Quantification and Monitoring of Inflammation in Murine Inflammatory Bowel Disease with Targeted Contrast-enhanced US[1]

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Purpose: To evaluate ultrasonography (US) by using contrast agent microbubbles (MBs) targeted to P-selectin ($MB_{P\text{-selectin}}$) to quantify P-selectin expression levels in inflamed tissue and to monitor response to therapy in a murine model of chemically induced inflammatory bowel disease (IBD).

Materials and Methods: All procedures in which laboratory animals were used were approved by the institutional administrative panel on laboratory animal care. Binding affinity and specificity of $MB_{P\text{-selectin}}$ were tested in cell culture experiments under flow shear stress conditions and compared with control MBs ($MB_{Control}$). In vivo binding specificity of $MB_{P\text{-selectin}}$ to P-selectin was tested in mice with trinitrobenzenesulfonic acid–induced colitis (n = 22) and control mice (n = 10). Monitoring of anti–tumor necrosis factor α antibody therapy was performed over 5 days in an additional 30 mice with colitis by using P-selectin-targeted US imaging, by measuring bowel wall thickness and perfusion, and by using a clinical disease activity index score. In vivo targeted contrast material–enhanced US signal was quantitatively correlated with ex vivo expression levels of P-selectin as assessed by quantitative immunofluorescence.

Results: Attachment of $MB_{P\text{-selectin}}$ to endothelial cells was significantly (P = .0001) higher than attachment of $MB_{Control}$ and significantly (ρ = 0.83, P = .04) correlated with expression levels of P-selectin on endothelial cells. In vivo US signal in mice with colitis was significantly higher (P = .0001) with $MB_{P\text{-selectin}}$ than with $MB_{Control}$. In treated mice, in vivo US signal decreased significantly (P = .0001) compared with that in nontreated mice and correlated well with ex vivo P-selectin expression levels (ρ = 0.69; P = .04). Colonic wall thickness ($P \geq .06$), bowel wall perfusion ($P \geq .85$), and clinical disease activity scoring ($P \geq .06$) were not significantly different between treated and nontreated mice at any time.

Conclusion: Targeted contrast-enhanced US imaging enables noninvasive in vivo quantification and monitoring of P-selectin expression in inflammation in murine IBD.

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Inflammatory bowel disease (IBD), which includes Crohn disease and ulcerative colitis, affects about 1.4 million people in the United States and is characterized by extensive inflammatory changes in the bowel wall (1). Because of the chronic nature of IBD with multiple relapses and long treatment phases, including administration of immunosuppressants and immunomodulators that are associated with substantial side effects, regular and accurate monitoring of disease activity is of paramount importance. However, clinical scores poorly correlate with histologic grades of inflammation (2), and although endoscopic monitoring is relatively invasive, it remains the reference standard. Thus, noninvasive quantitative methods with which to assess the grade of disease are desirable.

Contrast material–enhanced ultrasonography (US) with contrast agent microbubbles (MBs) targeted at molecular markers of inflammation is a promising technology that enables imaging of inflammation at the molecular level (3–6). MBs are gas-filled echogenic contrast agents that can be used to bind molecular markers by attaching binding ligands to their surface (7). After intravenous injection, these targeted MBs distribute themselves throughout the whole body and attach to tissue sites that overexpress specific molecular markers; this is followed by a local increase in the US signal (8,9). Because of their size (several microns), MBs remain within the vascular system. Thus, targeted contrast-enhanced US has the potential to exclusively depict intraluminal markers on vascular endothelial cells overexpressed in inflammation.

Inflammation in patients with IBD is associated with increased expression of cell adhesion molecules, such as P-selectin, on intestinal vascular endothelial cells. Glycoprotein P-selectin is one of the major endothelial adhesion molecules involved in leukocyte capture and rolling on the endoluminal surface of capillaries in inflammation (1,10,11). In capillaries of inflamed intestinal tissue, P-selectin expression levels have been shown to be substantially higher than those of normal tissue (12,13). P-selectin is highly expressed in the colonic mucosa in patients with active IBD (12,14). Furthermore, administration of an anti-P-selectin antibody has been reported to inhibit mucosal injury and neutrophil accumulation in rodents with trinitrobenzenesulfonic acid (TNBS)-induced colitis (15). In addition, rolling and adhesion of both the CD4+ T helper (Th) lymphocyte type Th1 (implicated in the development of Crohn disease) and the Th2 cells (associated with ulcerative colitis) are mediated by P-selectin (16). These findings indicate that quantitative assessment of P-selectin expression levels on intestinal vascular endothelial cells may be used as a promising imaging target with which to monitor inflammation at the molecular level.

US fulfills many of the criteria for an ideal noninvasive imaging tool, especially for use in longitudinal monitoring of disease activity, particularly in young patients with IBD. For instance, US does not expose the patient to irradiation; it is noninvasive and relatively inexpensive; it has high spatial resolution; it is a real-time examination that can be performed at the bedside; and it is routinely available in almost all clinical imaging departments throughout the world. Furthermore, recent technical improvements in clinical US imaging of the bowel wall that have resulted from the introduction of hydrosonography have overcome possible limitations of US caused by gas in the bowel lumen (17,18).

Thus, the purpose of our study was to evaluate US imaging by using contrast agent MBs targeted to P-selectin ($MB_{P\text{-selectin}}$) to quantify P-selectin expression levels in inflamed tissue and to monitor response to therapy in a murine model of chemically induced IBD.

Advances in Knowledge

- In vivo US signal with use of contrast agent microbubbles targeted to P-selectin quantitatively correlates ($\rho = 0.69$; $P = .04$) with P-selectin expression levels on inflamed intestinal vascular endothelial cells as assessed with ex vivo quantitative immunofluorescence.
- Targeted contrast-enhanced US enables longitudinal in vivo monitoring of inflammation at the molecular level in a chemically induced mouse model of inflammatory bowel disease (IBD).

Implication for Patient Care

- This study lays the foundation to further develop US as a noninvasive imaging tool with which to monitor inflammation in patients with IBD at the molecular level.

Materials and Methods

One author (M.S.) is an employee of Bracco. Authors who were not affiliated with commercial entities (N.D., A.M.L., Y.R., K.F., L.T., R.P., P.J.P., J.K.W.) had control of the data and materials submitted for publication.

Cell Lines, Preparation of $MB_{P\text{-selectin}}$, and Flow Chamber Experiments

Standard cell culture methods were used and are described in detail in Appendix E1 (online). Targeted contrast agent

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Abbreviations:
IBD = inflammatory bowel disease
MB = microbubble
$MB_{Control}$ = control MBs
$MB_{P\text{-selectin}}$ = MBs targeted to P-selectin
TNBS = trinitrobenzenesulfonic acid
TNF = tumor necrosis factor

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Guarantors of integrity of entire study, J.K.W., N.D.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; manuscript final version approval, all authors; literature research, N.D., A.M.L., Y.R., P.J.P., J.K.W.; experimental studies, N.D., Y.R., K.F., R.P., P.J.P., J.K.W.; statistical analysis, Y.R., L.T., J.K.W.; and manuscript editing, N.D., A.M.L., K.F., M.S., R.P., P.J.P., J.K.W.

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Potential conflicts of interest are listed at the end of this article.

See also Science to Practice in this issue.

MBs were prepared by using standard protocols, as described in detail in Appendix E1 (online). Binding affinity and specificity of $MB_{P\text{-selectin}}$ to the molecular target P-selectin were first assessed in cell culture experiments under flow shear stress conditions meant to simulate flow in capillaries by using a flow chamber experimental set-up, as detailed in Appendix E1 (online).

Murine Model of Chemically Induced IBD

All procedures in which laboratory animals were used were approved by the institutional administrative panel on laboratory animal care. Male BALB/c mice (6–8 weeks old) were divided into two groups. In the first group (n = 52), chemically induced inflammation of the colon was induced according to well-described methods (19). Briefly, during inhalation anesthesia (2% isoflurane in 2 L of oxygen per minute), a 5-cm catheter (PE 90; Becton Dickinson, Sparks, MD) was inserted carefully with lubrication into the colon (with the tip approximately 4 cm proximal to the anus), and the contact sensitizing allergen 2.4.6-TNBS (2.5 mg in 50% ethanol; total injection volume, 100 μl) was administered into the lumen of the colon via the catheter. In the control group (n = 10), only saline was administered via the catheter.

In Vivo US of Mice

Figure 1 summarizes the study design of all US experiments. Inhalation anesthesia was maintained in all mice with 2% isoflurane in room air (2 L/min) during scanning. Targeted contrast-enhanced US was performed by using nonlinear harmonics response from MBs with a US machine dedicated to small-animal imaging (Vevo 2100; VisualSonics, Toronto, Ontario, Canada). Images were collected in a transverse plane with high spatial resolution (lateral and axial resolution of 165 μm and 75 μm, respectively; focal length, 8 mm; transmit power, 10%; mechanical index, 0.2; dynamic range, 35 dB) by using a dedicated transducer (MS250, VisualSonics; center frequency of 21 MHz). All imaging settings were kept constant throughout imaging sessions for all animals. In each mouse, US was performed in a representative colon segment approximately 3 cm from the anus. In a subgroup of six additional mice with colitis, US was also performed 2 and 4 cm from the anus to confirm that P-selectin expression is elevated at different locations of the colon in this animal model of IBD.

In all mice, intraanimal comparisons of imaging signals after injection of $MB_{P\text{-selectin}}$ and control MBs ($MB_{Control}$) (for preparation of different MB types, please refer to Appendix E1 [online]) were performed by injecting both types of MBs in the same animal during the same imaging session. Mice were injected in random order twice with a bolus of 100 μL of saline containing either 5×10^7 $MB_{P\text{-selectin}}$ or 5×10^7 $MB_{Control}$ via an intravenous catheter placed into one of the two tail veins (injection time, 2 seconds). To allow MBs from previous injections to clear, we waited at least 30 minutes between injections (8). During the bolus injection, signal intensity–time curves were acquired over 20 seconds to assess perfusion in the colon wall from peak enhancement, as described previously (20). US imaging was then performed, as described previously (21–23): Four minutes after each MB bolus injection, 120 B-mode imaging frames were acquired over a 6-second period. This was followed by application of a destruction pulse of 3.7 MPa (transmit power, 100%; mechanical index, 0.63 for 1 second to destroy all MBs in the field of view). Nine seconds later, 120 frames were acquired again to capture the influx of freely circulating MB.

To further demonstrate specific binding of $MB_{P\text{-selectin}}$ to the target P-selectin in vivo, an additional subgroup of six mice with colitis was first imaged by using the US sequence described previously after administration of $MB_{P\text{-selectin}}$. After a 30-minute pause to allow clearance of the MB, in vivo blocking of P-selectin binding was performed by allowing 125 μg of rat antimouse P-selectin antibody (BD Pharmingen, San Diego, Calif), which was injected via the tail vein, to circulate for 30 minutes. Thereafter, US of the same colon segment was performed a second time after intravenous injection of $MB_{P\text{-selectin}}$.

Figure 1

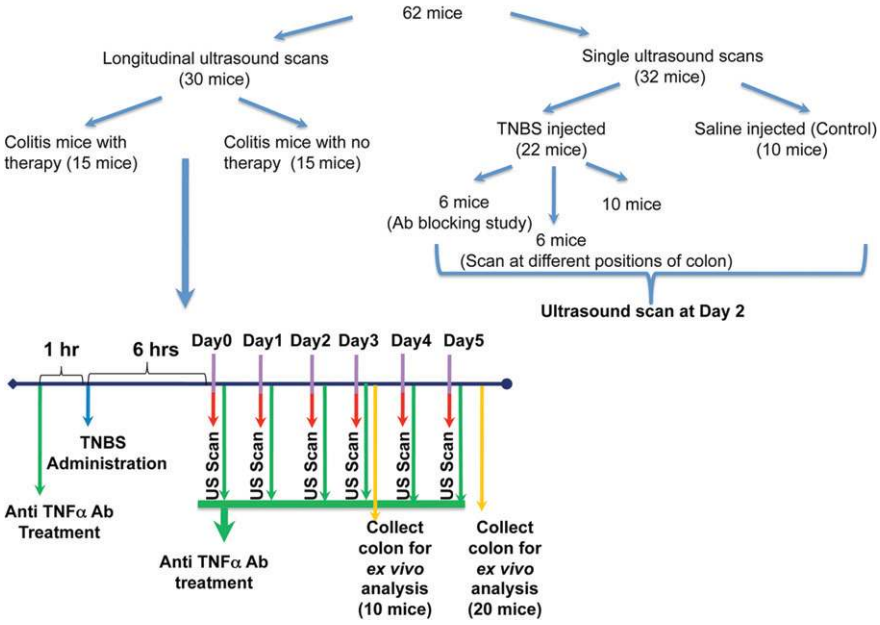

Figure 1: Flow diagram summarizes experimental design of in vivo targeted contrast-enhanced US. *Ab* = antibody.

Monitoring of Antiinflammatory Treatment

Longitudinal in vivo US imaging.—A longitudinal imaging study was performed

in an additional 30 mice with TNBS-induced colitis that were divided into two groups (Fig 1). Mice in group 1 (n = 15) did not receive antiinflammatory treatment (intravenous administration of saline only), while those in group 2 (n = 15) received antiinflammatory treatment. Antiinflammatory treatment consisted of daily intravenous administration of a clinically used anti–tumor necrosis factor (TNF) α monoclonal antibody (infliximab, Remicade; Centocor Ortho Biotech, Horsham, Pa) at a dose of 5 mg per kilogram of body weight. Targeted contrast-enhanced US with $MB_{P\text{-selectin}}$ was performed before TNBS injection (day −1), 6 hours after TNBS injection (day 0), and 1, 2, 3, 4, and 5 days after TNBS injection, as described previously. Mice were not imaged beyond 5 days after TNBS injection since it has been shown that mice recover spontaneously at about 5–7 days after rectal TNBS administration (19,24–26).

In vivo clinical assessment of colitis in mice.—A well-described disease activity index for mice was used to clinically evaluate grade and extent of intestinal inflammation (27,28). In brief, for all animals, weight, stool blood, presence of gross blood, and stool consistency were determined daily by combining scores of weight loss, stool consistency, and bleeding and then dividing the combined score by three. Each score was determined as follows: change in weight (0, <1%; 1, 1%–5%; 2, 6%–10%; 4, >10%), stool blood (0, negative; 2, positive or gross bleeding (5,6), and stool consistency (0, normal; 2, loose stools; 4, diarrhea), as described previously (27,28).

US Image Analysis

All imaging data sets were analyzed offline and in random order at a dedicated workstation with commercially available software (VevoCQ; Visualsonics, Toronto, Ontario, Canada) by a biologist (N.D.) and a radiologist (Y.R.) with 2 years and 1 year of experience in small-animal US image analysis of the large bowel, respectively, who were blinded to the type of MB used ($MB_{P\text{-selectin}}$ vs $MB_{Control}$) and the type of animal (mice with colitis vs control mice, mice that received treatment vs those that did not). First, the mean thickness of the bowel wall was measured on transverse B-mode images by using an electronic caliper available on the workstation by averaging the values obtained in four regions of the colon wall (3, 6, 9, and 12 o'clock). Regions of interest (area range, 5–9 mm^2) were then drawn over the colon wall. The peak signal intensity of the signal intensity–time curves, which is the maximal contrast enhancement in the region of interest, was calculated from the US data sets acquired after bolus injections to estimate perfusion in the colon wall, as described previously (20). The magnitude of imaging signal from attached MB was calculated by calculating an average for pre- and postdestruction imaging signals and subtracting the average postdestruction signal from the average predestruction signal, as described previously (22,29). Images representing the adherent MB were displayed as a color-coded signal overlaid on the B-mode image.

Ex Vivo Analysis of Colon Tissues

Ex vivo analysis of colon tissues for histopathologic, quantitative immunofluorescence, and microvessel density analyses was performed with standard techniques. Details are provided in Appendix E1 (online).

Statistical Analysis

Data are reported as means ± standard deviation. For flow chamber experiments, a Poisson regression model was used to compare groups regarding number of attached $MB_{P\text{-selectin}}$ and $MB_{Control}$ within one cell type, attachment of $MB_{P\text{-selectin}}$ and $MB_{Control}$ between two cell types (stimulated and nonstimulated vascular endothelial cells), and differences in $MB_{P\text{-selectin}}$ attachment before and after addition of blocking antibodies. Repeated measurements were accounted for by using a subject-specific random intercept at Poisson regression. Differences in expression levels of P-selectin between stimulated and nonstimulated vascular endothelial cells were also assessed with a Poisson regression model. The Pearson correlation coefficient (ρ) was calculated to measure the correlation between expression levels of P-selectin on vascular endothelial cells and the number of attached $MB_{P\text{-selectin}}$ per cell and the correlation between in vivo US signal and ex vivo expression levels of P-selectin. The nonparametric Wilcoxon rank sum test was used to compare in vivo imaging signal after administration of $MB_{Control}$ with that after administration of $MB_{P\text{-selectin}}$, to compare US signal, mean bowel wall thickness, and peak enhancement in normal mice with those in mice with colitis, and to compare US signal, mean bowel wall thickness, and peak enhancement in mice that received treatment with those in mice that did not. The nonparametric sign test was used to compare US signals before and after blocking with the anti-P-selectin antibody. The reliability of measurement of bowel wall thickness, peak enhancement, and US signal obtained by two independent readers was assessed with the intraclass correlation coefficient (ICC). ICCs were defined as follows: an ICC of 0–0.20 indicated no agreement; an ICC of 0.21–0.40, poor agreement; an ICC of 0.41–0.60, moderate agreement; an ICC of 0.61–0.80, good agreement; and an ICC greater than 0.80, excellent agreement (30). The sample sizes were selected to have adequate power to enable detection of the expected differences. For example, we selected 22 mice with induced inflammation and 10 control mice to have 80% power to detect a group difference of 1.10 standard deviations. We additionally selected 15 mice that underwent treatment and 15 mice with colitis that did not undergo treatment to have 80% power to detect a difference of 1.0 standard deviation, which is expected given the effectiveness of the antiinflammation treatment. All statistical analyses were performed with R2.10.1 software *(www.r-project.org)*. A P value of less than .05 was considered indicative of a significant difference.

Results

Flow Chamber Cell Culture Experiments

Vascular endothelial cells showed background expression of P-selectin (54375 receptors per cell ± 11640), which was significantly (P = .001) increased after

stimulation with TNFα (88788 receptors per cell ± 7871). Consequently, attachment of $MB_{P\text{-selectin}}$ was significantly higher (P = .001) in cells stimulated with TNFα than in nonstimulated cells (Fig 2, Table). Furthermore, attachment of $MB_{P\text{-selectin}}$ to stimulated (P = .001) and nonstimulated (P = .001) endothelial cells was significantly higher compared with attachment of $MB_{Control}$ (Fig 2, Table). Administration of blocking antibodies significantly decreased $MB_{P\text{-selectin}}$ attachment to stimulated (P = .001) and nonstimulated (P = .002) cells, further enabling us to confirm binding specificity of $MB_{P\text{-selectin}}$ to the target P-selectin in cell culture experiments. In addition, there was a significant correlation between the number of attached $MB_{P\text{-selectin}}$ and the expression levels of P-selectin on vascular endothelial cells as assessed with flow cytometry (ρ = 0.83, P = .04).

In Vivo US Imaging of Mice

In vivo US signal obtained from the colon wall in animals with colitis was significantly higher (P = .001) after administration of $MB_{P\text{-selectin}}$ (16.2 dB ± 2.8) than after administration of $MB_{Control}$ (3.5 dB ± 1.5). Furthermore, imaging signal in animals with colitis was significantly (P = .003) higher than that in control animals (3.3 dB ± 2.8) after $MB_{P\text{-selectin}}$ administration (Fig 3). These differences were significant at all three anatomic levels (2, 3, and 4 cm from the anus), and there was no significant difference (P = .06–.78) between in vivo molecular imaging signals after $MB_{P\text{-selectin}}$ administration at the three anatomic levels. Specificity of the observed binding of $MB_{P\text{-selectin}}$ to the target P-selectin in mice with colitis was further evaluated with in vivo blocking of the receptor with anti-P-selectin antibodies (in vivo imaging signal with $MB_{P\text{-selectin}}$ was significantly reduced [P = .03] after in vivo antibody blocking). Histopathologic analysis (Fig 3) enabled us to confirm the presence of colonic inflammation in mice after TNBS administration (mean histologic score, 2.0 ± 0.77 in mice with colitis after TNBS administration vs 0.3 ± 0.4 in mice with a normal colon; P = .003).

Figure 2

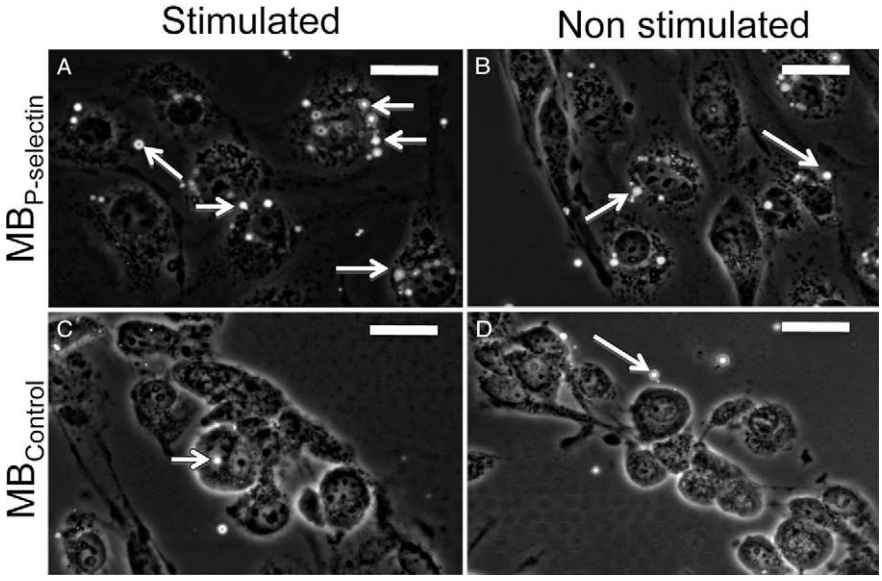

Figure 2: *A–D*, Phase-contrast bright-field micrographs (original magnification, ×100; scale bar = 20 μm) show binding of $MB_{P\text{-selectin}}$ (arrows in *A* and *B*) and $MB_{Control}$ (arrows in *C* and *D*) to stimulated and nonstimulated vascular endothelial cells.

Attachment of $MB_{P\text{-selectin}}$ and $MB_{Control}$ to Nonstimulated and Stimulated Vascular Endothelial Cells in Cell Culture Experiments

Cell Type	$MB_{P\text{-selectin}}$	$MB_{Control}$	*P* Value	$MB_{P\text{-selectin}}$ after Blocking	*P* Value
Nonstimulated endothelial cells	1.0 ± 0.1	0.4 ± 0.03	.001	0.4 ± 0.1	.002
Stimulated endothelial cells	1.7 ± 0.2	0.4 ± 0.1	.001	0.3 ± 0.1	.001

Note.—Data are means ± standard deviation of attached MBs per cell. *P* values were calculated by comparing data with $MB_{P\text{-selectin}}$ data.

Monitoring of Antiinflammatory Treatment

Longitudinal in vivo US imaging.—Before treatment was initiated (day −1), US signal was not significantly (P = .64) different between mice that received treatment and those that did not. US signal significantly (P = .001) increased as early as 6 hours after rectal TNBS administration in both groups of mice and remained high during the first 2 days of treatment (Fig 4). At day 3, US signal significantly (P = .001) decreased in mice that received treatment (10.2 dB ± 2.4) and remained lower at day 4 (8.2 dB ± 2.1) compared with US signal in mice that did not receive treatment (13.0 dB ± 3.8). At day 5, there was no significant difference (P = .14) between mice that received treatment and those that did not regarding US signal; this was likely due to known spontaneous recovery of mice that did not receive treatment (24–26). Histologic scoring enabled us to confirm that there was a significant (P = .02) decrease in grade of inflammation in mice that received treatment versus mice that did not at day 3 (mean histologic score, 0.9 ± 0.6 vs 2.0 ± 1.0). Overall, there was excellent interobserver agreement between both readers regarding $MB_{P\text{-selectin}}$ signal at all time points (overall ICC, 0.88).

In contrast, bowel wall thickness was not significantly different (P = .06) between treated mice (mean thickness, 0.46 mm; range, 0.09–0.81 mm)

Figure 3

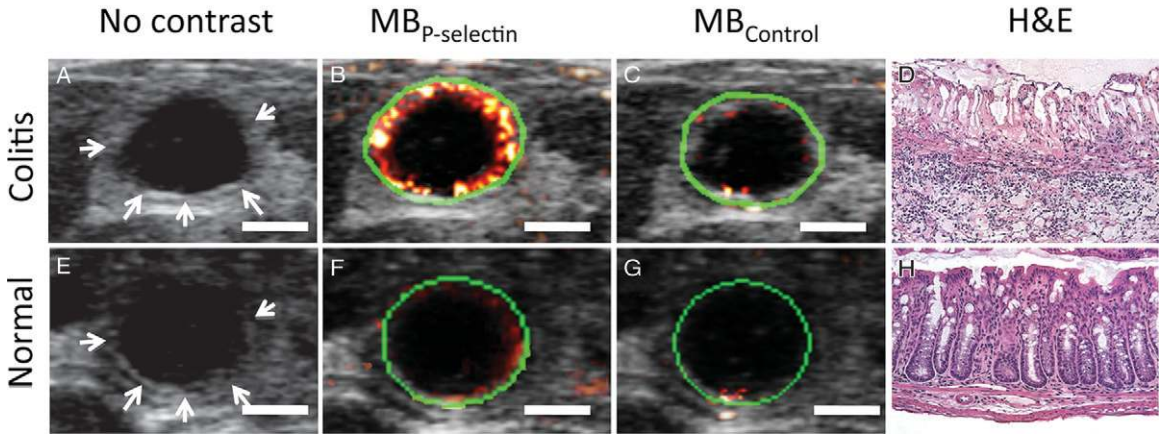

Figure 3: Transverse US images of the colon wall obtained in, *A–C*, a mouse with TNBS-induced colitis and, *E–G*, a healthy control mouse. US signal (overlaid as a color map on B-mode images, scale bar = 1 mm) in the colonic wall (arrows) after $MB_{P\text{-selectin}}$ injection was substantially higher in colitis *(B)* than in normal colon *(F)* and was significantly higher compared with $MB_{Control}$ used in colitis *(C)* and normal colon *(G)*. Hematoxylin-eosin *(H&E)* staining enabled us to confirm the presence of high-grade inflammation in the mouse with colitis *(D)* and normal histologic bowel wall appearance in the normal control mouse *(F)*. (Original magnification, ×100.)

and mice that were not treated (mean thickness, 0.46 mm; range 0.18–0.88 mm) at any time point. Similarly, peak enhancement as a measure of perfusion in the bowel wall was not significantly different ($P = .85$) between mice that received treatment (mean, 17.5 dB; range, 9–25 dB) and mice that did not (mean, 17.5 dB; range, 5–24 dB) at any time point. While interobserver agreement was poor regarding bowel wall measurements (ICC = 0.21), interobserver agreement was good regarding measurements of peak enhancement (ICC = 0.72).

In vivo clinical assessment of colitis in mice.—There was a trend toward lower clinical disease activity index scoring in mice that received treatment versus those that did not; however, the differences between groups were not significantly different ($P = .06$) at any time point.

Ex Vivo Analysis of Colon Tissues

There was good quantitative correlation between in vivo US signal with $MB_{P\text{-selectin}}$ and ex vivo expression levels of P-selectin as assessed with quantitative immunofluorescence ($\rho = 0.69$, $P = .04$), with higher P-selectin expression levels in mice with colitis versus those in mice with a normal colon and downregulation of P-selectin in mice that received treatment versus mice that did not (Fig 5). In addition, P-selectin staining colocalized with CD31-stained intestinal vascular endothelial cells (Fig 5) enabled us to confirm that the in vivo US signal was indeed generated by MBs attaching to P-selectin expressed on vascular endothelial cells in the colon wall. Microvessel density analysis revealed no significant difference ($P = .7$) between treated mice and those that were not treated (27.8 ± 2.7 vs 29.8 ± 7.8, respectively).

Discussion

Because of the poor correlation of clinical scores with histologic grades of inflammation (31) and the invasiveness of the reference standard, colonoscopy (with recognized limitations, such as procedure-related discomfort, risk of bowel perforation, and relatively poor patient acceptance), noninvasive quantitative methods with which to assess the grade of inflammation are critically needed for patients with IBD.

In our study, we explored the potential of US with a molecularly targeted contrast agent in the quantification and monitoring of inflammation at the molecular level in a murine model of IBD. We first confirmed binding affinity and specificity of $MB_{P\text{-selectin}}$ to the molecular target P-selectin overexpressed in IBD in flow chamber cell culture experiments by showing increased attachment of $MB_{P\text{-selectin}}$ to vascular endothelial cells stimulated to overexpress P-selection compared with nonstimulated cells. $MB_{P\text{-selectin}}$ attachment to stimulated vascular endothelial cells was significantly higher compared with $MB_{Control}$ and could be substantially blocked after administration of blocking antibodies. Furthermore, the attachment of $MB_{P\text{-selectin}}$ under flow shear stress conditions substantially correlated with the expression levels of P-selectin on vascular endothelial cells as assessed with flow cytometry. Similarly, in vivo imaging signal after administration of $MB_{P\text{-selectin}}$ was substantially higher compared with $MB_{Control}$ in the murine IBD model and could be substantially blocked with anti-P-selectin antibodies. These findings suggest that US imaging enables noninvasive assessment of P-selectin expression in murine IBD.

Targeted contrast-enhanced US has shown promise in the assessment of markers of inflammation in atherosclerosis (32,33), the myocardium (34,35), the kidneys (36), and the hindlimb and cremaster muscles (37,38). However, there is limited experience with targeted

Figure 4

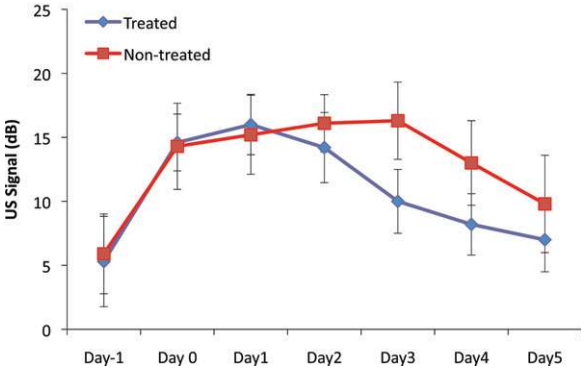

Figure 4: Graph summarizes mean US signals with $MB_{P\text{-selectin}}$ in longitudinal US experiments. Mice were scanned before (day −1) and at days 0 (6 hours), 1, 2, 3, 4, and 5 after rectal TNBS administration. US signal significantly decreased at day 3 in mice that received treatment, while it remained elevated in mice that did not receive treatment. Note spontaneous recovery of mice that did not receive treatment beginning at day 4. Error bars = standard deviations.

Figure 5

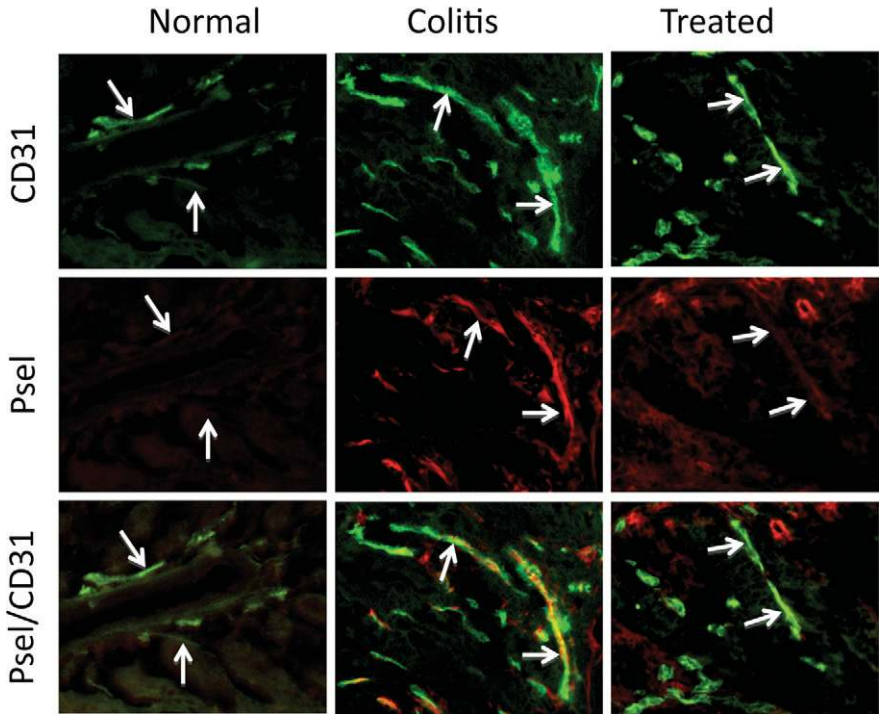

Figure 5: Representative colon sections in a control mouse, a mouse with TNBS-induced colitis, and a mouse with colitis after anti-TNF-α treatment. Sections were stained for the endothelial cell marker CD31 (green) and the inflammation marker P-selectin (red, *Psel*). Merged image *(Psel/CD31)* shows coexpression of P-selectin and CD31 on vascular endothelial cells in the mouse with colitis, as indicated by the yellow band. There is only background expression of P-selectin on vascular endothelial cells of normal colon and downregulation of vascular P-selectin expression in colitis after anti-TNFα treatment. Arrows = vessels.

contrast-enhanced US of inflammation in subjects with IBD. A study (39) showed feasibility of inflammation imaging in the SAMP1/*Yit Fc* mouse strain, a model of Crohnlike ileitis that uses a mucosal addressin cellular adhesion molecule-1–targeted contrast agent MB. Our results take this observation to several different levels: The first is by quantitative correlation of the in vivo US signal with the ex vivo expression levels of the inflammation marker P-selectin, as assessed with quantitative immunofluorescence. The second is by comparing in vivo imaging signal with morphologic and functional imaging criteria, including bowel wall thickness and bowel perfusion, as well as with an established clinical disease activity index in mice. The third is by exploring the potential of US imaging in monitoring antiinflammatory treatment in a longitudinal in vivo imaging trial. The fourth is by assessing interobserver agreements when analyzing morphologic, functional, and molecular parameters in a murine model of colitis.

Our study showed that in vivo targeted contrast-enhanced US imaging with $MB_{P\text{-selectin}}$ substantially correlated with ex vivo expression levels of P-selectin, suggesting that this US approach may be used as a noninvasive surrogate in the quantification of molecular tissue biomarkers of inflammation, such as P-selectin. This was further underscored by our longitudinal antiinflammatory treatment trial with a clinically approved anti-TNFα antibody. Three days after treatment initiation, the in vivo US signal substantially dropped; however, in mice that were not treated, the imaging signal remained elevated. The decreased signal correlated well with decreased P-selectin expression as quantified by immunofluorescence. In contrast, we did not observe a significant difference between the clinical disease activity score in mice that underwent treatment and that in mice that did not in the present study at any time point. Furthermore, there was no significant difference between mice that underwent treatment and those that did not regarding bowel wall thickness and bowel wall perfusion. Thus, this approach may be further developed as an imaging surrogate end point in the study of therapeutic approaches to IBD in preclinical experiments and may ultimately be used in future clinical trials to monitor inflammation at the molecular level in patients with IBD.

We acknowledge the following limitations of our study. In this proof-of-principle

preclinical study, we used streptavidin-biotin binding chemistry to make MBs functional, which can cause allergic reactions in patients. Ongoing research explores next-generation contrast agent MBs by using binding chemistry that enables a clinical translation of targeted MB technology into patients (40,41). Furthermore, three-dimensional US approaches could substantially improve imaging over the currently limited field of view of two-dimensional US of the intestine. Finally, although TNBS-induced colitis is a well-established murine IBD model, it is a relatively short-term model, and mice recover spontaneously without treatment about 5–7 days after rectal TNBS administration (24–26). Thus, future studies are warranted in which researchers use animal models of more chronic IBD that enable longer longitudinal US imaging experiments to confirm our in vivo findings.

The results of our study suggest that targeted contrast-enhanced US imaging with $MB_{P\text{-selectin}}$ enables accurate and reliable quantification and monitoring of inflammation at the molecular level in a chemically induced murine IBD model. Our study lays the foundation for an eventual future translation of US imaging and monitoring of inflammation in patients with IBD.

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