The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization

Mike C. L. Wu[a], Faith H. Brennan[a], Jason P. L. Lynch[a], Susanna Mantovani[a], Simon Phipps[a], Rick A. Wetsel[b], Marc J. Ruitenberg[a,c], Stephen M. Taylor[a], and Trent M. Woodruff[a,1]

[a]School of Biomedical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia; [b]Research Center for Immunology and Autoimmune Diseases, Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Health Science Center at Houston, Houston, TX 77030; and [c]Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia

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C3a is a key complement activation fragment, yet its neutrophil-expressed receptor (C3aR) still has no clearly defined role. In this study, we used a neutrophil-dependent mouse model of intestinal ischemia-reperfusion (IR) injury to explore the role of C3aR in acute tissue injuries. C3aR deficiency worsened intestinal injury, which corresponded with increased numbers of tissue-infiltrating neutrophils. Circulating neutrophils were significantly increased in $C3aR^{-/-}$ mice after intestinal ischemia, and $C3aR^{-/-}$ mice also mobilized more circulating neutrophils after granulocyte colony-stimulating factor infusion compared with WT mice, indicating a specific role for C3aR in constraining neutrophil mobilization in response to intestinal injury. In support of this role, $C3aR^{-/-}$ mice reconstituted with WT bone marrow reversed IR pathology back to WT levels. Complement C5a receptor (C5aR) antagonism in $C3aR^{-/-}$ mice also rectified the worsened pathology after intestinal IR injury but had no effect on circulating neutrophils, highlighting the opposing roles of C3a and C5a in disease pathogenesis. Finally, we found that using a potent C3a agonist to activate C3aR in vivo reduced neutrophil mobilization and ameliorated intestinal IR pathology in WT, but not $C3aR^{-/-}$, mice. This study identifies a role for C3aR in regulating neutrophil mobilization after acute intestinal injury and highlights C3aR agonism as a potential treatment option for acute, neutrophil-driven pathologies.

therapeutic | leukocyte | bone-marrow | intestine

Ischemic injury to the intestine frequently occurs during abdominal surgery for aortic aneurysm repair or cardiopulmonary bypass (1). Other clinical complications, including acute superior mesenteric artery occlusion and mesenteric venous thrombosis, also lead to intestinal ischemia (2). Prolonged intestinal ischemia rapidly causes significant and irreversible tissue damage. Although restoration of blood flow to ischemic tissue is critical for tissue salvage, reperfusion also introduces inflammatory changes that exacerbate injury (3). This intestinal ischemia-reperfusion (IR) injury is associated with local and systemic inflammatory responses that ultimately progress to multiorgan failure and often to death (2).

Inflammatory changes associated with the reperfusion of ischemic tissues involve activation of the innate immune complement system and subsequent infiltration of neutrophils, the latter being central to IR pathology (4). Complement activation triggers the generation of cleavage products, including the anaphylatoxins C3a and C5a (5). C5a plays a particularly pivotal role in the modulation of the immune response during IR injuries by inducing chemotaxis and activation of granulocytes, with subsequent release of reactive oxygen species and other tissue-destructive mediators (3, 6, 7). In contrast to C5a, the biological actions of C3a in IR injuries have received limited attention. Progress here has been stymied by the complex and multifarious, context-dependent roles of C3a (5), as wells as the dearth of selective inhibitors to block its signaling (8).

The G protein-coupled C3a receptor (C3aR) is predominantly distributed on granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages, and mast cells (9, 10). Previous studies have shown that C3a can readily degranulate eosinophils, basophils, and mast cells (11, 12) while also activating and modulating cytokine release from monocytes/macrophages (13). However, despite high expression of C3aR on neutrophils and a clear Ca^{2+} mobilization response in response to C3a (or other C3aR ligands) (9, 14), a clear role for C3a has not yet been ascribed to neutrophils (15), which form the vast majority of the granulocytic population.

In the present study, we used a mouse model of intestinal IR injury to elucidate C3aR functions in neutrophil-driven pathologies. We report that $C3aR^{-/-}$ mice were more susceptible to IR injury despite their reduced inflammatory cytokine expression. Mechanistically, this worse IR pathology resulted from increased mobilization of $C3aR^{-/-}$ neutrophils into the blood and subsequent recruitment of these cells to the inflamed intestine via a C5a-dependent process. We also found that selective targeting of C3aR using a potent C3a agonist reduced neutrophil mobilization in response to intestinal IR injury, which ameliorated pathology significantly. This study highlights a role for C3aR as an inhibitor of neutrophil mobilization after intestinal IR injury, and identifies C3aR agonists as a potential future therapy to modulate neutrophil mobilization and reduce tissue damage during the acute inflammatory phase of IR injuries.

Results

C3a and Neutrophil Mobilizers, Along with Circulating Neutrophils, Are Increased During Intestinal IR. To ascertain the involvement of C3a and known neutrophil-mobilizing factors [i.e., granulocyte colony-stimulating factor (G-CSF), keratinocyte chemoattractant (KC/CXCL1/IL-8), and stromal cell-derived factor-1 (SDF-1/CXCL12)] in intestinal IR injury, we measured their tissue and plasma levels, along with circulating neutrophil counts, in WT mice before and during IR. Intestinal C3a/desarginated C3a (C3a-desArg) levels increased significantly after a 30-min reperfusion, whereas plasma C3a/C3a-desArg increased progressively during IR, with a 2.5-fold increase after a 150-min reperfusion (Fig. 1*A*). Circulating neutrophil numbers initially decreased during ischemia, most likely as a result of early recruitment, but rapidly and progressively increased thereafter during the reperfusion phase (Fig. 1*B*). Circulating G-CSF, KC, and SDF-1 levels also

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[1]To whom correspondence should be addressed. E-mail: t.woodruff@uq.edu.au.

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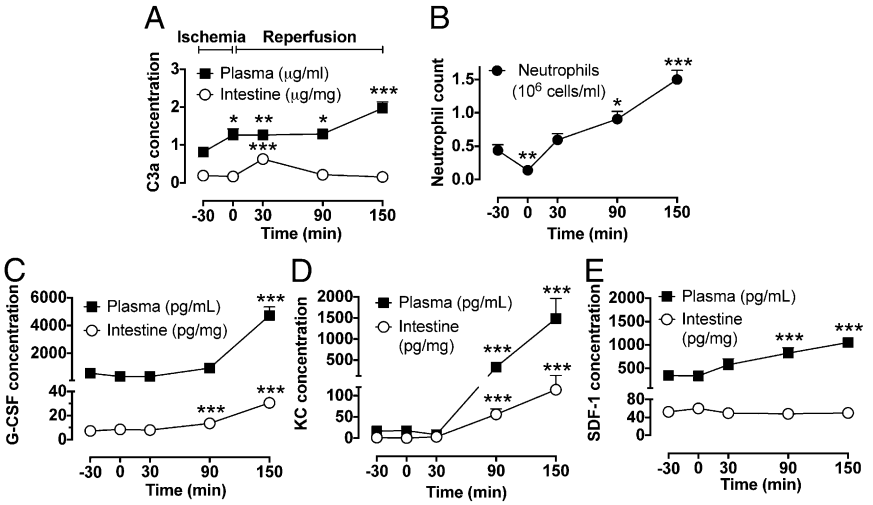

Fig. 1. Intestinal IR up-regulates C3a, neutrophil mobilizers, and circulating neutrophil numbers. Intestinal ischemia was induced by clamping the superior mesenteric artery in WT mice for 30 min, followed by reperfusion for 0, 30, 60, 90 or 150 min. (*A*, *C*, *D*, and *E*) Levels of C3a/C3a-desArg (*A*), G-CSF (*C*), KC (CXCL1/IL-8) (*D*), and SDF-1 (CXCL12) (*E*) were increased at endpoint IR, as measured by ELISA. (*B*) Circulating neutrophil numbers also increased progressively during the reperfusion phase. $n = 6$–8 per time point. Data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with preischemia (−30 min).

dramatically increased during the period of reperfusion after intestinal ischemia (Fig. 1 *C*–*E*); intestinal G-CSF and KC (but not SDF-1) were also increased after reperfusion (Fig. 1 *C*–*E*).

C3aR Deficiency Exacerbates Local Tissue Damage in Intestinal IR. WT and C3aR$^{-/-}$ mice were then subjected to intestinal IR, and the degree of intestinal injury after 150 min of reperfusion examined. Intestinal sections were stained with H&E and examined for morphological changes in mucosal villi (Fig. 2 *A*–*C*). In sham-operated mice, normal mucosal villi were present, as anticipated (Fig. 2*A*). In sharp contrast, WT mice subjected to IR displayed numerous swollen and hemorrhagic villi, along with extensive development of subepithelial spaces (Fig. 2*B*). Interestingly, mucosal damage was significantly exacerbated in C3aR$^{-/-}$ mice after IR injury, with greater digestion of lamina propria and shortening of villi (Fig. 2*C*). Semiquantitative assessment of histopathology confirmed that C3aR$^{-/-}$ mice had significantly higher mean gross pathological scores after intestinal IR compared with their WT counterparts (Fig. 2*G*). Apoptotic cell death in the intestinal lamina propria was also increased in C3aR$^{-/-}$ mice after IR injury (see Fig. 2 *D*, *E*, *F*, and *H*). The degree of intestinal injury was also assessed by

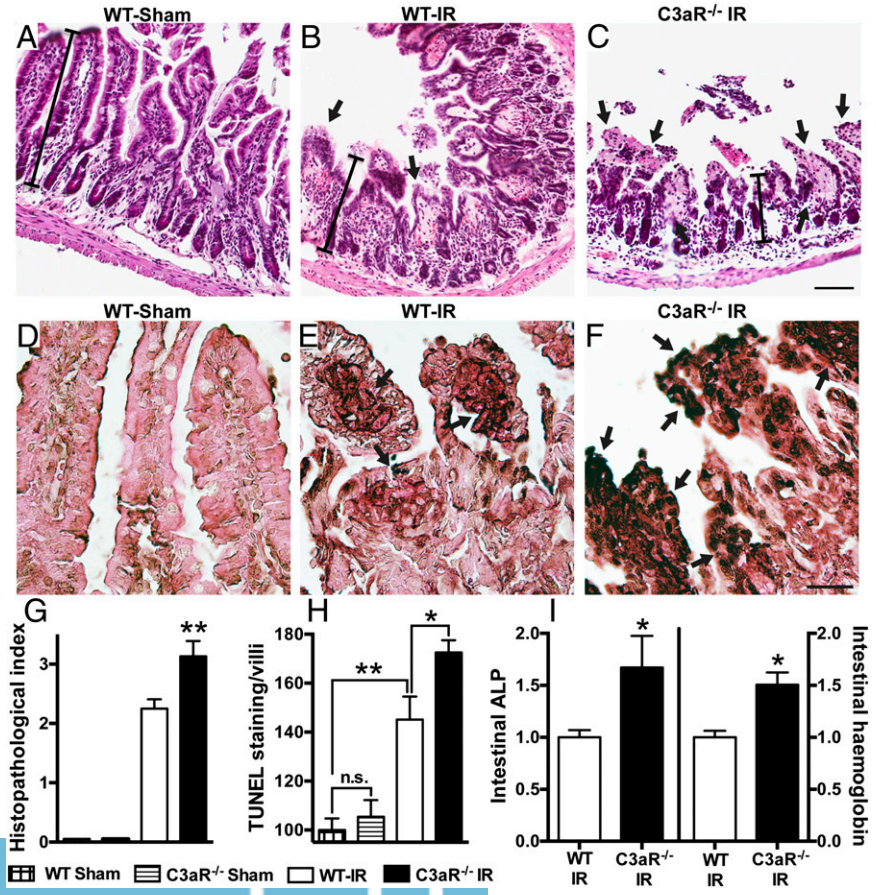

Fig. 2. Genetic ablation of C3aR exacerbates intestinal injury after IR. (*A*–*C*) Representative H&E staining of cross-sections of ileum from WT sham-operated (*A*), WT IR (*B*), and C3aR$^{-/-}$ IR (*C*) mice, demonstrating considerably increased damage to villi after IR in the absence of C3aR. Arrows indicate swelling of villi, and bars indicate approximate length of villi. (*D*–*F*) Representative TUNEL-stained ileum sections from WT sham-operated (*D*), WT IR (*E*), and C3aR$^{-/-}$ IR (*F*) mice, demonstrating increased apoptosis (arrows) with IR, which was worsened in C3aR$^{-/-}$ mice. (*G* and *H*) Histopathological scores of H&E-stained intestinal sections (*G*) and TUNEL staining intensity (*H*), showing worsened mucosal injury and elevated apoptosis in C3aR$^{-/-}$ mice compared with WT mice after IR injury. (*I*) Intestinal ALP (*Left*) and hemoglobin (*Right*) levels were also significantly higher in C3aR$^{-/-}$ mice compared with WT mice after IR. $n = 4$–6 mice/group for sham-operated, $n = 10$ mice/group for IR. Data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. (Scale bars: 50 μm in *A*–*C*; 20 μm in *D*–*F*.)

measuring intestinal hemoglobin and intestinal alkaline phosphatase (ALP), markers of tissue hemorrhage and intestinal tissue damage, respectively. These methods confirmed worse intestinal hemorrhage and injury in C3aR$^{-/-}$ mice after intestinal IR compared with WT mice (Fig. 2*I*). C3aR$^{-/-}$ sham-operated mice showed no differences in pathology compared with WT sham-operated mice (Fig. 2 *G* and *H*).

Elimination of C3aR Enhances Neutrophil Infiltration into Ischemic Gut Tissue After Intestinal IR. Given the key role of infiltrating neutrophils in driving intestinal IR injury (4, 16), we next examined the infiltration of these cells into the injured small intestine by measuring myeloperoxidase (MPO) activity and counting esterase-stained neutrophils (17). MPO levels were significantly elevated in WT mice subjected to IR and increased further with C3aR deficiency (Fig. 3*A*). Intestinal sections from WT mice subjected to IR displayed a moderate number of stained neutrophils in the lamina propria of the villi. Consistent with our MPO data, however, C3aR deficiency led to a significantly greater number of stained neutrophils in the villi (Fig. 3 *B* and *C*).

Circulating Neutrophil Numbers Are Elevated in C3aR-Deficient Mice in Response to Intestinal IR or G-CSF Infusion. Based on the increase in infiltrating granulocytic neutrophils detected in the intestines of C3aR$^{-/-}$ mice subjected to IR, we evaluated whether their numbers were also disproportionally increased in the circulation. Baseline (i.e., preinjury) neutrophil populations were not altered in C3aR$^{-/-}$ mice compared with WT mice (Fig. 4*A*). In contrast, intestinal IR led to a significant elevation of blood neutrophils in C3aR$^{-/-}$ mice after 150 min of reperfusion compared with WT mice (Fig. 4*A*). Examination of other leukocyte populations in these mice revealed significant elevations in monocyte numbers in response to IR, but, in contrast to neutrophils, these were not affected by C3aR deficiency (Fig. 4*B*). To confirm our neutrophil counts, we also assessed the relative populations of leukocytes after IR by flow cytometry. We prepared scatterplots of three main leukocyte populations: granulocytes, monocytes, and lymphocytes (Fig. 4 *C* and *D*). Granulocytes composed 51 ± 1% (n = 9) of the total leukocytes in WT mice and 69 ± 3% (n = 9; $P < 0.001$) in C3aR$^{-/-}$ mice, in correlation with our circulating cell count data.

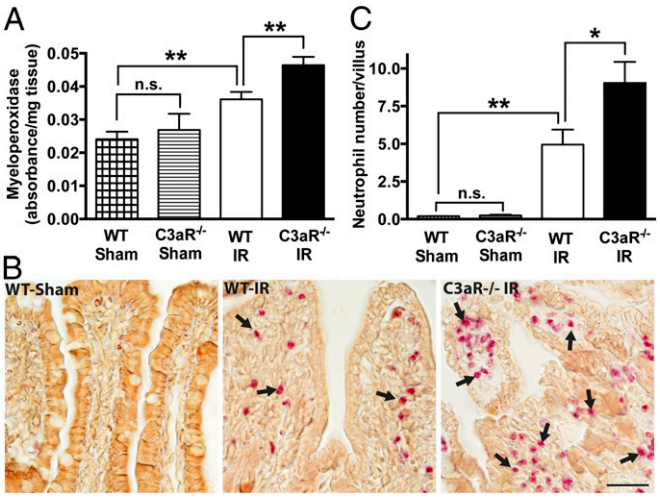

Fig. 3. Elimination of C3aR increases neutrophil infiltration after intestinal IR. (*A*) IR increased intestinal MPO activity in WT mice and even more significantly in C3aR$^{-/-}$ mice. (*B*) Representative sections of ileum showing infiltrating neutrophils in the villi of small intestine (red/pink, arrows). Note the enhanced IR-induced neutrophil infiltration in C3aR$^{-/-}$ mice. (*C*) Quantitative counts of the stained cells per villus, showing significant increases in C3aR$^{-/-}$ mice compared with WT mice after intestinal IR. n = 6–12/group. Data in *A* and *C* are mean ± SEM. *$P < 0.05$; **$P < 0.01$. n.s., not significant ($P > 0.05$). (Scale bar: 20 μm.)

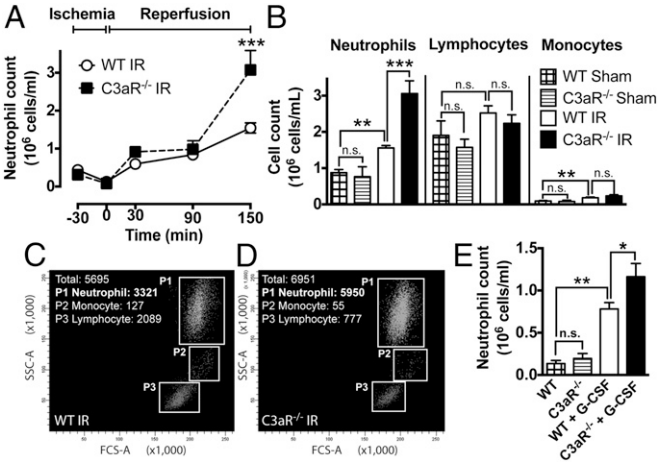

Fig. 4. Elimination of C3aR increases granulocytic neutrophil mobilization in response to intestinal IR or human G-CSF infusion. (*A*) Quantitative counts showing a significant elevation of circulating neutrophils with C3aR deficiency after a 150-min reperfusion period following intestinal IR. (*B*) Only neutrophils were selectively increased in C3aR$^{-/-}$ mice in response to IR. (*C* and *D*) Representative forward/side scatter (FSC/SSC) plots showing the major blood leukocyte populations (i.e., granulocytes, monocytes, and lymphocytes) in WT (*C*) and C3aR$^{-/-}$ (*D*) mice after intestinal ischemia and 150 min of reperfusion. Note the increase in granulocytic neutrophils in C3aR$^{-/-}$ mice. (*E*) C3aR$^{-/-}$ mice also mobilized more neutrophils into the circulation in response to recombinant G-CSF infusion. n = 7–14 mice/group. Data in *A*, *B*, and *E* are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.s., not significant ($P > 0.05$).

Finally, we assessed whether C3aR deficiency can also enhance neutrophil mobilization in the absence of tissue injury, by injecting WT and C3aR$^{-/-}$ mice with human recombinant G-CSF, a potent bone marrow neutrophil mobilizer (18). C3aR$^{-/-}$ mice mobilized significantly greater numbers of neutrophils compared with WT mice (Fig. 4*E*). Collectively, these findings indicate that C3aR acts as a negative regulator of neutrophil mobilization.

To rule out the possibility that the phenotype of C3aR$^{-/-}$ mice in IR resulted from altered complement activation as a result of their genetic deficiency, we assessed plasma C3a/C3a-desArg and C5a/desarginated C5a (C5a-desArg) levels after reperfusion. We found no significant differences in these anaphylatoxins between WT and C3aR$^{-/-}$ mice after intestinal IR (Fig. S1*A*). We also examined the degree of C3b immunostaining as a proxy for intestinal complement activation. Whereas IR induced a significant degree of C3b deposition in the injured intestines, we observed no differences between genotypes (Fig. S1 *B*–*E*). In addition, we found equal expression of C5a receptors (C5aR) on neutrophils from WT and C3aR$^{-/-}$ mice after intestinal IR (Fig. S1*F*).

To also eliminate the possibility that reduced neutrophil sequestration in the lung (19) accounted for the increase in circulating neutrophils in C3aR$^{-/-}$ mice after intestinal IR, we processed lung tissue and counted neutrophils by flow cytometry. As expected, these mice exhibited significantly greater levels of total (Ly6G^{+}, CD11b^{-}) and activated (Ly6G^{+}, CD11b^{+}) lung neutrophils compared with sham-operated animals, yet again there were no significant differences between genotypes (Fig. S2). Taken together, these results demonstrate that the worsened pathology in C3aR$^{-/-}$ mice with IR cannot be accounted for by increased complement activation, C5a generation, and/or C5aR neutrophil expression, or by reduced neutrophil sequestration in the lung.

Restoring C3aR Expression in Bone Marrow Rescues the Phenotype of C3aR-Deficient Mice After Intestinal IR Injury. To evaluate whether C3aR influences IR pathology through local expression on cells resident to the intestine, or systemically via circulating blood leukocytes, we generated WT:C3aR$^{-/-}$ bone marrow chimeric

mice in which C3aR expression was selectively restored in the peripheral immune compartment. When intestinal IR was induced in these animals, no significant differences were observed in histopathology, MPO activity, neutrophil accumulation, or circulating neutrophil counts compared with WT:WT controls (Fig. S3). These results indicate that a WT bone marrow transplantation can rescue the phenotype of C3aR$^{-/-}$ mice, again emphasizing a key role for infiltrating neutrophils in further exacerbating mucosal damage under C3aR-deficient conditions.

C5aR Antagonism Inhibits Infiltration of Neutrophils After Intestinal IR and Reduces Intestinal Mucosal Damage in C3aR-Deficient Mice. The interplay between C3a and C5a during neutrophil mobilization and recruitment after intestinal IR was examined by inhibiting the C5a–C5aR axis in C3aR$^{-/-}$ mice with the specific C5aR antagonist PMX53 (20). Consistent with previous observations in WT animals (3, 6), PMX53-treated C3aR$^{-/-}$ mice with intestinal IR had significantly reduced intestinal MPO and histopathological scores compared with vehicle-treated controls (Fig. 5 *A* and *B*). These findings again confirm the detrimental role of neutrophils in this pathology, as well as the ability of C5aR antagonism to prevent neutrophil egress into the ischemic tissue. Thus, the blocking of C5aR can reverse the exacerbated intestinal IR pathology seen in C3aR$^{-/-}$ mice. Interestingly, neutrophil mobilization into the circulation was not significantly affected by treatment with PMX53 (Fig. 5*C*). These observations indicate that whereas C5a promotes neutrophil tissue infiltration, it plays a redundant role in neutrophil mobilization into the circulation in this setting.

Mobilization of Leukocytes After C-X-C Chemokine Receptor Type 4 (CXCR4) Antagonism Does Not Affect Outcomes After Intestinal IR Injury. To corroborate the specificity of our observations of increased neutrophil mobilization and more severe intestinal injury in C3aR$^{-/-}$ mice, we next inhibited CXCR4, another innate immune receptor that negatively regulates the mobilization of various leukocyte populations, including neutrophils (18). Indeed, administration of the CXCR4 antagonist AMD3100 before IR injury significantly increased the total number of leukocytes after reperfusion, including neutrophil and mononuclear cell populations. However, in contrast to C3aR deficiency, CXCR4 antagonism did not enhance neutrophil infiltration into the injured intestine, and accordingly, the observed IR pathology was similar to that in controls (Fig. S4). Although this finding requires further confirmation, we postulate that the absence of increased neutrophil infiltration after AMD3100 treatment is most likely related to the impaired functional activity of mobilized cells (21).

C3aR Deficiency Increases Plasma and Intestinal G-CSF but Reduces Expression of Select Cytokines After Intestinal IR. Given the significant elevations of granulocyte-mobilizing factors G-CSF, KC, and SDF-1 in intestinal IR, we compared their expression in WT and C3aR$^{-/-}$ mice after IR injury. C3aR deficiency increased both plasma and intestinal G-CSF levels, as well as intestinal SDF-1 level, but not KC level, after IR compared with WT mice (Fig. S5). To determine whether C3aR has any direct effect on G-CSF generation in the absence of tissue injury, we injected mice i.p. with zymosan and measured G-CSF levels. G-CSF was dramatically and equally increased in both WT and C3aR$^{-/-}$ mice (Fig. S5), suggesting that the slightly increased generation of G-CSF (and SDF-1) seen in C3aR$^{-/-}$ mice after intestinal IR is most likely related to the enhanced tissue damage in these mice and not a direct consequence of C3aR deficiency. Previous studies have also demonstrated that C3aR activation influences the production of various proinflammatory cytokines, and these mediators have been implicated in local tissue damage after intestinal IR injury (13). Thus, we explored the effect of C3aR deficiency on cytokine expression in our model. Intestinal IR induced significant but equal increases in circulating TNF-α and IL-6 in both WT and C3aR$^{-/-}$ mice (Fig. S6). In the intestine, C3aR deficiency abrogated the injury-induced increases in intestinal TNF-α, IL-1β, and IL-17 compared with WT mice (Fig. S6).

Selective Activation of C3aR in WT Mice Using a Potent C3a Agonist Reduces Intestinal IR-Induced Pathology. Finally, given that C3aR appeared to be a crucial negative regulator of neutrophil mobilization after intestinal IR, which in turn inhibits mucosal injury, we aimed to enhance C3aR activation to identify the therapeutic potential of this receptor in ameliorating intestinal IR pathology. Using a highly potent linear peptide "super-agonist" of C3aR, WWGKKYRASKLGLAR (22), we first demonstrated that this compound is indeed a selective agonist of the mouse C3aR through an intracellular Ca^{2+} mobilization assay in isolated WT and C3aR$^{-/-}$ peritoneal macrophages (Fig. 6*A*). After the administration of the super-agonist in vivo, WT animals demonstrated significantly reduced numbers of both circulating (Fig. 6*B*) and intestinal neutrophils (Fig. 6*C*) after reperfusion compared with vehicle-treated mice. C3a agonist treatment also was associated with reduced intestinal tissue damage compared with vehicle treatment (Fig. 6 *D* and *E*). To control for any potential off-target activities of the C3a agonist in vivo, we also administered this compound to C3aR$^{-/-}$ mice, and found no reduction in intestinal pathology with IR ($P > 0.05$).

Discussion

Activation of complement along with neutrophil infiltration are widely recognized consequences of intestinal IR injury in both animal and clinical studies (4, 23–25). Whereas C5a has clearly been shown to recruit tissue neutrophils and to be a pathogenic factor in numerous IR injury models (3), the role of the upstream anaphylatoxin C3a has been relatively unexamined in IR injuries, owing in part to a lack of selective C3aR inhibitors (8, 26, 27). Given this situation, and considering the paucity of data on the role of C3a in IR injuries and neutrophil function, we used genetic elimination of C3aR to evaluate its impact on the development of intestinal IR injury in a neutrophil-dependent injury model.

Global elimination of C3a-C3aR signaling significantly increased local intestinal damage to the mucosal layer after reperfusion, with extensive destruction of villi. Consistent with the idea that neutrophils play a key role in tissue destruction after acute intestinal injury, inducing cell damage and apoptosis (4, 7, 16, 28), we confirmed the widespread presence of neutrophils within the intestine as a result of IR. Interestingly, neutrophil infiltration

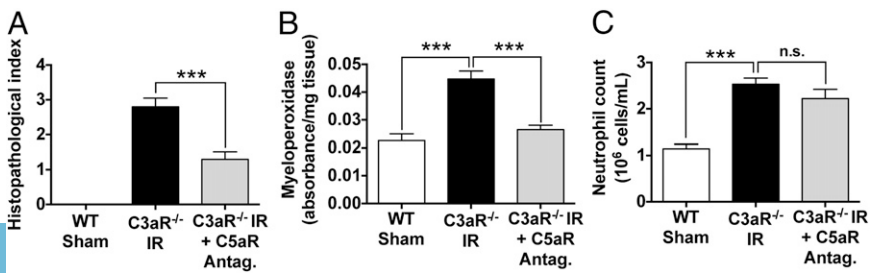

Fig. 5. Blockade of C5aR reduces intestinal IR injury in C3aR$^{-/-}$ mice. C3aR$^{-/-}$ mice were treated with the selective C5aR antagonist PMX53 (1 mg/kg i.p.) or vehicle [5% (wt/vol) glucose] before intestinal ischemia. (*A* and *B*) Histopathological scores (*A*) and intestinal MPO activity (*B*) were significantly reduced in C3aR$^{-/-}$ IR mice after PMX53 treatment. (*C*) In contrast, the number of circulating neutrophils was not altered by C5aR antagonism. $n = 7$–11 mice/group. Data are mean ± SEM. ***$P < 0.001$. n.s., not significant ($P > 0.05$).

was markedly increased in C3aR$^{-/-}$ mice and associated with increased apoptotic cell death compared with WT mice after intestinal IR. Thus, elimination of C3aR leads to greater numbers of neutrophils infiltrating the intestine, which in turn may contribute to the exacerbation of tissue damage.

Intriguingly, although C3aR is highly expressed on neutrophils (10), no clear physiological function has been ascribed to its presence on these cells, despite rapid rises in intracellular calcium after the addition of low nanomolar concentrations of C3a (9, 14). Based on previous studies identifying C3aR as a negative regulator of hematopoietic stem/progenitor cell egress and mobilization (29), we hypothesized that the enhanced neutrophil infiltration in C3aR$^{-/-}$ mice could have been driven by increased mobilization of neutrophils into the circulation after IR injury, particularly because a large proportion of the body's mature neutrophils reside in the bone marrow, from where they can be rapidly mobilized in response to infection or trauma (18, 30). C3aR$^{-/-}$ mice indeed showed a striking increase in the number of circulating neutrophils over WT mice after injury, but neither monocyte nor lymphocyte levels were increased with IR. Given that IR led to a rapid rise in G-CSF level, a well-known mediator of neutrophil egress from the bone marrow (18), we also directly confirmed that C3aR deficiency can induce enhanced neutrophil mobilization after G-CSF infusion. These findings thus support the view that elimination of C3aR selectively causes mature neutrophils to egress from the bone marrow in response to intestinal injury, which in turn leads to exacerbated pathology. We further tested this hypothesis by generating WT:C3aR$^{-/-}$ chimeric mice. Our results show that selective restoration of C3aR expression in the bone marrow leukocyte pool and circulation eliminates the sensitivity of C3aR$^{-/-}$ mice to intestinal IR-induced neutrophil mobilization and tissue pathology. This confirms that C3aR plays a key role in preventing neutrophil egress into the circulation, thereby reducing acute tissue injury after intestinal ischemia.

Whereas C3aR deficiency leads to a greater number of neutrophils in the circulation after IR injury, our data also show that signaling through C5aR is critical for their recruitment to the injured intestine. Specifically, we found that administration of a selective C5aR antagonist, PMX53 (20), reduced neutrophil infiltration at the tissue level. This finding is consistent with the well-established role of C5a as a chemoattractant factor for the recruitment of circulating leukocytes into injured tissues (5). Importantly, C5aR antagonist treatment also reduced the exacerbated IR pathology in C3aR$^{-/-}$ mice; however, C5aR antagonism did not affect the increase in circulating neutrophil numbers in response to IR injury and/or C3aR deficiency, demonstrating a clear distinction of pathophysiological roles for C3a and C5a in this model. Previous studies have postulated a key role for C3a and C5a anaphylatoxins in regulating hematopoietic stem/progenitor cell mobilization (29). Our present findings suggest a similar role for C3a-C3aR in neutrophil mobilization. On the other hand, signaling through the C5a-C5aR axis appears to play a redundant role in neutrophil mobilization in this model, although it is critically important for promoting the infiltration of neutrophils into the injured tissue. Collectively, our data indicate that these anaphylatoxins work in concert to provide an integrated, neutrophil-based response to acute tissue injury.

To our knowledge, no previous study has shown a clear role for C3aR expression on neutrophils (9). In contrast to activation of the C5a-C5aR axis, C3a signaling through C3aR does not lead to chemotaxis or degranulation of neutrophils (15). Analogous to the role of C3aR in hematopoietic stem/progenitor cells (29), our results suggest that a major function of C3aR expression on neutrophils is in regulating bone marrow-derived neutrophil mobilization after acute tissue injury. Previously identified endogenous factors influencing neutrophil mobilization include G-CSF, SDF-1, KC, and C5a (18). We now add C3a to this biological repertoire as another, negative modulator of neutrophil mobilization, similar to SDF-1. In contrast to CXCR4, the receptor for SDF-1 (31), elimination of C3aR does not affect the number of circulating neutrophils under normal physiological conditions. Increases in circulating neutrophils in C3aR-deficient mice were seen only after intestinal IR injury or G-CSF infusion. Also in contrast to CXCR4, C3aR deficiency did not influence mobilization of the mononuclear cell population after IR injury. Taken together, these findings suggest that a key facet in C3aR-mediated protection of intestinal IR is the selective inhibition of neutrophil mobilization after tissue injury.

Finally, we also report that in vivo administration of a C3a agonist can have therapeutic merit, in this case by reducing IR-induced intestinal injury. The agonist that we used, a highly potent peptide analog of C3a (22, 32), reduced neutrophil mobilization in response to intestinal injury and subsequently their presence in inflamed tissue. Thus, exogenous administration of a C3a agonist analog can augment any constituent activity of C3a generated during the disease process, perhaps because of the extremely short half-life of endogenously generated C3a (33). As such, appropriate C3a agonism may be a potential future therapy of IR injuries and other acute traumas in which neutrophils play a central and injurious role. It would now be worthwhile to investigate whether the therapeutic efficacy provided by acute C3aR agonism in this study can also afford long-term protection after intestinal IR injury. On the other hand, C3aR antagonism still likely has therapeutic validity in chronic, cytokine-driven pathologies, such as asthma, where C3aR ablation has clearly proven efficacious (5). Consistent with this, our study demonstrates that several key proinflammatory cytokines (TNF-α, IL-1β, and IL-17) were in fact reduced in the injured intestine as a result of C3aR deficiency. However, this reduction did not have a beneficial impact on injury outcomes in our model. Thus, the increased mobilization of neutrophils in response to intestinal IR in C3aR$^{-/-}$ mice appears to outweigh the benefits of locally reduced inflammatory cytokines in this model.

Collectively, our findings provide experimental evidence that C3aR inhibits neutrophil mobilization after acute intestinal IR injury or G-CSF infusion. C5aR signaling, on the other hand, plays a major role by trafficking these circulating neutrophils into the inflamed tissue, thereby exacerbating the resulting tissue injury. Thus, in this setting, C3a acts essentially as a retardant of the tissue destructive activity of C5a via distinctly different regulatory activities of the very same cell, albeit at different times. In this

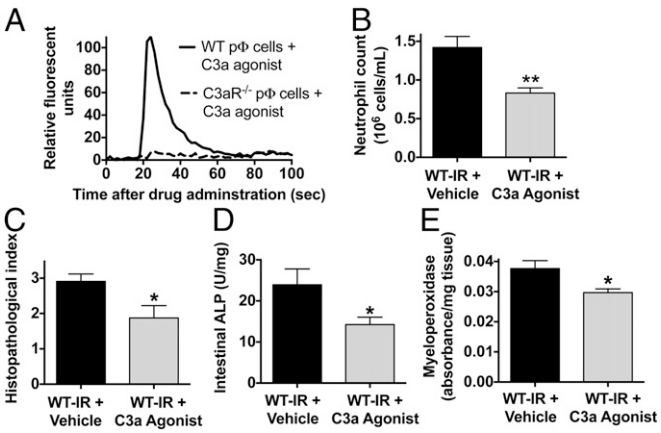

Fig. 6. Specific pharmacologic activation of C3aR reduces neutrophil mobilization, infiltration, and tissue injury after intestinal IR. (*A*) Peritoneal macrophages (pϕ cells) from WT mice showed a strong calcium mobilization response after the addition of the C3a agonist WWGKKYRASKLGLAR (1 μM); no such response was seen in C3aR$^{-/-}$ macrophages. (*B–E*) WT or C3aR$^{-/-}$ mice were treated with WWGKKYRASKLGLAR (10 μg/kg i.v.) or vehicle (saline) before intestinal ischemia. Note that the numbers of circulating neutrophils (*B*), histopathological scores (*C*), intestinal ALP levels (*D*), and intestinal MPO activity (*E*) were all significantly reduced in mice treated with the C3a agonist. n = 9–12 mice/group. Data are mean ± SEM. *$P < 0.05$; **$P < 0.01$.

context, C3a might be best described as a biological modifier of C5a on the neutrophil. Although blocking the classical proinflammatory C5a-C5aR axis may be a viable therapeutic option for many diseases (20), our data indicate that C3aR agonism offers an alternative or complementary therapeutic avenue for acute neutrophil-driven pathologies by reducing the mobilization of these cells into the circulation in response to injury.

Materials and Methods

Animals. $C3aR^{+/+}$ (WT) and $C3aR^{-/-}$ mice (34) on a C57BL/6J genetic background (>12-generation backcross) were maintained at the University of Queensland's Biological Resources Animal Facilities under specific pathogen-free conditions. Male mice aged 10–12 wk and weighing 20-25 g were used for all experiments. The experimental protocols were approved by the University of Queensland's Animal Ethics Committee.

Intestinal IR Injury Model. In anesthetized mice, the superior mesenteric artery was exposed and occluded by a loop of ligature, inducing nontraumatic intestinal ischemia. After 30 min, the ligature was removed to allow for a reperfusion period of 150 min. Sham-operated mice underwent the same surgical procedure except for the superior mesenteric artery occlusion. Mice were then killed, and small intestinal tissue or whole blood [collected in 1 mg/mL EDTA and 0.1 mg/mL nafamostat mesylate (FUT-175)] was collected for further analysis.

Intestinal IR Experimental Design. For our initial studies, we used WT and $C3aR^{-/-}$ mice to compare and contrast pathology as a result of intestinal IR injury and C3aR deficiency. In a separate group of experiments, $C3aR^{-/-}$ mice were given the C5aR antagonist PMX53 (1 mg/kg i.p.) (20) or vehicle (5% glucose) 1 h before superior mesenteric artery occlusion. A third cohort of WT mice was treated with the CXCR4 antagonist AMD3100 (5 mg/kg s.c.; Merck) or vehicle (water), 1 h before ischemia. A final group of animals were given the selective C3a agonist WWGKKYRASKLGLAR (10 μg/kg i.v) (22, 32) or vehicle (saline) 10 min before ischemia. Detailed descriptions of various methods used to measure IR pathology are provided in *SI Materials and Methods*.

G-CSF Neutrophil Mobilization. WT and $C3aR^{-/-}$ mice received a single i.v. bolus of 3 μg human recombinant G-CSF (Lenogastrim) in 100 μL of saline to induce neutrophil mobilization, and circulating neutrophils were counted 2 h later (*SI Materials and Methods*).

Bone Marrow Chimeras. C3aR activation on resident intestinal cell populations was selectively disarmed by transplantation of WT bone marrow into $C3aR^{-/-}$ recipients (WT:$C3aR^{-/-}$) as detailed previously (35). As a control, WT bone marrow was transplanted into WT recipients (WT:WT). Bone marrow chimeras were allowed to rest for 8 wk before intestinal surgery.

Zymosan-Induced Peritonitis. Zymosan (20 μg in 0.5 mL of saline) was injected i.p. into WT and $C3aR^{-/-}$ mice. Peritoneal lavage fluid (5 mL ice-cold saline) was collected 2 h later, and G-CSF levels were analyzed by ELISA (BD Biosciences).

Calcium Mobilization Assay. Thioglycollate-elicited peritoneal macrophages were obtained from WT and $C3aR^{-/-}$ mice. Cells pooled from three mice were plated and preincubated with Fluo4 calcium dye (Invitrogen), after which calcium mobilization following the addition of C3a agonist (or vehicle) was measured using a Flexstation 3 plate reader (Molecular Devices).

Statistical Analyses. All data are expressed as mean ± SEM and were analyzed using Graphpad Prism version 6.0. One-way ANOVA with Dunnett's post-hoc test was used for experiments containing more than two groups. For all other data, the Student *t* test (2-tailed distribution) was used. For each test, a P value < 0.05 was considered to indicate statistical significance.

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