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Relationship among circulating leukocytes, platelets, and microvascular responses during induction of chronic colitis

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Abstract

The mechanisms by which microvascular alterations contribute to the pathogenesis of the inflammatory bowel diseases (IBD; Crohn's disease, ulcerative colitis) have not been clearly delineated. The purpose of the current study was to characterize the inflammatory events, microvascular alterations, and blood cell changes that occur in a mouse model of IBD. In this model, CD4⁺ T-lymphocytes obtained from interleukin-10-deficient mice were injected intraperitoneally into lymphopenic, recombina-activating gene-1 deficient (RAG^{-/-}) mice. Two groups of control mice were also included: RAG^{-/-} mice and C57BL/6 mice that were injected with phosphate-buffered saline but did not receive the T-cells. Four weeks later, the RAG^{-/-} mice that had received the T-cell transfer showed significant signs of colonic inflammation, but without significant decreases in either body weight or mean arterial blood pressure. T-cell transfer increased the volume % of circulating platelets, while decreasing the number of circulating red blood cells. Additionally, the T-cell transfer tended to increase the circulating numbers of both lymphocytes and neutrophils when compared to unmanipulated RAG^{-/-} mice. First-order colonic arterioles and venules tended to dilate in the colitic mice; however, the dilation was considerably more substantial with higher numbers of circulating leukocytes. The possibility that circulating inflammatory cells initiate the microvascular alterations in colitis warrants further investigation.

Keywords

Crohn's Disease; colitis; microcirculation; blood cells; mice

1. Introduction

Changes in the intestinal vasculature are thought to accompany and contribute to the pathogenesis of inflammatory bowel disease (IBD; ulcerative colitis and Crohn's disease) [1-4]. As described by Hulten et al. [3], colonic blood flow rates are highly elevated (three-

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fold) in cases of severe ulcerative colitis, but are decreased by 25-30% in mild colitis. In the same study, blood flow rates in the ileum were found to be close to normal in early exudative stages of Crohn's disease, but decreased by 60-70% in the late fibrosing stage.

These changes in blood flow can influence the progression of inflammation, with effects ranging from altered nutrient delivery, perfusion pressure driving filtration and edema, changes in vascular wall shear forces regulating leukocyte adhesion and nitric oxide production, and delivery of inflammatory blood cells. To establish the mechanisms and effects of the altered perfusion, it will be helpful to investigate the microvascular responses in animal models of IBD, and others and we have previously published several studies examining microvascular alterations in the dextran sodium sulfate model of colitis [5-9], as well as in T-cell transfer models of colitis [10-12].

In one of these T-cell transfer models, lymphocytes are obtained from interleukin-10 knockout mice, and sorted for naïve T-cells expressing CD4. These CD4⁺ T-cells are injected into lymphocyte-deficient mice, with the resulting intestinal pathology having several similarities to what is found clinically in IBD patients [13], including goblet cell loss, abnormal crypt architecture, and inflammatory cell infiltrate. In the current study, we further characterize this model, using a relatively early time point of 4-weeks post-transfer, at which time the mice have a spectrum of mild-to-severe inflammation. Included in the current characterization are measurements not only of pathology, but also of colonic vasodilation and changes in circulating blood counts.

2. Materials and Methods

2.1 Animals

Recombinase-activating gene-1 deficient (RAG^{-/-}), interleukin-10-deficient (IL-10^{-/-}), and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were maintained on 12-hr light/12-hr dark cycle in standard animal cages with filter tops under specific pathogen-free (SPF) conditions in our animal care facility at Louisiana State University Health Sciences Center, Shreveport (LSUHSC-S). The mice were provided standard laboratory rodent chow and water ad libitum. All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of LSUHSC-S and performed according to the criteria outlined by the National Institutes of Health.

2.2 Induction of Chronic Colitis

Chronic colonic inflammation was induced in mice by the adoptive transfer of IL-10^{-/-}CD4⁺ T-cells into RAG^{-/-} recipients as previously described [11-13]. Briefly, RAG^{-/-} recipient mice were injected with 10⁶ CD4⁺ T-cells obtained from IL-10^{-/-} donor mice, with the T-cells enriched (>90%; from single cell suspensions of splenocytes) by negative selection using a commercially available kit (Dyna; Invitrogen; Carlsbad, CA, USA).

Another RAG^{-/-} group, along with C57BL/6 mice, were treated identically except for the injection of vehicle alone (phosphate-buffered saline, PBS) instead of the T-cells. These three groups are identified as IL-10^{-/-}CD4⁺ → RAG^{-/-} (N=17 total), PBS → RAG^{-/-} (N=16 total), and PBS → C57BL/6 (N=17 total). At 4 weeks post-injection, the mice were used for experimental measurements.

2.3 In Vivo Measurements

Mice were anesthetized with 150 mg/kg ketamine and 10 mg/kg xylazine, with the carotid artery cannulated to measure mean arterial blood pressure. The proximal colon was

exteriorized and kept moist with warmed saline, and a Doppler flow probe (Vasamedics LaserFlo Blood Perfusion Monitor BPM2; St. Paul, MN, USA) was positioned over the exposed tissue for measurements of blood flow rate (obtained as the median of 10 measurements). A stereomicroscope (Zeiss; 6.4× magnification) with an attached video camera (Sony DXC-390) was used to capture images and diameters of the first-order arterioles and venules leading into and out of the colon. After obtaining a blood sample for complete blood count analysis (Beckman Coulter LH750; Brea, CA, USA), the mice were euthanized (150-200 mg/kg pentobarbital) and the colons and spleens were excised and weighed, along with a measurement of the colon length. After these measurements were obtained, the colon was then processed for tissue staining.

2.4 Tissue Staining

The excised colons were placed in cold PBS, and processed as we have described previously [11,12, 14], with the colon cut lengthwise into two halves, with each half rolled from the proximal to the distal end forming a Swiss roll. One of the Swiss rolls was fixed in 10% buffered formalin and embedded in paraffin to be used for histopathological evaluation, whereas the other half was frozen for immunohistochemical determination of endothelial cell density.

Frozen sections on the slides were processed in the same manner as we have published previously to immunostain platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) – for further details please see reference [12]. As previously described, we used a 1:200 dilution of the primary antibody, rat antimouse CD31 (550274; BD Biosciences, Franklin Lakes, NJ, USA): as a negative control, sections also were incubated using an equal concentration of an isotype control rat IgG (559073; BD Biosciences). The secondary antibody (FITC-conjugated) was a goat polyclonal antibody to rat IgG (ab7093-100, Abcam, Cambridge, MA, USA) at a dilution of 1:250. The sections were mounted with a coverslip and Vectashield mounting medium containing propidium iodide as a nuclear counterstain. Pictures from the slides were taken with a NanoZoomer Digital Pathology System (Hamamatsu) at the Pennington Biomedical Research Center (Baton Rouge, LA, USA). The number of pixels stained for PECAM-1 was divided by the number of pixels stained for propidium iodide for a tissue-normalized ratio. The other half of the colon that was fixed in formalin and embedded in paraffin was cut into 5-μm thick cross-sections. For histological scoring of inflammation and injury, the sections were stained with hematoxylin and eosin (H&E). The slides were scored by a pathologist (S.Z.), who was blinded to the treatment groups. The scoring was performed using our previously published criteria (8), based on the degree of inflammatory infiltrate in the lamina propria (0-3 scale), Goblet cell loss (0-2), neutrophils per 40× field (0-4), abnormal crypt architecture (0-3), number of crypt abscesses (0-1), mucosal to frank erosion (0-1), and submucosal to transmural involvement (0-3) for a total score ranging from 0-17.

2.5 Statistics

Statistical analysis of variance (ANOVA) was performed with Student-Newman-Keuls post-hoc corrections, using GraphPad Instat software (San Diego, CA, USA). The same software was used for linear regression analysis. Values are presented as means ± standard error, with $p < 0.05$ used as a criterion to determine statistical significance.

3. Results

After 4 weeks following T-cell transfer, the $IL-10^{-/-}CD4^{+} \rightarrow RAG^{-/-}$ mice exhibited substantial colonic inflammation. Colon weight per unit length increased ~two-fold compared with the values in PBS $\rightarrow$ C57BL/6 mice and PBS $\rightarrow$ $RAG^{-/-}$ mice (Fig 1A), and

histologic pathology scores in the colitic mice (Fig. 1B) averaged ~50% of the maximum score of 17. The spleens of all RAG^{-/-} mice were substantially smaller than in C57BL/6 mice (Fig. 1C), but the IL-10^{-/-}CD4⁺ T-cell reconstitution increased the spleen size by more than 40% compared with the PBS → RAG^{-/-} mice (Fig. 1C). At this early stage in the progression of colitis, there were no significant differences among groups in the body weight change during the 4-week protocol (Fig. 1D), nor any differences in mean arterial blood pressure on the day of the experimental measures (Fig. 1E).

Within the data collected from the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice, we observed that the histopathology scores correlated with the increase in colon weight per unit length (Fig. 2A; $p < 0.01$; $r^2 = 0.44$), although the relationship tends to plateau as the histological scores approach the maximum. Spleen weights also positively correlated with colonic inflammation in the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice, as shown in Figure 2B ($p < 0.05$; $r^2 = 0.43$). Despite the fact that the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice gained weight at the same average rate as in the control groups at 4-weeks of colitis, there was considerable variability in the weight gain, with the changes at this time point inversely related to colonic inflammation (Fig. 2C; $p < 0.05$; $r^2 = 0.28$).

Complete blood counts were obtained from the three groups of mice. The PBS → RAG^{-/-} mice had less than 50% total circulating leukocytes compared with the PBS → C57BL/6 mice (Fig. 3A), with this decrease due to lymphopenia as expected, rather than neutropenia (Fig. 3B & 3C). The numbers of neutrophils and lymphocytes were somewhat elevated in the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice compared with the PBS → RAG^{-/-} mice (Fig. 3B & 3C; no statistical differences). However, the IL-10^{-/-}CD4⁺ T-cell reconstitution did not alter the ratio of neutrophils or lymphocytes to the total number of circulating leukocytes compared with the PBS → RAG^{-/-} mice (Fig. 3D & 3E). The leukocyte distributions in RAG^{-/-} mice (neutrophils comprising ~70% of the population) were significantly different ($p < 0.001$) from those in C57BL/6 mice (neutrophils comprising ~35%).

Platelet numbers and volumes also were obtained from the complete blood count analysis. The number of circulating platelets (Fig. 4A) and mean platelet volume (Fig. 4B) both tended to increase in the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice, with the combination of these two changes leading to a statistically significant 37% increase in the percent blood volume occupied by platelets compared with PBS → C57BL/6 mice ($p < 0.01$; Fig. 4C).

The IL-10^{-/-}CD4⁺ → RAG^{-/-} mice had fewer circulating red blood cells (RBCs) than PBS → C57BL/6 mice (Fig. 5A). However, the decrease at the 4-week time point was relatively small, and since the RBCs were somewhat enlarged in the RAG^{-/-} mice ($p < 0.01$; Fig. 5B), the overall decrease in hematocrit was not statistically significant (Fig. 5C). The larger RBCs in the RAG^{-/-} mice were found to have slightly more hemoglobin than in the PBS → C57BL/6 mice ($p < 0.001$; Fig. 5D).

We have previously found that first-order colonic arterioles and venules (entering and exiting from the colon from the mesentery) are significantly enlarged at this early 4-week time point of the IL-10^{-/-}CD4⁺ T-cell reconstitution protocol [11]. By obtaining the complete blood counts in the current study, we were able to determine the relationship between these vascular changes and the numbers of circulating leukocytes in IL-10^{-/-}CD4⁺ → RAG^{-/-} mice. As shown in Figure 6, the diameters of the arterioles (Fig. 6A) correlate with the numbers of circulating neutrophils ($p < 0.05$; $r^2 = 0.57$), and the diameters of the venules (Fig. 6B) correlate ($r^2 = 0.84$) with both neutrophils ($p < 0.05$) and even more strongly with lymphocytes ($p < 0.01$).

The primary colonic arterioles and venules in the IL-10^{-/-}CD4⁺ → RAG^{-/-} mice had 20-25% larger diameters than in the PBS → C57BL/6 mice (Fig. 7A & 7B). If this extent of dilation

extended throughout the microcirculation, we would expect more than a 100% increase in blood flow through the colon, given that flow resistance is inversely proportional to the 4th power of diameter. However, since we found that flow does not change appreciably (Fig. 7C), the dilation of the larger vessels is likely offset by an increased resistance of smaller vessels. Although loss of vasculature would be one potential cause of increased downstream resistance, immunostaining for the endothelial cell density (using PECAM-1 as a marker) appeared to be unaffected by the IL-10^{-/-}CD4⁺ → RAG^{-/-} reconstitution protocol at this early time point of 4 weeks (Fig. 7D).

4. Discussion

The current study provides a characterization of several pathological changes occurring in IL-10^{-/-} CD4⁺ → RAG^{-/-} mice. At 4 weeks following T-cell transfer, several changes were present in the circulating blood counts of red blood cells, white blood cells, and platelets, with the alterations in circulating white blood cells correlating with the microvascular dilations occurring in the primary arterioles and venules feeding and draining the colon. These correlations are a first step toward future studies that can attempt to establish the mechanistic details of the vascular changes occurring in, and possibly contributing to, chronic colitis.

The extent of dilation of the primary arterioles and venules in this study was approximately 20-25% compared with the control groups, which is slightly lower than the 30-50% dilation that we have previously observed in this same model at the same time point [11]. This level of variability is not surprising given the wide range of inflammatory states induced by the IL-10^{-/-}CD4⁺ T-cell transfer (evaluated as a pathology score between 0-17). It should be noted that in our previous study, 60% of the mice had histologic pathology scores in the 13-16 range (with the remaining 40% having a score ≤ 5). In contrast, the pathology scores in the present study were more evenly distributed throughout a range of 3-15 (see Fig. 2A), with only 27% of the mice having scores in the 13-16 range. This relationship between severe inflammation and more dramatic vascular dilation is consistent with our previous findings that colonic (and ileal) blood flow rates are near or slightly above normal with severe inflammation, but substantially lower than normal with mild inflammation.

The relationship in our model between inflammatory state and vascular dilation could have a clinical correlate to human IBD, where colonic blood flow rates are elevated (three-fold) in cases of severe ulcerative colitis, but are decreased by 25-30% in mild colitis [3]. However, in our two studies of the 4-week time point in the IL-10^{-/-}CD4⁺ T-cell transfer model (current study and reference [11]), colonic blood flow rates are not elevated substantially above controls even with the 20-50% arteriolar dilations that are present. With flow resistance inversely proportional to the fourth power of diameter, we could potentially observe 100-400% increases in blood flow rate if not for what appears to be a counteracting constriction further downstream in the microcirculation. The other potential explanation for the lack of hyperperfusion is a loss of capillary density, which does not appear to be likely based on our very similar levels of PECAM-1 staining among groups (Fig. 7D), and on the evidence for *increased* capillary density at a later time point in a similar T-cell transfer model [7].

On the one hand, the wide range of inflammatory states in the IL-10^{-/-}CD4⁺ T-cell transfer model makes statistical comparisons more challenging, but an advantage of this variability is the opportunity to evaluate the differences that occur with mild vs severe inflammation, as would be seen clinically. Besides the variation in blood flow rates with the extent of inflammation, we also have previously published another interesting difference, that is, elevated colonic hypoxia occurring only in the subset of IL-10^{-/-}CD4⁺ → RAG^{-/-} mice with

mild inflammation, and no signs of hypoxia with severe inflammation [12]. In the current study, this variability was used to uncover the relationship between the number of circulating neutrophils and lymphocytes and the vascular dilation. Future studies could be performed to further investigate this relationship, for example, by determining whether the adhesion of these blood cells to the vessel walls is responsible for the vasodilatory response.

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References

1. Hatoum OA, Binion DG, Otterson MF, Gutterman DD. Acquired microvascular dysfunction in inflammatory bowel disease: Loss of nitric oxide-mediated vasodilation. *Gastroenterology*. 2003; 125:58–69. [PubMed: 12851871]
2. Hatoum OA, Miura H, Binion DG. The vascular contribution in the pathogenesis of inflammatory bowel disease. *Am J Physiol Heart Circ Physiol*. 2003; 285:H1791–1796. [PubMed: 14561675]
3. Hulten L, Lindhagen J, Lundgren O, Fasth S, Ahren C. Regional intestinal blood flow in ulcerative colitis and Crohn's disease. *Gastroenterology*. 1977; 72:388–396. [PubMed: 832785]
4. Thornton M, Solomon MJ. Crohn's disease: in defense of a microvascular aetiology. *Int J Colorectal Dis*. 2002; 17:287–297. [PubMed: 12172921]
5. Harris NR, Whatley JR, Carter PR, Specian RD. Venular constriction of submucosal arterioles induced by dextran sodium sulfate. *Inflamm Bowel Dis*. 2005; 11:806–813. [PubMed: 16116314]
6. Petersson J, Schreiber O, Steege A, Patzak A, Hellsten A, Phillipson M, Holm L. eNOS involved in colitis-induced mucosal blood flow increase. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G1281–1287. [PubMed: 17947450]
7. Chidlow JH Jr, Langston W, Greer JJ, Ostanin D, Abdelbaqi M, Houghton J, Senthikumar A, Shukla D, Mazar AP, Grisham MB, Kevil CG. Differential angiogenic regulation of experimental colitis. *Am J Pathol*. 2006; 169:2014–2030. [PubMed: 17148665]
8. Mori M, Stokes KY, Vowinkel T, Watanabe N, Elrod JW, Harris NR, Lefer DJ, Hibi T, Granger DN. Colonic blood flow responses in experimental colitis: time course and underlying mechanisms. *Am J Physiol Gastrointest Liver Physiol*. 2005; 289:G1024–1029. [PubMed: 16081759]
9. Harris NR, Specian RD, Carter PR, Morgan GA. Contrasting effects of pseudoephedrine and papaverine in dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis*. 2008; 14:318–323. [PubMed: 17941078]
10. Harris NR, Whatley JR, Carter PR, Morgan GA, Grisham MB. Altered microvascular hemodynamics during the induction and perpetuation of chronic gut inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G750–754. [PubMed: 19221013]
11. Harris NR, Carter PR, Lee S, Watts MN, Zhang S, Grisham MB. Association between blood flow and inflammatory state in a T-cell transfer model of inflammatory bowel disease in mice. *Inflamm Bowel Dis*. 2010; 16:776–782. [PubMed: 19821506]
12. Harris NR, Carter PR, Yadav AS, Watts MN, Zhang S, Kosloski-Davidson M, Grisham MB. Relationship between inflammation and tissue hypoxia in a mouse model of chronic colitis. *Inflamm Bowel Dis*. 2011; 17:742–746. [PubMed: 20878754]
13. Ostanin DV, Bao J, Koboziev I, Gray L, Robinson-Jackson SA, Kosloski-Davidson M, Price VH, Grisham MB. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G135–146. [PubMed: 19033538]
14. Lee S, Carter PR, Watts MN, Bao JR, Harris NR. Effects of the endothelin-converting enzyme inhibitor SM-19712 in a mouse model of dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis*. 2009; 15:1007–1013. [PubMed: 19202571]

Figure 1. Colon weight per unit length (A), histologic pathology score (B), spleen weight (C), change in body weight (D), and mean arterial pressure (E) in the three groups of mice. *** $p < 0.001$ vs PBS → C57BL/6 mice; +++ $p < 0.001$ vs PBS → RAG^{-/-} mice; + $p < 0.05$ vs PBS → RAG^{-/-} mice. N=12-16 per group.

Figure 2. Relationship between histologic pathology score (A), spleen weight (B), and change in body weight (C) and the colon weight to length ratio, with the data obtained from IL-10^{-/-} CD4⁺ → RAG^{-/-} mice.

Figure 3.

The numbers of circulating leukocytes (A), neutrophils (B), and lymphocytes (C), as well as the % circulating neutrophils (D) and lymphocytes (E) in the three groups of mice. *p<0.05 and ***p<0.001 vs PBS →C57BL/6 mice. N=12-13 per group.

Figure 4.

The number of circulating platelets (A), the mean platelet volume (B), and the platelet to blood volume percentage (C) in the three groups of mice. **p<0.01 vs PBS → C57BL/6 mice; +p<0.05 vs PBS → RAG^{-/-} mice. N=12-13 per group.

Figure 5. The number of circulating RBCs (A), mean RBC volume (B), hematocrit (C), and mean RBC hemoglobin (D) in the three groups of mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs PBS →C57BL/6 mice. N=12=13 per group.

IL-10^{-/-}CD4⁺ → RAG^{-/-}

Figure 6. Relationship between arteriolar (A) and venular (B) colonic diameters and the number of circulating leukocytes. Panel A provides the correlation between arteriolar diameter and the number of circulating neutrophils, while panel B demonstrates the combined relationship of both neutrophils (PMN) and lymphocytes to the changes in venular diameter.

Figure 7. Arteriolar diameter (A), venular diameter (B), colon blood flow (C), and the PECAM-1 to DAPI immunostaining ratio (D) in the three groups of mice. *p<0.05 vs PBS → C57BL/6 mice; +p<0.05 vs PBS → RAG^{-/-} mice. N=13-15 for panels A-C and N=8-11 for panel D.