CD44v6 has been reported in inflamed cervical and intestinal epithelial cells (7, 20). The importance of CD44v6 as a marker of epithelial stress is

further highlighted by studies showing that CD44v6 expression is restricted to inflamed or squamous epithelia (21, 22), and that epithelial CD44v6 expression strongly associates with disease activity in ulcerative colitis (23, 24).

Importantly, in addition to its use as a marker of inflamed intestinal epithelium, here we show that systemic treatment with mAb GM35 reduces ulceration associated with DSS induced colitis in mice. This is consistent with previous studies showing protection of epithelial barrier *in vivo* downstream of sialyl Lewis glycan targeting (7). In support of the importance of glycan mediated signaling events during colitis, others have reported that plant derived polysaccharides ameliorate colitis *in vivo* by suppressing NF-kB signaling and NLRP3 inflammasome activation (25, 26). The relevance of glycans as targets to better understand and control mechanisms of intestinal inflammation is further highlighted by studies demonstrating increased levels of highly sialylated glycans in plasma of individuals with IBD (27, 28). Furthermore, mice with impaired fucosyltransferase gene expression or deficiencies in core-1 derived O-glycans develop an IBD-like spontaneous colitis (29). Chronic inflammation in the gastrointestinal tract is also associated with microbiota induced changes in host glycosylation that can result in enhanced entry of bacteria and dietary lectins into host tissues (30). Taken together, these studies demonstrate that glycans play a key role in processes fundamental to IBD pathogenesis and that sialylated O glycans on CD44v6 represent inflammation induced druggable targets to ameliorate intestinal inflammation.

In addition to protective effects on disease course during colitis, we demonstrated increased epithelial proliferation and migration and improved intestinal epithelial wound closure downstream of targeting sialylated Lewis glycans on CD44v6 *in vivo* and *in vitro*. Critically no effects on epithelial migration were observed downstream of targeting CD44v6 with anti-peptide mAbs highlighting the importance of CD44v6 expressed sialylated glycans in mediating pro-repair

effects in the intestinal mucosa. We observed increased expression and co-localization of sialyl Lewis glycans/CD44v6 specifically in epithelial crypts directly adjacent to biopsy induced wounds *in vivo*. Consistent with these findings, other studies have demonstrated that, after mechanical damage of bronchial epithelial cells, CD44v6 expression increased in areas close to the wound edge (30). A similar localized increase in CD44v6 expression has been reported in damaged areas of lung tissue in asthmatic individuals (31). Injury induced expression of CD44v6 has also been reported in liver stellate cells and, after activation, these cells engage hyaluronic acid in a CD44v6 dependent manner to enhance cellular momentum (32). In addition to increasing epithelial migration, expression of CD44v6 also correlates with higher proliferative activity in human bladder and pancreatic epithelial cells (33) (34).

Here we report changes in post translational glycosylation in addition to increased protein expression of CD44v6 following colonic epithelial injury. The importance of glycosylation to wound repair is highlighted by previous studies reporting glycan mediated increases in epithelial proliferation and wound repair. Specifically, plant root polysaccharides have been shown to promote proliferation, migration and cell cycle progression in murine and human models of skin wounding (35). Similarly, β-glucan polysaccharides promote migration of dermal fibroblasts resulting in improved healing of murine skin wounds (36). Previous studies have also highlighted a role for sialylated glycans in enhancing epithelial mobility. As such, treatment of mammary carcinoma cells with sialidase to remove terminal sialic acids resulted in decreased epithelial cell matrix adhesion interactions and reduced cell motility (37). Other studies have shown that cell surface sialyloglycans on human colon cancer cells regulate intercellular adhesion events and play an important role in tumor migration. Taken together, these studies support the importance of

sialylated Lewis glycan/CD44v6 mediated cellular adhesion events in promoting epithelial migration and proliferation and increasing mucosal wound healing in the intestine.

It is well documented that CD44v6 ligation activates multiple downstream intracellular signaling molecules to modulate epithelial cell function (38, 39). Furthermore, several kinases including Akt, Src and FAK have been identified as being downstream signaling mediators of CD44v6 (40-44). In support of these previous reports, we show intestinal epithelial activation of Src, FAK and Akt downstream of GM35-mediated ligation of sLe^a on CD44v6. Our observations are consistent with previous work reporting that increased CD44v6 expression results in Akt activation in oral epithelial cells (43) and another study with pancreatic epithelial cells reporting downstream signaling through FAK and PI3K following engagement of CD44v6 (40).

The data presented here demonstrate inflammation induced increases in expression of immature sialylated Lewis glycans on intestinal epithelial expressed CD44v6, and that selective antibody mediated targeting of these glycans on colonic epithelial cells activates intracellular signaling pathways known to promote epithelial proliferation, migration and wound closure (40, 43). While commercial antibodies for sLe^a are available, they have a much lower affinity than mAb GM35 (7) and, importantly, do not recognize the murine glycan sLe^c (http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_3256). Taken together, these findings suggest that targeting sialylated Lewis glycans on epithelial CD44v6 may represent a promising new therapeutic strategy for improved recovery of epithelial barrier function and restitution of intestinal homeostasis following inflammation or injury.

Methods

Animals

Experimental studies were performed in C57BL/6 WT mice bred at University of Michigan Animal resource facilities or purchased from Jackson Laboratories. Animals (10-14 weeks, both sexes) were maintained under specific pathogen-free conditions with 12-hours day/night cycle and access to food and water ad libitum.

Epithelial cell lines and human colonoids.

T84 IECs were grown under standard conditions as previously described (45). Human colonoids were generated from biopsies of normal human colonic mucosa as previously described (46, 47). All human colon sample collection was performed in accordance with the University of Michigan Institutional Review Board regulations. Isolated colonoids were re-suspended in matrigel and cultured in growth media (50% L-WRN conditioned media:50% Advanced DMEM/F-12, 10% FBS, 2mM GlutaMax, 10mM HEPES, N-2 media supplement, B-27 Supplement, 1mM N-Acetyl-L-cysteine, 50ng/mL huEGF, 100 units/mL Penicillin, 0.1 mg/mL streptomycin, 500nM A83-01, 10μM SB202190, 10mM Nicotinamide, 10nM Gastrin). To generate 2D monolayers, colonoids grown as described above were spun out of matrigel and dissociated into a single cell suspension according to published protocols (48). Following one day in complete growth media (as described above) epithelial cells were switched to differentiation media (growth media minus Wnt3a, R-Spondin, Nicotinamide and SB202190 and with a 50% reduction in Noggin) for 4-5 days of differentiation into confluent monolayers of colonoid epithelium.

Antibodies

mAb GM35 was isolated as described previously (6, 7). Monoclonal anti-human CD44v6 mAb was purchased from R&D systems (Minneapolis, MN, catalog # BBA1) and anti-mouse CD44v6 mAb was ordered from eBioscience (Waltham, MA, catalog # BMS145). Monoclonal anti-sLe^a mAb NS19-9 was purchased from Origene (Rockville, MD, catalog # 190083). Anti Muc-2 mAb was purchased from Abcam (Cambridge, MA catalog # ab90007).

DSS-induced colitis and histological scoring

For DSS colitis experiments, mice were intraperitoneal injected with 250µg of mAb GM35 or isotype control mAb (BD Biosciences, 554721) one day before and 2 days after administration of Dextran Sodium Sulfate (DSS). 2.5% DSS was added to drinking water and disease activity was calculated daily for 8 days by measuring body weight, stool consistency and the presence of occult blood (49). For H&E staining, six-micron sections were used. Two independent investigators analyzed histology for total length as well as areas of inflammation and ulceration by Aperio ImageScope 12 (Leica Biosystems). Subsequently, percentages of inflamed and ulcerated areas were calculated and a final score generated using a validated scoring system (50).

For DSS colitis experiments with no mAb pretreatment, mice were placed on 3% DSS for 5 days followed by 4 days of water. Mice were intraperitoneal injected with 250µg of mAb GM35 or isotype control mAb (BD Biosciences, 554721), or PBS alone on days 1, 3, 6 and 8 after administration of DSS. Disease activity and mucosal injury scores were calculated as described above.

PMN TEpM into the colon in vivo

Colon loop experiments were performed with C56BL/6 WT mice (8-12 weeks, both sexes) which were maintained under standard conditions with 12-hour day/night cycles and ad libitium

access to food and water. To quantify PMN TEpM into the proximal colon *in viv*o, a recently published protocol was applied (12). Briefly, mice were pre-treated with proinflammatory cytokines before a 2cm loop of vascularized proximal colon was injected with 1nM LTB4 with GM35 or an IgG ctrl mAb before migration of PMNs into the intestinal lumen was quantified by flow cytometry.

In vivo wounding of colonic mucosa

Biopsy wounding of colonic mucosa in mice was performed as previously described (51). Briefly, mice were anesthetized by an intraperitoneal injection of a ketamine (100mg/kg)/xylazine (10mg/kg) solution. Biopsy-induced injuries of the colonic mucosa were made along the mesenteric artery using a high-resolution, miniaturized colonoscope system equipped with a biopsy forceps (Colorview Veterinary Endoscope; Karl Stortz). 6-10 lesions were generated per animal. Endoscopic procedures were viewed on days 1, 2 and 3 post-injury with high resolution images (1,024 x 768 pixels) on a flat-panel monitor. 250µg mAb GM35 or isotype control antibodies (BD Biosciences, catalog #554721) were intraperitoneal injected one day after biopsy wounding. For wound bed injections 10µg of mAb GM35 or isotype control mAb were directly injected into wound beds using a modified 30-gauge needle (30G½; Becton Dickinson). To analyze proliferation, antibodies were intraperitoneal injected immediately after wounding. Subsequently, mucosal wounds and intact tissues were harvested for immunofluorescence labeling, immunoblotting and flow cytometry analyses.

Lamina propria isolation and immune cell staining

To isolate immune cells from wounds, five wounds per animal were removed by punch biopsy (3mm, Disposable Biopsy Punch, Integra Miltex, catalog #33-32) and pooled together.

Tissues were minced and digested with Liberase TM (2.5 mg/ml; Roche Diagnostics) and DNAse I (MilliporeSigma) under mechanical agitation. After filtering, cell suspensions were transferred to a 96-well plate and stained for indicated immune cell populations. For quantification, a NovoCyte Flow Cytometer (ACEA Bioscience) was used and data analyses were performed using FlowJo v10 software. After exclusion of cell doublets by gating FSC area (FSC-A) versus FSC height (FSH-H), cells were initially gated for CD45+ events as well as CD11b expression to only include myeloid cells. Following that, cells were gated for Ly6G-1A8 (PMNs) and SiglecF (eosinophils). Ly6G-/SiglecF- double negative cells were further gated for f4/80 (macrophages) and Ly6C (monocytes).

Immunofluorescence and Immunohistochemistry

For immunofluorescence labeling with mAb GM35 and anti-CD44v6 mAbs (eBiosciences, catalog # BMS145), tissues were formalin fixed and embedded in paraffin. Sections were cut at 5µm-thickness followed by deparaffinization, rehydration and antigen retrieval with Antigen Unmasking Solution (1:100, Vector laboratories, catalog # H3301). After blocking in 1% Bovine Serum Albumin (Sigma-Aldrich, catalog # A3059), sections were incubated with primary antibody at 4°C overnight. The next day, slides were incubated with secondary antibody (1:500) for 45 minutes and Hoechst stain (1:1000, Invitrogen, catalog # H3570) to visualize nuclei before being mounted with ProLong Gold antifade reagent (Invitrogen, catalog# P36934). For Ki67 staining (Abcam, ab15580), OCT-embedded sections (6µm) were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100 (Millipore Sigma). After blocking, sections were incubated with primary antibody Ki67 (1:100) at 4°C overnight. Subsequently, slides were incubated with a secondary antibody (1:500) and Hoechst stain (1:1000) before mounting with ProLong Gold antifade reagent. Immunofluorescence images were taken by confocal microscopy

(Leica SP5 Inverted 2-Photon FLIM Confocal). ImageJ (NIH) was used for image processing. For human tissue staining, paraffin embedded sections (6μm) slides of discarded resections from patients with UC, CD or negative colorectal screens were obtained with approval from the University of Michigan Institutional Review Board (IRB). Tissue sections were incubated with GM35 at 10μg/ml followed by hematoxylin and eosin staining. Images were captured using an IX73 microscope system.

Human IEC monolayer wounding

Migration of T84 IECs and human colonoid derived monolayers was assessed using a scratch wound assay as previously described (52). 10μg/ml GM35, NS19-9, anti-CD44v6 mAb or IgG ctrl mAbs were added directly after wounding and epithelial migration/wound healing measured after 24 hours. For immunoblot analysis, confluent T84 cell monolayers grown in 6-well tissue culture plates were grid wounded to enrich for migrating and spreading cells as previously described (51).

Epithelial cell proliferation Assays

Confluent T84 IEC monolayers were scratch wounded with a sterile pipette tip (as described above) before 18h treatment with 10μg/ml GM35, NS19-9, or IgG ctrl mAbs. One hour before fixing of cells, EdU was added to the media at a concentration of 10μM. Proliferating cells were detected with the Click-iT EdU Alexa Fluor 594 Imaging kit (Life Sciences, Thermo Fisher Scientific; catalog # C10639) according to manufacturer's instructions and captured using a Leica SP5 confocal microscope (Leica Microsystems).

Cell lysis and Immunoblotting

For cell lysis, IECs were harvested in RIPA buffer (0.5% Triton X-100, 0.5% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 150mM NaCl, 1mM EGTA [pH 8.0], 0.2mM sodium orthovanadate, 20mM Tris [pH 7.4]) supplemented with protease and phosphatase inhibitors. Immunoblotting was performed as described previously (7, 53).

Statistics

Statistical analyses were performed using Prism software (GraphPad Software Inc.). 2-tailed Student's t-test was used in case of parametric parameters. For non-parametric data, differences were evaluated by Mann-Whitney U test or 1-way ANOVA followed by Tukey post-hoc test. $p<0.05$ was considered statistically significant. Data are presented as means ± SEM. All results show data from at least three independent experiments.

Study approvals

All experimental procedures involving animals were conducted in accordance with NIH guidelines and protocols approved by the University Committee on Use and Care of Animals at the University of Michigan.

Author contributions:

MK, MQ, VA, KB and JCB designed the study, performed data collection and data analysis/interpretation. JCB wrote the manuscript. RDC provided assistance with interpretation of glycan binding data. AN and CAP provided assistance in writing the manuscript.

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Figure Legends

Figure 1. Increased expression of CD44v6 and Sialyl Lewis glycans in inflamed murine and human intestinal mucosa. Immunohistochemical analysis of healthy colonic mucosa and colonic mucosa from patients with IBD. Representative images from normal uninflamed mucosa (A) is compared to active UC (B). Non-inflamed mucosa (C) and adjacent inflamed tissue (D) from Crohn's disease are also compared. (E) Quantification of intensity of staining from n = 3 normal colonic mucosa or colonic mucosa from n = 3 patients with UC or CD. Data are means ± SEM and were analyzed by one- 1-way ANOVA followed by Tukey post hoc testing. **, $p<0.01$. Expression of CD44v6 (red), Muc-2 (red) or sialyl Lewis glycans stained with GM35 (green) in representative images of normal colonic mucosa (F) and inflamed colonic mucosa (G) of WT C57BL/6J mice given 2.5% DSS for 5 days. Images are representative of n = 3 independent experiments. Scale bars are 10μm.

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Figure 3. GM35 improves healing of biopsy-induced colonic wounds *in vivo*.

(A) Schematic overview of experimental design timeline. Animals were wounded on Day 0 and treated with GM35 or a control IgG on Day 1 via intraperitoneal injection (250μg/animal) or microinjection directly into the wound bed (10μg/wound). (B) Percentage of wound healing in animals injected with IgG (blue) or GM35 (red) directly into wound beds. Data are means ± SEM; n = 3 independent experiments. ** $p<0.01$, ****, $p<0.0001$. Mann-Whitney test for Day 2 and unpaired t-test for Day 3. (C) Percentage of wound healing in animals intraperitoneal injected with 250μg control IgG (blue) or GM35 (red). Data are means ± SEM (n = 3 independent experiments) and were analyzed by 1-way ANOVA followed by Tukey post hoc testing. ****, $p<0.0001$. (D) Representative images of wounds at Day 1 and Day 3 following intraperitoneal injection with IgG ctrl Ab or GM35. (E) Representative immunofluorescence images of colonic wounds stained with anti-CD44v6 mAb (red) and GM35 (green). Scale bars are 10μm. Magnification of area adjacent to wound (yellow square) demonstrates expression and co-localization of CD44v6 (red) and sialyl glycan epitopes (GM35, green). (F) Representative immunofluorescence images with proliferation marker Ki67 (pink) in wound adjacent epithelial crypts following 24 hours of treatment with IgG or GM35. Nuclei are stained in blue. Graph shows quantification of Ki67-positive cells in wound adjacent crypts following treatment with IgG or GM35. Scale bars are 5μm. Data is mean ± SEM

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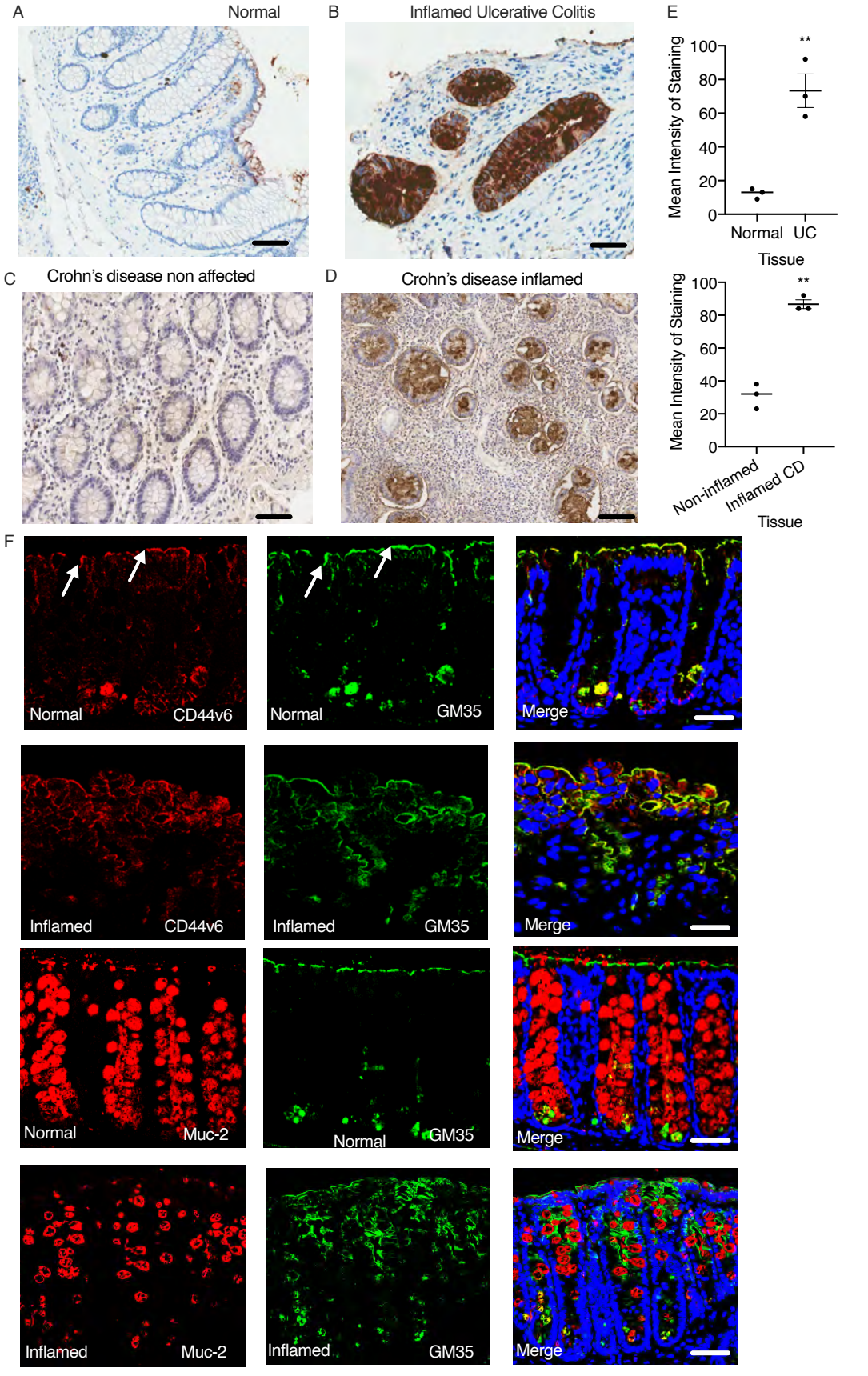

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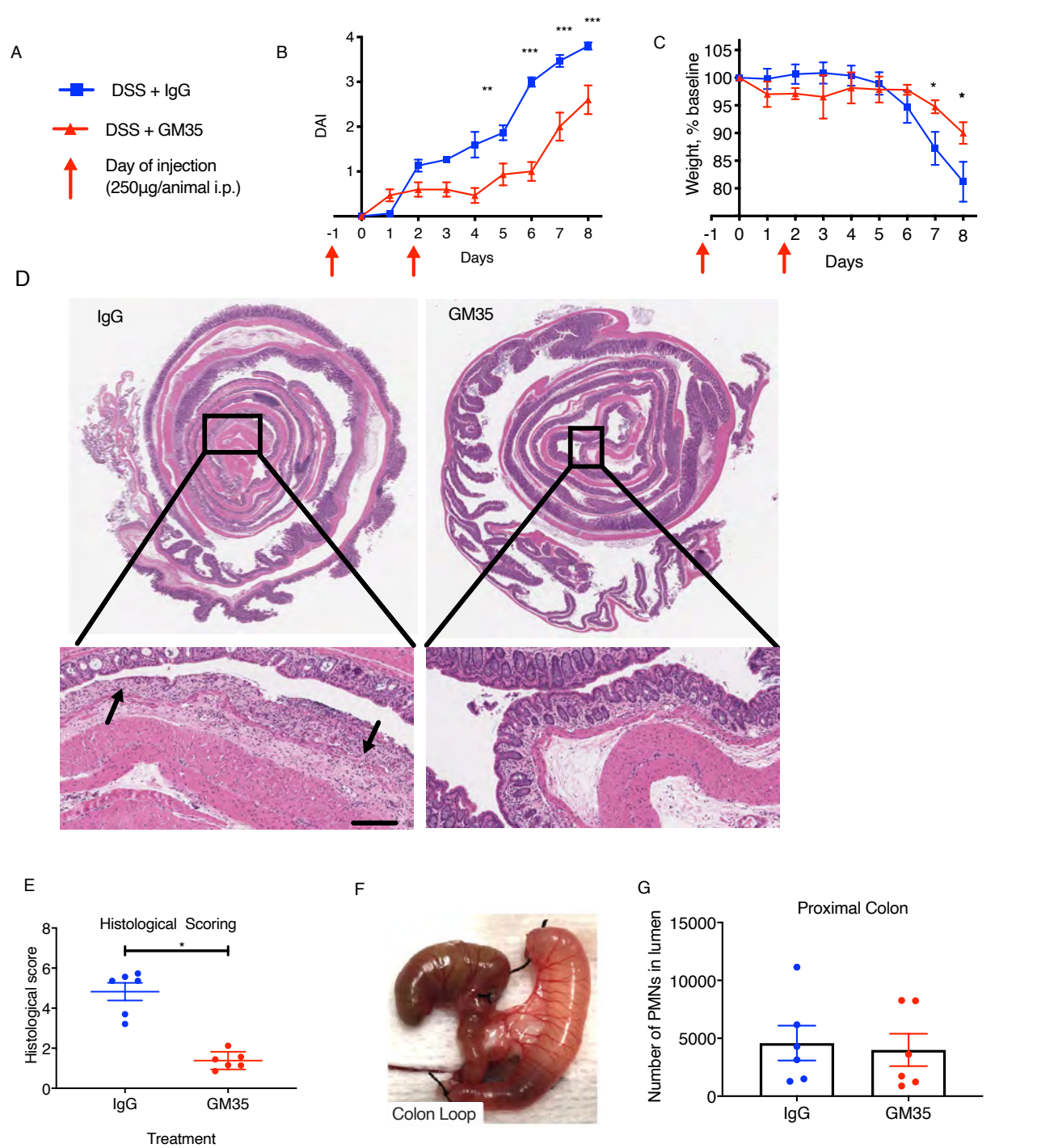

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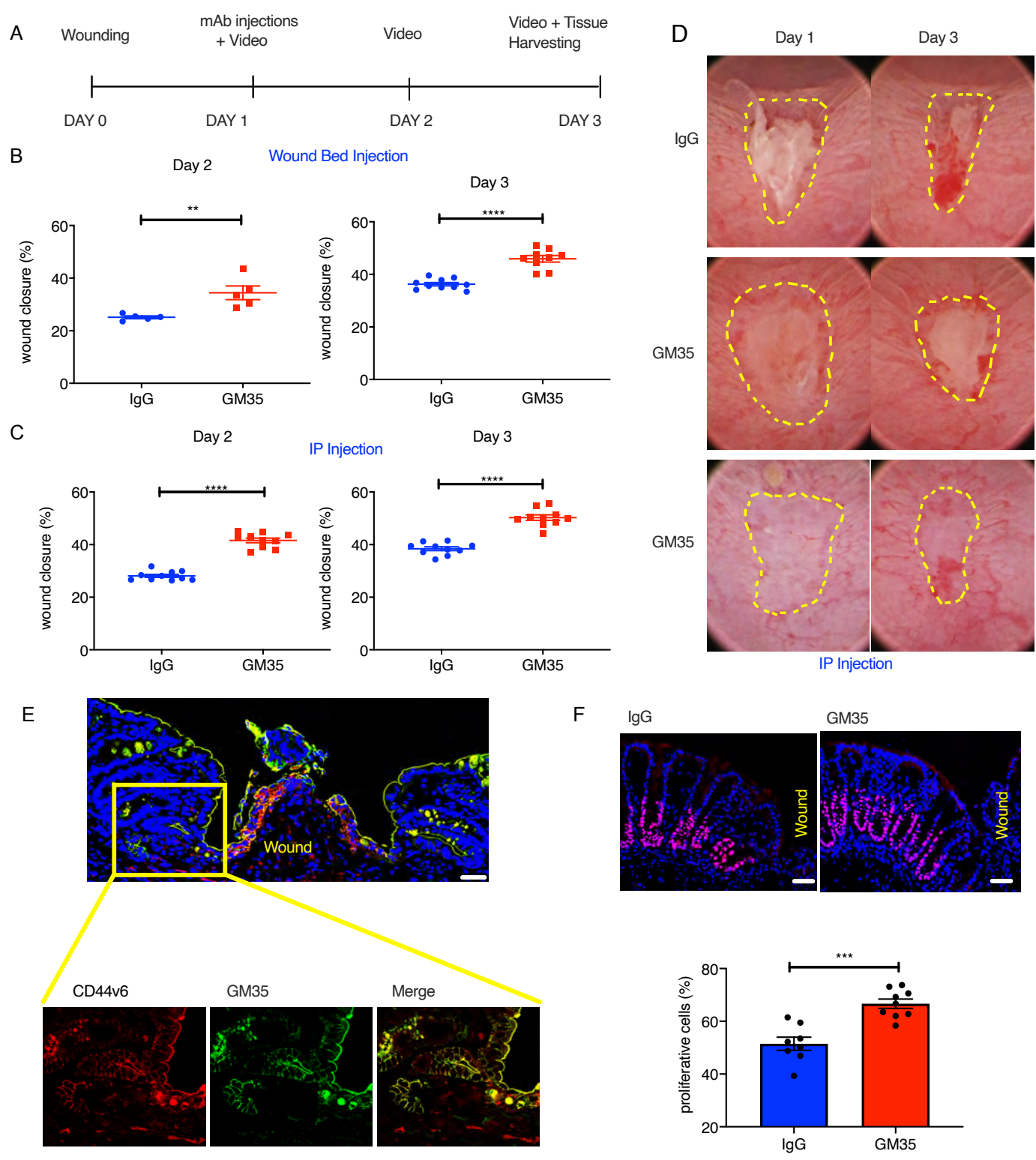

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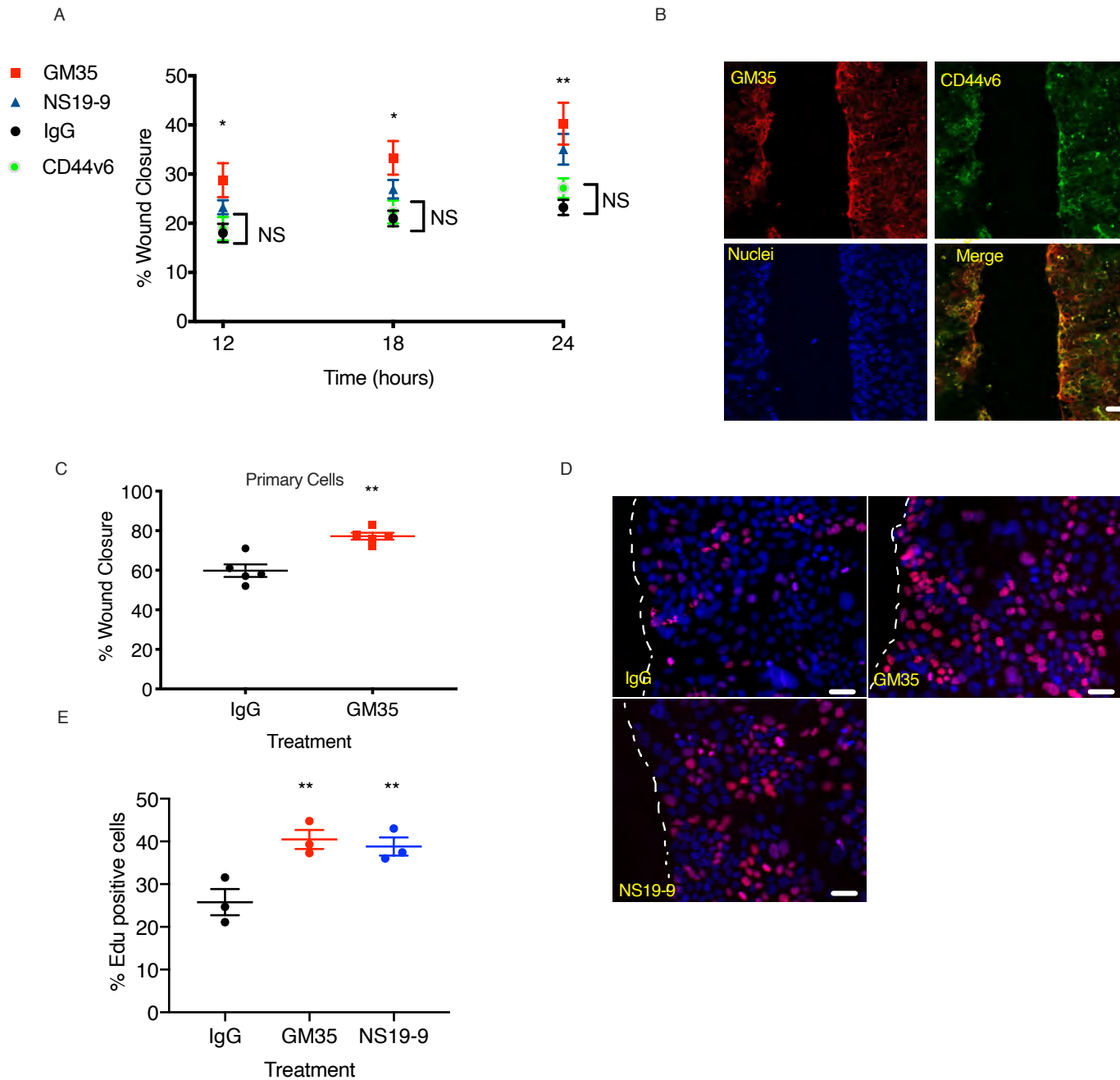

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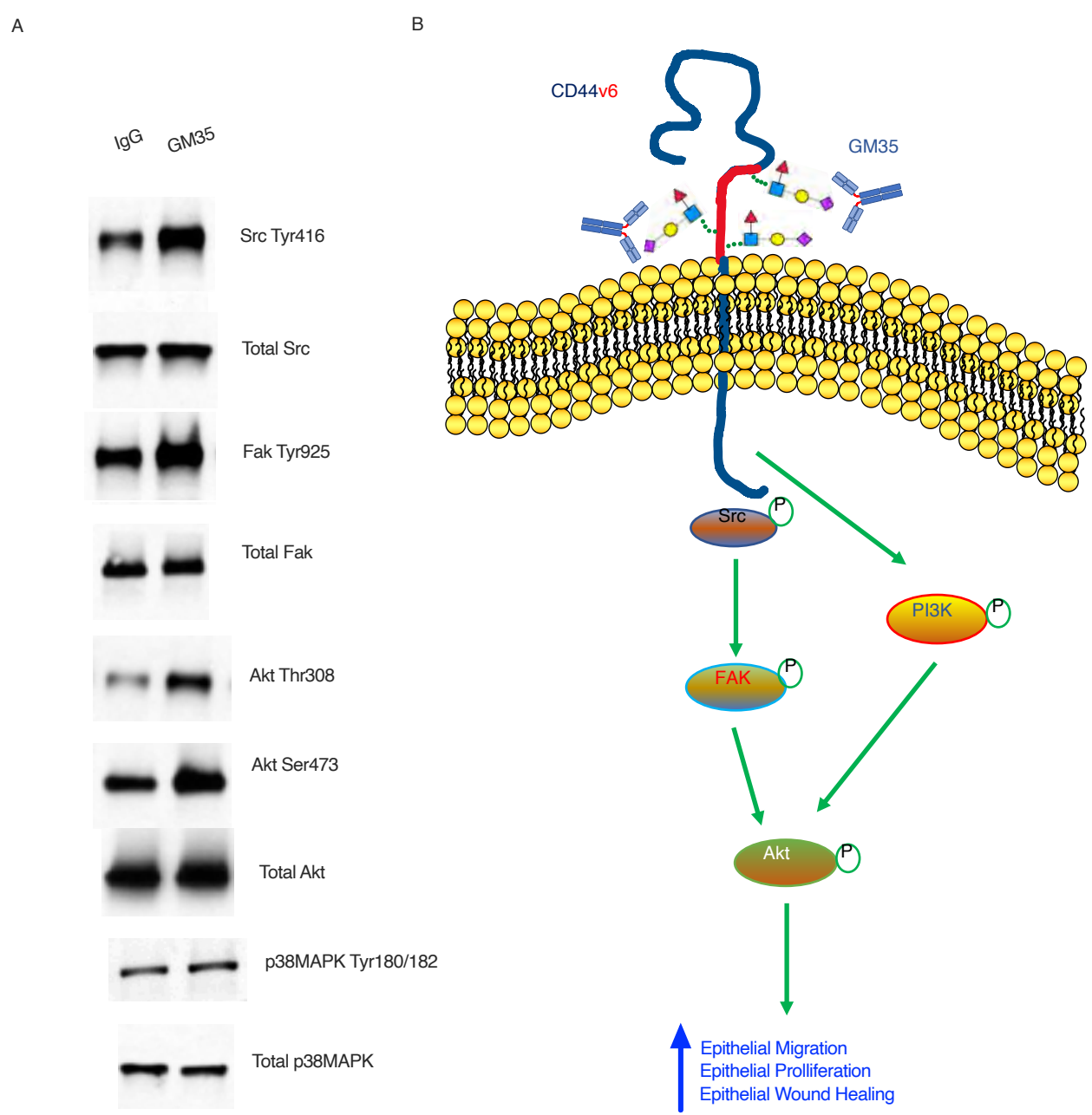

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