Published in final edited form as:

J Autoimmun. 2009 ; 32(0): 195–200. doi:10.1016/j.jaut.2009.02.021.

B Cells Contribute to Ischemia/Reperfusion-Mediated Tissue Injury

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Abstract

Multiple elements are known to participate in ischemia reperfusion (I/R)-mediated tissue injury. Amongst them, B cells have been shown to contribute by the production of antibodies that bind to ischemic cells and fix complement. It is currently unknown whether B cells participate through antibody-independent mechanisms in the pathogenesis of I/R. In a mesenteric I/R model we found that B cells infiltrate the injured intestine of normal and autoimmune mice 2 hours after reperfusion is established. B cell depletion protected mice from the development of I/R-mediated intestinal damage. The protection conferred by B cell depletion was significantly greater in MRL/*lpr* mice. Finally, we show that ischemic tissue expressed the B cell-attractant CXCL13 and infiltrating B cells expressed the corresponding receptor CXCR5. Our data grants B cells an antibody-independent role in the pathogenesis of intestinal I/R and suggests that B cells accumulate in the injured tissue in response to the chemokine CXCL13.

Keywords

B cell; CXCL13; inflammation; ischemia/reperfusion

Introduction

An intense inflammatory reaction is produced in tissues transiently deprived of blood flow during reperfusion. The mechanisms that trigger inflammation in this model known as ischemia/reperfusion (I/R) and the pathways involved in its development and amplification have been identified only partially [1]. The rapid kinetics with which inflammation and tissue damage occur in organs affected by I/R indicates that the involved elements are triggered directly by signals originated in the ischemic cells and implies that more complex mechanisms such as antigen uptake and presentation followed by expansion of clones of specific lymphocytes do not participate. Indeed, constituents of the innate immune system including reactive oxygen species, cytokines and chemokines, complement, natural antibodies, and neutrophils have been shown to be key players in I/R-induced tissue injury [1]. Interestingly, T cells have also been implicated in the pathogenesis of I/R [2]. They accumulate in damaged areas of the intestine of mice subjected to mesenteric artery occlusion as early as one hour into the reperfusion phase [3]. The ability of infiltrating T

cells to cause tissue damage in the setting of I/R has been demonstrated to depend on the production of the pro-inflammatory cytokine IL-17 [3]. This suggests that T cells amplify the inflammatory response by producing factors that attract and activate effector cells such as neutrophils.

B cells have been shown to play central roles in the development of human and murine autoimmune inflammatory diseases [4]. Likewise, they have been implicated in the pathogenesis of I/R-mediated tissue injury [5]. Mainly regarded as cells that differentiate into antibody-producing cells, B lymphocytes perform several tasks aside from producing antibodies. They detect and process soluble antigens and present them to T cells; produce cytokines and express surface molecules that activate other cells. Moreover, they are equipped with receptors that allow them to become activated in the presence of inflammatory signals. B cells have been shown to be able to instigate the inflammatory response by mechanisms not limited to antibody production even in settings such as murine lupus in which antibodies are considered a pathogenic element [6]. Antibodies and complement are known to be central in the pathogenesis of I/R [1]. In fact, antibody-containing serum has been shown to restore disease in B cell-deficient animals in models of renal [7] and intestinal I/R [8,9]. In this work we investigated whether B cells contribute to I/R injury in an antibody-independent manner in normal and autoimmune mice. We demonstrate that ischemia up-regulates the expression of the B cell chemoattractant CXCL13 which induces B cell recruitment to damaged areas of the intestine and we show that B cell depletion in mice with intact levels of circulating antibodies ameliorates tissue injury in a model of mesenteric I/R.

Methods

Mice

Adult male B6.MRL *Tnfrsf6^{pr}* (B6.MRL/*lpr*) and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and housed in the animal facility of the Beth Israel Deaconess Medical Center.

Reagents

The following antibodies were used for immunofluorescence studies: FITC-labeled anti-mouse B220 (BD Pharmingen) was used in flow cytometry studies. Anti-mouse CXCL13 (R&D systems), rat anti-mouse B220 (BD Pharmingen), rat anti-mouse CXCR5 (BD Pharmingen) were used in immunohistochemistry studies. FITC-labeled anti-B220 and PE-labeled anti-CXCR5 (BD Pharmingen) were used for immunofluorescence and confocal microscope imaging.

Ischemia reperfusion protocol

After an acclimation period of at least 7 days, mice were prepared for surgery. Anesthesia was induced with ketamine (10mg/kg), xylazine (2mg/kg), and acepromazine (0.3mg/kg), administered by i.p. injection. Anesthesia was maintained during the entire experiment. All procedures were performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water-circulating heating pad. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Animals were subjected to I/R as previously described [10]. Briefly, a midline laparotomy was performed before a 30-min equilibration period. The superior mesenteric artery was identified and isolated, and a small non-traumatic vascular clamp was applied for 30 min. After this ischemic phase, the clamp was removed and the intestine was allowed to reperfuse for 3 hours. As sham procedure, animals were subjected to the same surgical intervention without artery occlusion. The laparotomy incisions were sutured and the animals were monitored during the reperfusion period. Additional ketamine and xylazine was administered by i.m. injection immediately before euthanasia. There was no significant difference in survival between treatment and control groups. After euthanasia, the small intestine (20 cm distal to the gastroduodenal junction) was removed for histological and immunofluorescence analysis as described below.

B cell depletion

In some experiments, B cells were depleted using a monoclonal anti-mouse CD20 antibody as previously described [11]. Briefly, anti-mouse CD20 or a control IgG2a monoclonal antibody were administered in a single i.p. injection 9 days prior to the I/R procedure. Control C57Bl/6 mice received 20 µg (per mice) of antibody. In MRL/lpr mice the antibody dose had to be escalated to 250 µg (per mice) to achieve comparable B cell depletion.

Histology and immunofluorescence

To prepare specimens for histological analysis, 20-cm segments of small intestine specimens were fixed in 10% buffered Formalin phosphate immediately after euthanasia. Next, tissues were embedded in paraffin, sectioned transversely in 5µm sections, and stained with Giemsa. In each section, 50–100 villi were graded on a six-tiered scale, as previously described [10]. Briefly, a score of 0 was assigned to a normal villus; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Guggenheims' spaces were scored as 2; villi with patchy disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were assigned a score of 4; villi in which the lamina propria was exuding were scored as 5; and finally, villi displaying hemorrhage or denudation were scored as 6. All histological analysis was performed in a blinded manner.

B cell infiltration into intestinal tissue was quantified using Nikon NIS-Elements software in four to six fields per animal. Pictures of the immunohistochemistry stained (anti-B220) and control sides of each slide were taken at 200× magnification. Objects above threshold level were counted as square pixels in areas of the same size. These numbers were entered into a Microsoft Excel spreadsheet for each animal and treatment group.

For immunofluorescence, small intestine sections were snap-frozen to –70°C, and sections were cut with a cryostat and fixed in acetone. Samples were blocked in PBS + 10% FCS. Sections were incubated overnight with the primary antibodies (1:100). After thorough washing, secondary antibodies were incubated for one hour. Sections were counterstained with DAPI (0.5 µg/mL) and mounted using anti-fade solution (Slowfade Gold, Invitrogen). Finally, slides were scanned in a Nikon Eclipse Ti confocal microscope. Images were analyzed with EZ-C1 v.3.6 software.

Reverse transcription PCR

Total RNA was isolated from intestine tissue using Trizol (Invitrogen). It was reverse transcribed into cDNA using Superscript preamplification kit and amplified with specific primers (CXCL13, sense 5'-TCTCTCCAGGCCACGGTATTCT-3', anti-sense 5'-ACCATTTGGCAGGAGGATTCAC-3'; CXCR5, sense 5'-GCTGTCGTCTGTCACTGGTC-3', antisense 5'-ACATCTTCCTGTTTTTATTT-3').

Statistical analyses

Data are presented as mean $\pm$ SEM. All data were subjected to statistical analysis using Microsoft Excel software and GraphPad Prime 4 software. A $p < 0.05$ was considered significant.

Results

Increased numbers of B cells enter intestinal tissue subjected to I/R injury

To investigate the trafficking pattern of B cells during I/R intestinal injury, we subjected C57Bl/6 mice to 30 min mesenteric artery occlusion followed by either 1 or 3 hour reperfusion. Significant ($p < 0.001$) numbers of B220⁺ B cells were detected in the intestinal tissue 3 hours after the initiation of the reperfusion phase. B cell accumulation was mostly observed in the basal portions of the intestinal villi and within Payer's patches (Fig. 2A). The numbers of intestinal B cells did not differ between animals subjected to sham procedure and animals allowed to reperfuse for only one hour (Fig. 1C).

In order to corroborate the histological data, we isolated Payer's patches from mice subjected to I/R and sham procedures and quantified the percentage of cells positive for the B cell marker B220 with flow cytometry. As shown in Fig. 2B and 2C, the B220⁺ Payer's patch cell fraction increased from 36.8 ± 5.5 in sham operated mice to 60.0 ± 7.0 in mice subjected to ischemia and 2 hour reperfusion and to 66.7 ± 6.1 in mice after ischemia and 3 hour reperfusion. These results confirm that I/R increases the number of intestinal B cells and indicate that B cells infiltrate ischemic intestine after 2 hours of reperfusion.

I/R induces B cell infiltration in to I/R injured intestinal tissue in MRL/lpr mice

I/R-mediated tissue injury has been shown to be amplified in autoimmune-prone MRL/lpr mice [3,8]. Circulating autoantibodies [8], as well as increased infiltration of IL-17-producing T cells [3] has been reported to contribute to this phenomenon. In order to determine whether MRL/lpr mice exhibit increased B cell infiltration into tissue subjected to I/R, we subjected twenty month-old MRL/lpr mice to sham or I/R procedures. Kinetics and intensity of B cell tissue infiltration were similar to those observed in normal C57Bl/6 mice suggesting that B cell infiltration into ischemic tissue is guided by the same mechanism in both normal and autoimmunity-prone mice (Fig. 2D).

B cell depletion reduces I/R-mediated intestinal injury

To establish whether B cell infiltration has pathogenic significance in intestinal I/R, we depleted B cells from C57Bl/6 and MRL/lpr mice. B cells were depleted with a single injection of an anti-CD20 antibody, 9 days before I/R [11]. Complete B cell depletion was achieved in peripheral blood, Payer's patches, peritoneum, and spleen of C57Bl/6 mice using 20 μ g per mouse. In contrast, total B cell depletion was not observed in spleen and Payer's patches from MRL/lpr mice, even when a much higher antibody dose (250 μ g per mouse) was used (Fig. 3 and data not shown).

As shown in Fig. 4A, B cell depletion significantly reduced intestinal tissue damage. Injury score in B cell sufficient C57Bl/6 mice was 5.1 ± 0.04 . After B cell depletion, tissue injury was reduced to 3.9 ± 0.11 ($p < 0.01$). Intestinal injury was also reduced in MRL/lpr mice from 5.75 ± 0.07 to 3.58 ± 0.23 after B cells were depleted ($p < 0.01$). Albeit complete B cell depletion could not be achieved in MRL/lpr mice, the reduction in tissue injury score was significantly greater in MRL/lpr mice than in control littermates ($p < 0.05$). Antibody titers are not immediately modified by anti-CD20 treatment [11]. Thus, these results indicate that B cells play an antibody-independent role in I/R-induced intestinal injury.

CXCL13 expression is locally induced by I/R injury

We hypothesized that the B cell chemoattractant CXCL13 could be involved in the migration of B cells into areas of injured intestine. CXCL13 is known to guide the entry of B cells into follicles and inflamed tissues [12,13]. Thus, we sought the expression of this chemokine by immunohistochemistry in intestinal tissue from mice subjected to sham and I/R procedures. CXCL13 was not observed in intestine not subjected to ischemia (sham). In contrast, CXCL13 was abundantly expressed in injured areas of intestine damaged by I/R (Fig. 5A). To confirm this finding, we isolated total RNA from intestinal tissue. We were able to detect specific CXCL13 and CXCR5 transcripts only in lysates from mice subjected to I/R. Congruent with the histological findings, CXCL13 and CXCR5 mRNA were completely absent in control intestine (Fig 5C).

To confirm the importance of this finding, we stained intestinal tissue with an antibody against the receptor of CXCL13 (CXCR5). As expected, CXCR5 was exclusively present in intestinal sections from mice subjected to I/R (Fig. 5B). In order to exclude the expression of CXCR5 in cells different than B cells, we performed dual color immunofluorescence with FITC-labeled anti-B220 and PE-labeled anti-CXCR5. As shown in Fig. 6, confocal microscope scanning demonstrated colocalization of B220 and CXCR5 in intestine of animals subjected to I/R.

Taken together, our results indicate that intestine affected by I/R expresses the chemokine CXCL13 that attracts CXCR5+ B cells into damaged areas. B cell-infiltration is involved to some extent in the tissue injury process, as demonstrated by B cell depletion experiments.

Discussion

Experiments reported in this communication grant B cells an antibody-independent role in I/R-mediated intestinal injury. Further, we describe the kinetics with which they infiltrate the ischemic tissue and identify a chemokine involved in their recruitment. Our data indicate that an analogous process occurs in MRL/*lpr* mice with similar kinetics and intensity.

B cells contribute to tissue pathology in immune-mediated disorders not only by producing antibody but also through other mechanisms which may involve antigen presentation and cytokine production [4,14]. The development of anti-B cell therapies that spare plasma cells and thus circulating antibodies has demonstrated that B cells are involved in autoimmune and inflammatory conditions in an antibody-independent fashion [6,15]. Although antibodies, particularly cationic auto-antibodies capable of binding negatively-charged phospholipids exposed after cell injury, have been considered the main mechanism by which B cells contribute to I/R-induced tissue injury [7–9,16–18], our data demonstrate that B cell depletion decreases tissue injury in mice which carry intact levels of circulating antibodies [19]. Interestingly, the protective effect of B cell depletion was significantly greater in the autoimmune mouse strain suggesting that B cells in the MRL/*lpr* mouse are capable of causing more damage than in the normal mouse (Fig. 4B).

Our results indicate that B cells appear in the ischemic tissue between 2 and 3 hours after circulation is restored. This behavior contrasts with the earlier time in which T cells are observed to infiltrate intestine subjected to I/R [3]. Interestingly, we have previously shown that infiltrating T cells produce IL-17 [3]. This suggests that T cells might contribute to B cell infiltration by producing the B cell-chemoattractant CXCL13. This chemokine has been shown to be a product of IL-17-producing T cells [20]; this fact, added to the kinetics of infiltration of each cell type suggests that products secreted by early infiltrating T cells play a role in the amplification of the inflammatory response by recruiting effector cells. The whole process seems to be enhanced in autoimmune-prone mice. Should our results be

reproduced in additional models of I/R injury and particularly in those closely resembling myocardial infarction and organ transplant, B cell depletion may prove of clinical value in multiple related clinical conditions.

Acknowledgments

This work was supported by Medical Research and Material Command grants W81XWH-07-1-0286, W81XWH-06-1-0486 and by NIH grants CA105001 and AI56363. We are grateful to Dr. Tong Shi for help with animal surgery and data analysis.

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A**Sham** **I/R** **B**

Figure 1. B cells infiltrate ischemic intestine during reperfusion

A. C57Bl/6 mice were subjected to sham procedure or I/R. Immunohistochemistry was performed with an anti-B220 antibody. Magnification 200 $\times$. *B.* B cell infiltration was quantified with image analysis software. * $p < 0.001$ when compared to sham mice.

Figure 2. I/R leads to an increase in the number of B cells within Payer's patches

A. The number of B220⁺ cells in Payer's patches increases following I/R (magnification 200×). *B.* Payer's patches were isolated and cells were stained with anti-B220 antibody. B cells were quantified in a flow cytometer. *C.* Cumulative data of flow cytometry experiments (*n*=7 per group). **p*<0.001 when compared to Sham.

Figure 3. MRL/lpr mice are resistant to complete B cell depletion

A depleting anti-mouse CD20 antibody was administered in a single dose to mice 9 days before I/R was induced. C57BL/6 mice received 20 μg per mice; MRL/lpr received 250 μg per mice. Cellular suspensions from blood and spleen were stained with anti-B220 and analyzed by flow cytometry.

Figure 4. B cell depletion reduces I/R-induced intestinal tissue injury

A. Tissue injury score was significantly reduced in C57Bl/6 (left panel) and MRL/lpr (right panel) mice depleted of B cells (black bars) when compared to B cell-sufficient mice (white bars). * $p < 0.01$. **B.** The reduction in tissue injury scores was significantly greater in MRL/lpr mice than in control mice. * $p < 0.05$.

Figure 5. CXCL13 expressed by injured tissue recruits CXCR5⁺ cells

Anti-CXCL13 (A) and anti-CXCR5 (B) antibodies were used to stain frozen sections from C57Bl/6 and MRL/*lpr* mice subjected to I/R (left panels) or sham (right panel) procedures ($n=5$). Magnification 200 $\times$. C. Total RNA was extracted from intestinal tissue of mice that had undergone I/R or sham (Sh) procedures. CXCL13 and CXCR5 transcripts were amplified by reverse transcriptase-PCR.

Figure 6. CXCR5 expression is limited to B cells

Intestinal sections C57Bl/6 mice subjected to I/R were stained with FITC-labeled anti-B220 and PE-labeled anti-CXCR5 antibody and scanned in a confocal microscope.