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ORIGINAL ARTICLE

$Fc_{\gamma}RIIb$ protects from reperfusion injury by controlling antibody and type I IFN-mediated tissue injury and death

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Abstract

Intestinal ischemia and reperfusion (I/R) is accompanied by an exacerbated inflammatory response characterized by deposition of IgG, release of inflammatory mediators, and intense neutrophil influx in the small intestine, resulting in severe tissue injury and death. We hypothesized that $Fc_{\gamma}RIIb$ activation by deposited IgG could inhibit tissue damage during I/R. Our results showed that I/R induction led to the deposition of IgG in intestinal tissue during the reperfusion phase. Death upon I/R occurred earlier and was more frequent in $Fc_{\gamma}RIIb^{-/-}$ than WT mice. The higher lethality rate was associated with greater tissue injury and bacterial translocation to other organs. $Fc_{\gamma}RIIb^{-/-}$ mice presented changes in the amount and repertoire of circulating IgG, leading to increased IgG deposition in intestinal tissue upon reperfusion in these mice. Depletion of intestinal microbiota prevented antibody deposition and tissue damage in $Fc_{\gamma}RIIb^{-/-}$ mice submitted to I/R. We also observed increased production of ROS on neutrophils harvested from the intestines of $Fc_{\gamma}RIIb^{-/-}$ mice submitted to I/R. In contrast, $Fc_{\gamma}RIII^{-/-}$ mice presented reduced tissue damage and neutrophil influx after reperfusion injury, a phenotype reversed by $Fc_{\gamma}RIIb$ blockade. In addition, we observed reduced IFN-β expression in the intestines of $Fc_{\gamma}RIII^{-/-}$ mice after I/R, a phenotype that was also reverted by blocking $Fc_{\gamma}RIIb$. $IFNAR^{-/-}$ mice submitted to I/R presented reduced lethality and TNF release. Altogether our results demonstrate that antibody deposition triggers $Fc_{\gamma}RIIb$ to control IFN-β and IFNAR activation and subsequent TNF release, tailoring tissue damage, and death induced by reperfusion injury.

KEYWORDS
autoantibodies, gut, inflammation, microbiota, neutrophil

Camila Bernardo de Brito and Fernando Roque Ascenção contributed equally to this study.

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INTRODUCTION

Intestinal ischemia is a serious clinical condition associated with elevated mortality rates (60%–80%). Intestinal ischemia is also secondary to several other clinical situations, including during hypovolemic shock. During ischemia, hypoxanthine and succinate levels build up in the affected tissue [1, 2]. The therapeutic option to revitalize ischemic tissue is the restoration of blood flow and reperfusion. However, reperfusion intensifies tissue damage because the large supply of oxygen provided by reperfusion reacts with metabolites produced during ischemia, leading to reactive oxygen species (ROS) production and inducing severe intestinal inflammation. Reperfusion injury is characterized by an intense influx of neutrophils and production of inflammatory mediators that play pivotal roles in damage induced by intestinal ischemia and reperfusion [3–6]. Type I interferons (IFN-Is) have been described as important inflammatory mediators in reperfusion injury of the liver, kidneys, or brain. However, the role of IFN-Is in reperfusion injury depends on their production time and the vascular bed involved [7–10]. Further studies are required to understand the role of IFN-Is and the mechanisms involved in their induction during intestinal reperfusion injury [11].

Several studies have demonstrated that natural antibodies play an important role in I/R-induced inflammatory response. MμT$^{-/-}$ or RAG$^{-/-}$ mice, which are lacking circulating antibodies, are protected from intestinal reperfusion injury [12–14], probably because self-reactive antibodies are required to promote the production of proinflammatory mediators [15]. Notably, the injection of serum from wild-type mice restored the pro-inflammatory response in MμT$^{-/-}$ mice after reperfusion injury [12]. Self-reactive antibodies have been shown to recognize several auto-antigens, such as nucleic acids, carbohydrates, proteins, and phospholipids, which are displayed during reperfusion injury and to deposit in intestinal tissue, leading to complement activation and exacerbation of reperfusion injury [13–17]. Recently, we demonstrated that the production of self-reactive IgG antibodies that drive intestinal reperfusion injury is promoted by the indigenous microbiota [12].

IgG plays several effector functions, including complement activation, neutralization of micro-organisms, and engagement of Fc$_{\gamma}$Rs on immune cells [18]. Fc$_{\gamma}$Rs are cell surface glycoproteins that bind to the Fc portion of IgG. Fc$_{\gamma}$R activation triggers several biological responses and exerts pro-inflammatory, anti-inflammatory, and immunomodulatory roles depending on the type of Fc$_{\gamma}$R involved in the response [18, 19]. In mice, there are five different Fc$_{\gamma}$Rs: Fc$_{\gamma}$RI, Fc$_{\gamma}$RIIb, Fc$_{\gamma}$RIII, Fc$_{\gamma}$RIV, and Fc$_{\gamma}$Rn. Fc$_{\gamma}$RIIb is an inhibitory low-affinity IgG receptor involved in the inhibition of antibody production, as demonstrated by the uncontrolled production of IgG observed in mice deficient for the Fc$_{\gamma}$RIIb receptor, making these mice more susceptible to autoimmune diseases such as systemic lupus erythematosus and Goodpasture syndrome [20, 21]. Fc$_{\gamma}$RIIb contains an immunoreceptor phosphotyrosine-based inhibitory motif (ITIM) in its cytoplasmic region. The activation of Fc$_{\gamma}$RIIb leads to the recruitment of phosphatases to the ITIM motif, inhibiting signal transduction from other activating receptors, such as Fc$_{\gamma}$RIII. Fc$_{\gamma}$RIII is a low-affinity IgG receptor that consists of a ligand-binding chain and a signal-transducing adaptor molecule that contains immunoreceptor phosphotyrosine-based activating motifs (ITAMs) in its cytoplasmic domain. After Fc$_{\gamma}$RIII is linked by immune complexes, subsequent ITAM-mediated signalling triggers cellular responses such as phagocytosis, pro-inflammatory mediator release and cellular activation [20]. Currently, the role played by Fc$_{\gamma}$Rs during reperfusion injury is not well understood. Therefore, we sought to investigate the role of Fc$_{\gamma}$RIIb during intestinal ischemia–reperfusion injury in mice.

MATERIALS AND METHODS

Animals

C57BL/6 (WT) mice were obtained from the Biotério Central from UFMG. Fc$_{\gamma}$RIII$^{-/-}$, Fc$_{\gamma}$RIIb$^{-/-}$, MμT$^{-/-}$, SV129, and IFNAR$^{-/-}$ mice were obtained from the Immunopharmacology Laboratory Animal Facility at the Institute of Biological Sciences (UFMG). Fc$_{\gamma}$RIII$^{-/-}$ and Fc$_{\gamma}$RIIb$^{-/-}$ mice were acquired from Jackson Laboratory, stock numbers #003171 and #002848 respectively, and matrices were regularly genotyped as recommended by Jackson Laboratories. All animals were 6-12-week-old males and females and were housed under standard conditions in separate cages with 12-hr night/day cycles and free access to commercial chow and water. Experiments received prior approval by the animal ethics committees of the UFMG (338/2016 and 174/2017).

Intestinal ischemia and reperfusion

Intestinal ischemia and reperfusion (I/R) was induced after mice were anaesthetised with a solution containing xylazine (80 mg/kg) and ketamine (20 mg/kg) (intraperitoneal). After laparotomy, the superior mesenteric artery (SMA) was isolated, and ischemia was induced by totally occluding the SMA for 30 min. In experiments of lethality rates, reperfusion was conducted by re-establishing blood

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flow through the SMA, and mice were monitored for 24 hr after reperfusion. For other parameters, mice were euthanized 1, 3, or 5 hr after reperfusion. Sham-operated animals were used as controls. In the serum transfer experiments, $M\mu T^{-/-}$ mice received 400 µl of serum from WT mice or $Fc_{\gamma}RIIb^{-/-}$ mice 12 hr prior to ischemia.

Gut microbiota depletion

Six-week-old $Fc_{\gamma}RIIb^{-/-}$ mice were given a cocktail of antibiotics for microbiota depletion. Drinking water flasks were supplemented with ampicillin 1 g/L. Twice a day for 6 weeks, an antibiotic cocktail was administered by gavage at 100 µl per dose per mouse containing vancomycin (50 mg/kg), neomycin (100 mg/kg), metronidazole (100 mg/kg), and nystatin (0.03 mg/mouse). Ciprofloxacin (20 mg/mouse) was given by intraperitoneal injection once a day. Bedding and cages were changed periodically, and the whole experiment was conducted under aseptic conditions to prevent the reinfection of mice.

Myeloperoxidase (MPO) assay

The neutrophil influx was evaluated indirectly by assaying MPO activity. After I/R induction, duodenum samples were collected and snap-frozen in liquid nitrogen. After thawing and processing, the tissue was assayed for MPO activity by measuring the change in OD at 450 nm using tetramethylbenzidine and hydrogen peroxide [22]. The results were expressed as OD at 450 nm.

Histopathologic analysis

Intestinal samples from adult euthanized mice submitted to intestinal I/R were obtained. They were immediately fixed in 10% buffered formalin for 48 hr, processed, and embedded in paraffin. Tissue sections (5 µm thickness) were stained with H&E and evaluated under a microscope (Olympus) adapted with a digital camera. An experienced pathologist analysed the samples in a blinded manner and scored the degree of lamina propria inflammatory infiltrate, based on intensity and extension (score: 0 absent, 1 mild, 2 moderate, 3 intense, and 4 severe), oedema (score: 0 present and 1 absent), erosion (score: 0 absent, 1 present, 2 erosion + ulceration, and 3 ulceration), haemorrhage (score: 0 absent, 1 moderate and 2 severe) and hyperaemia (score: 0 absent and 1 present). The histopathological score was calculated as the sum of the score for each of the described parameters.

Immunohistochemistry for IgG deposition in gut tissue

Immunohistochemistry was performed to evaluate IgG deposition after I/R, as previously described [12]. Paraffin-embedded intestinal samples were cut into sections of 5 µm. Sections were treated with 3% vol/vol H_2O_2 diluted in PBS (pH 7.4) for 30 min and were then immersed in citrate buffer (pH = 6,0) for 20 min at 95° celsius for antigen retrieval. Unspecific reactions were blocked by incubating the slides with 2% BSA for 30 min at room temperature. Rat anti-mouse CD16/CD32 (Fc Block; BD Pharmingen) was used to block Fcγ receptors at a concentration of 0.5 mg/ml. Anti-mouse IgG Abs (Advance HRP; Dako) were used to detect mouse IgG in intestinal tissue samples. Next, sections were rinsed with a solution of PBS and DAB Chromogen and counterstained with haematoxylin. IgG+ cells present in intestinal villi were counted in 20 consecutive microscopic fields (×400) using ImageJ software (National Institutes of Health). Tissue sections not incubated with anti-mouse IgG antibodies were used as negative controls. The results are expressed as the number of IgG^{+} cells per square millimetre of tissue.

Measurement of TNF and CXCL1 concentrations in intestines

The concentration of TNF and CXCL1 were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits from R&D Systems, and the procedures were performed according to the manufacturer's recommendations. Thus, 100 mg of the small intestine of sham-operated and reperfused animals were homogenized in 1000 µl of PBS (0.4 M NaCl and 10 mM NaPO4) with anti-proteases (0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM ethylenediaminetetraacetic acid, and 20 KI aprotinin A). Then, the samples were centrifuged, and the supernatant was collected for ELISA at a 1:2 dilution in PBS containing 0,1% BSA. The results are expressed as the concentration of the cytokine per milligram of protein.

Measurement of IgG reactivity towards protein extracts from faecal microbiota or intestinal epithelial cells

Pools of faecal pellets obtained from WT and $Fc_{\gamma}RIIb^{-/-}$ mice were collected, homogenized, washed with 1X phosphate buffer, and centrifuged for 5 min at 1500g. This procedure was performed until the isolated bacterial layer was obtained. After washing, pellets were resuspended in 1 ml

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lysis buffer solution (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.002% wt/vol NaN3, 1 mM PMSF, 0.1 mM EDTA, 10 μM aprotinin, leupeptin 20 μM, 0.5 mM DTT, 25 mM NaF, DNAseI 100 U/ml, RNAse 25 μg/ml, and lysozyme 200 μg/ml). After this process, using a sonicator (MSE Soniprep), the suspension was sonicated. The samples were then incubated on ice for 15 min and then centrifuged at 4000*g* for 20 min at 4° celsius. The supernatant was collected, and proteins were quantified by the Bradford method. ELISA plates (Nunc Immunosorb) were coated with 1 μg/well of the extract. Intestinal epithelial cells (IECs) were isolated as previously described [23]. After isolation, proteins were extracted by incubating the cell pellets in lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.002% wt/vol NaN_3, 1 mM PMSF, 0.1 mM EDTA, 10 μM aprotinin, leupeptin 20 μM, 0.5 mM DTT, 25 mM NaF) on ice. After this process, proteins were quantified by the Bradford method. ELISA plates were coated with 10 μg/well of the extract. After overnight incubation with faecal microbiota antigens or intestinal antigens, plates were washed and blocked with 1% albumin in PBS. Serum samples diluted 1:100 in PBS containing 0.1% BSA were added, and after incubation and washing, bound IgG was detected using biotinylated anti-mouse IgG and streptavidin-conjugated HPR in the presence of OPD chromogen and hydrogen peroxide in citrate buffer (pH = 5). Wells loaded with MμT$^{-/-}$ sera were used as negative controls.

Measurement of mRNA expression by quantitative RT-PCR

Gene expression of *Ifnb*, *Ifna4*, *Isg15*, and *Isg20* were assessed by RT-qPCR from small intestine samples. Samples were processed, and mRNA was extracted using TRIzol reagent according to the manufacturer's guidelines. cDNA synthesis was performed with SuperScriptIII reverse transcriptase according to manufacturer guidelines. qPCR assays were performed with specific primers designed for each gene on 7500 Fast equipment (Applied Biosystems). The results were analysed following the $2^{-\Delta\Delta Ct}$ method using 18s as the housekeeping gene parameter. The results are expressed as the mRNA fold increases over the same genotype sham-operated animals.

Haemoglobin assay

Determination of haemoglobin concentration in the tissue was used as an index of tissue haemorrhage. For this, the intestines of sham-operated and reperfused animals were washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) to remove blood in the intravascular space. After washing, tissue was homogenized with the Drabkin colour reagent according to the manufacturer's recommendations (Analisa). Then, the suspension was centrifuged for 15 min at 3000*g* at 4° celsius and filtered using a 0.2×10^{-1} m syringe filter. After filtration, 100 μl of the final solution was added to a 96-well plate, and the absorbance was read at 520 nm using a microplate spectrophotometer. To determine the haemoglobin concentration, the absorbance was then compared to a standard haemoglobin curve.

Bacterial translocation

For analysis of bacterial translocation, the lungs and mesenteric lymph nodes of sham-operated and reperfused animals were collected and diluted 1:10 wt/vol in PBS and plated in Mueller–Hinton culture medium. Peritoneal lavage was performed with 1 ml of phosphate buffer, diluted 1:10 vol/vol in PBS, and plated in Mueller–Hinton culture medium. The plates were incubated for 24 hr at 37° celsius in aerobiosis.

Evaluation of ROS production in cells of intestinal lamina propria

Initially, mice were submitted to the I/R protocol, and cells from the lamina propria from the whole small intestine were isolated as previously described [24]. Isolated lamina propria cells were incubated in RPMI 1640 containing 10% FBS with 5 μM CellROX Deep Red Reagent (Invitrogen) at 37° celsius for 30 min. Then, cells were incubated with Pe-Cy5-conjugated anti-CD45 and Alexa 488-conjugated anti-Ly6G antibodies for 20 min at 4° celsius followed by analysis in FACScanto II flow cytometer. Results are expressed in percentage and number of CellROX^{+} neutrophils. CellROX Mean of fluorescence intensity in this population is also shown.

Statistical analysis

The results are shown as the mean ± SEM. Data were evaluated according to distribution and variances, and differences were compared using analysis of variance (ANOVA), followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. Results with a p value <0.05 were considered significantly different. GraphPad Prism 5.01 software (GraphPad) was used for the analyses.

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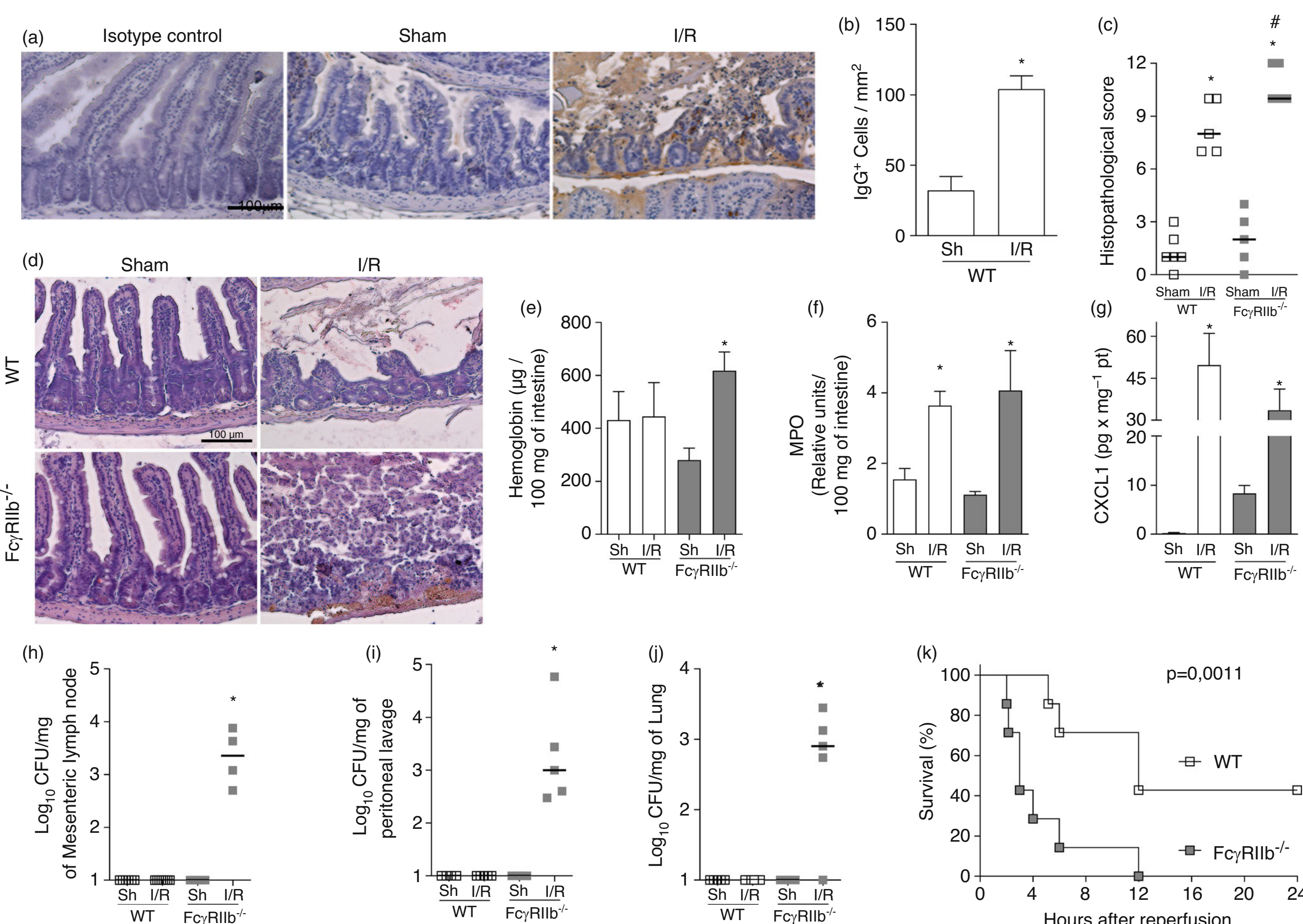

FIGURE 1 $Fc_{\gamma}RIIb$ protects mice from ischemia/reperfusion mediated intestinal injury. WT mice were submitted to ischemia of the SMA for 30 min, followed by reperfusion, which lasted for 3 h. subsequently, animals were euthanized, and small intestine were harvested for the following analyses: (a, b) quantification of IgG+ cells in intestines histological board with representative images of the tissues after staining for IgG deposition and counterstaining with haematoxylin. WT and $Fc_{\gamma}RIIb^{-/-}$ mice were submitted to the same protocol of I/R and small intestine were harvested for the following analyses: (c) H&E staining and (d) histopathological score, (e) haemoglobin quantification and (f) MPO activity and (g) CXCL1 production. After I/R induction the (h) mesenteric lymph nodes, (i) peritoneal lavage and (j) lung were collected for analysis of bacterial translocation. (k) After 30 min of ischemia by SMA occlusion, the blood flow was restored, and animals were monitored for up to 24 h to evaluate lethality rates. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. $^{*}p < 0.05$ versus the respective sham-operated control group. $\#p < 0.05$ versus WT mice that were submitted to I/R. Experimental N: 3–7, except for (f) and (g) where N: 5–15. MPO, myeloperoxidase; SMA, superior mesenteric artery

RESULTS

There is enhanced tissue damage and greater bacterial translocation upon intestinal reperfusion injury in $Fc_{\gamma}RIIb$-deficient mice

First, we evaluated whether our model of reperfusion injury induces IgG deposition in gut tissue. Our results demonstrated that histological sections from sham-operated mice presented, on average, 32.1 ± 17.1 IgG^{+} cells/mm^2. On the other hand, mice subjected to 30 min of ischemia and 3 hr of reperfusion had on average 104.1 ± 20.9 IgG+ cells/mm^2 (Figure 1a,b). Next, we evaluated the role of the IgG receptor $Fc_{\gamma}RIIb$ in the cascade of events involved in reperfusion injury.

In WT mice, the histopathologic analysis revealed marked changes in tissue architecture with great inflammatory infiltrate, as shown by the score and the histopathological analysis. However, in $Fc_{\gamma}RIIb^{-/-}$ mice subjected to I/R, tissue damage was more severe than in WT mice (Figure 1c,d). Histological analysis in $Fc_{\gamma}RIIb^{-/-}$ mice showed areas with intense haemorrhage, inflammatory infiltrate, erosion, and ulceration

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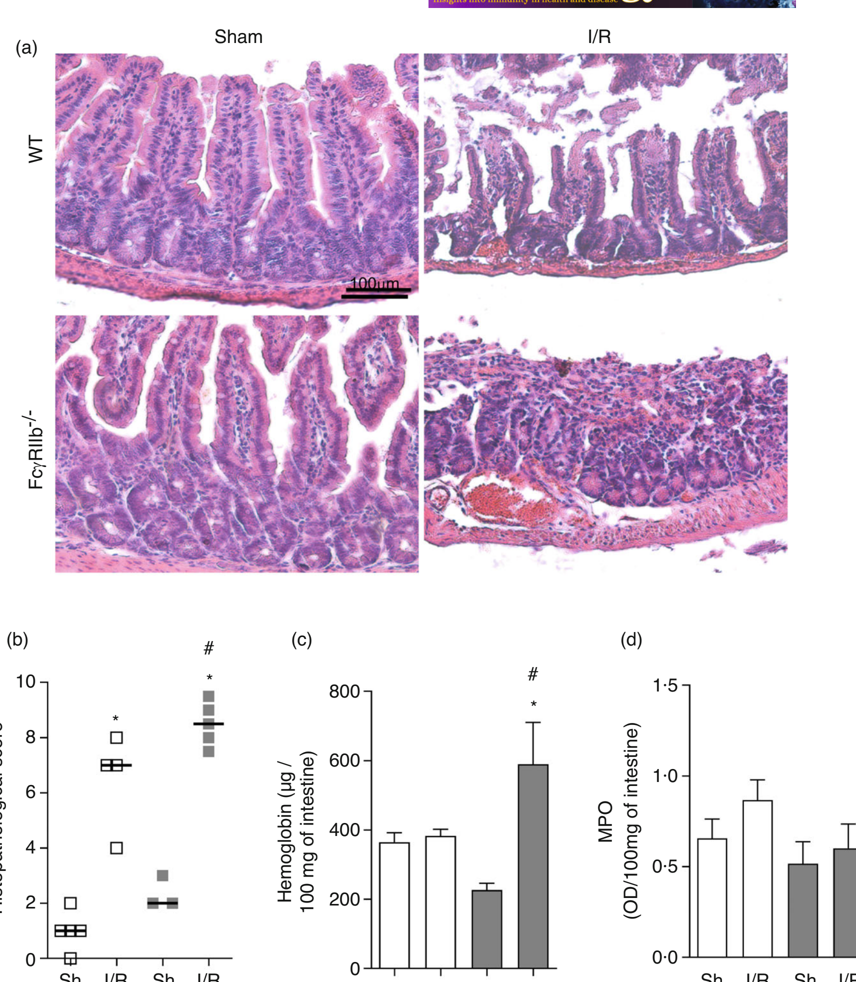

FIGURE 2 Fc$_{\gamma}$RIIb$^{-/-}$ mice presented early tissue injury upon intestinal ischemia/reperfusion. WT and Fc$_{\gamma}$RIIb$^{-/-}$ mice were submitted to ischemia of the SMA for 30 min and reperfusion was allowed for 1 h. Subsequently, animals were euthanized, and small intestine were harvested for the following analyses: (a) H&E staining and (b) histopathological score, (c) haemoglobin quantification and (d) MPO activity. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. *$p < 0.05$ versus the respective sham-operated control group. *$p < 0.05$ versus the respective sham-operated control group. #$p < 0.05$ versus WT mice that were submitted to I/R. Experimental *N*: 3–7, except for (d) where *N*: 13–14. MPO, myeloperoxidase; SMA, superior mesenteric artery

resulting in loss of intestinal architecture (Figure 1c,d). Fc$_{\gamma}$RIIb$^{-/-}$ mice presented a significant increase in haemoglobin levels, an index of haemorrhage, in the intestinal tissue in comparison with WT mice (Figure 1e). However, after I/R, there was a similar increase in MPO activity (Figure 1f) and CXCL1 (Figure 1g) production in both WT and Fc$_{\gamma}$RIIb$^{-/-}$ mice compared to sham mice.

In addition, intense tissue damage in Fc$_{\gamma}$RIIb$^{-/-}$ intestines induced by I/R was followed by bacterial translocation to mesenteric lymph nodes (Figure 1h), peritoneum (Figure 1i), and lung (Figure 1j), which was not observed in WT mice. In addition, lethality induced by I/R was higher in Fc$_{\gamma}$RIIb$^{-/-}$ mice than in WT mice (Figure 1k), as 60% of WT mice succumbed to death, but all Fc$_{\gamma}$RIIb$^{-/-}$ mice died until 12 hr of reperfusion.

Next, to understand how the absence of Fc$_{\gamma}$RIIb influenced early tissue damage induced by I/R, WT, or Fc$_{\gamma}$RIIb$^{-/-}$ mice were subjected to 30 min of ischemia and 60 min of reperfusion (I30R60). After I30R60, WT mice presented mild tissue injury, while Fc$_{\gamma}$RIIb$^{-/-}$ mice presented intense intestinal damage (Figure 2a,b). In accordance with these results, Fc$_{\gamma}$RIIb$^{-/-}$ mice presented intense levels of haemoglobin in the intestines when compared to the sham group (Figure 2c) after I30R60. However, after this time of reperfusion, there was no significant change in MPO activity in any of the groups evaluated (Figure 2d). Therefore, our results clearly indicate the important role played by Fc$_{\gamma}$RIIb in intestinal reperfusion injury.

Control of IgG production by Fc$_{\gamma}$RIIb is important to regulate reperfusion injury

Fc$_{\gamma}$RIIb$^{-/-}$ mice showed higher levels of IgG that bind to IECs protein extracts at the eighth week of life than WT mice. As a negative control, we can observe that MµT$^{-/-}$ mice (lacking circulating Igs), present low levels of IgG that bind to IECs protein extracts (Figure 3a). After I30R60, Fc$_{\gamma}$RIIb$^{-/-}$ mice, but not WT mice, presented an increase in the number of IgG-positive cells in the intestines (Figure 3b,c), suggesting that altered antibody repertoire led to early IgG deposition in Fc$_{\gamma}$RIIb$^{-/-}$ mice submitted to I/R. To evaluate whether the changes in the

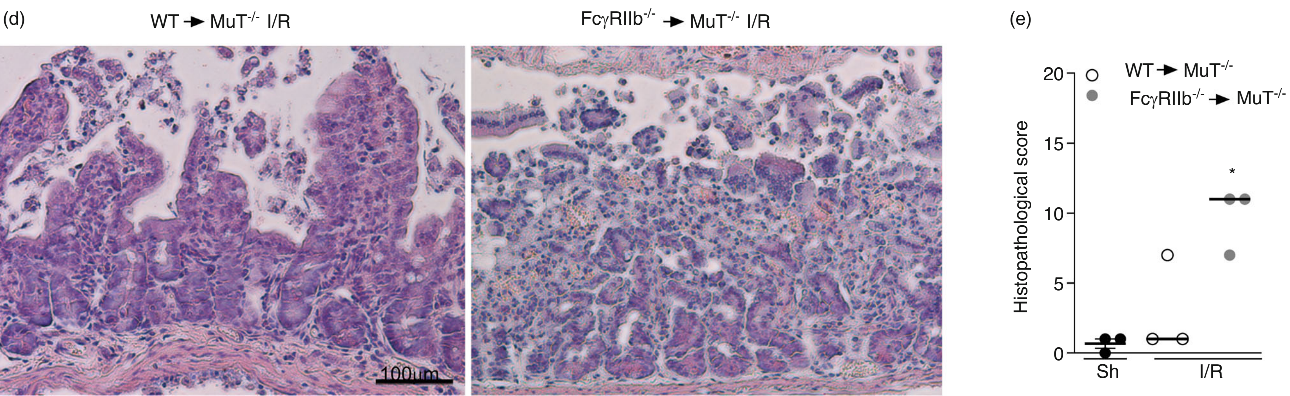

FIGURE 3 The alteration of IgG repertoire in $Fc_{\gamma}RIIb^{-/-}$ mice is important for the severe reperfusion injury in these animals. Serum was collected of 8 weeks old $Fc_{\gamma}RIIb^{-/-}$, WT, and $M\mu T^{-/-}$ mice in order to evaluate IgG reactivity towards intestinal antigens (a). Then, $Fc_{\gamma}RIIb^{-/-}$ mice were submitted to 30 min of ischemia by SMA occlusion and 1 h of reperfusion. After this, animals were euthanized, and small intestines were harvested for quantification of IgG+ cells in intestines; (b, c) histological boards with representative images of the tissues after staining for IgG deposition and counterstaining with haematoxylin. (d, e) $M\mu T^{-/-}$ mice received 400 μl of WT mouse serum or $Fc_{\gamma}RIIb^{-/-}$ mouse serum 12 h before ischemia. After 30 min of ischemia by SMA occlusion, reperfusion was performed, which lasted for 1 h. posteriorly, mice were euthanized, and the small intestines were harvested for H&E staining and histopathological scoring. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. *$p < 0.05$ versus the respective sham-operated control group. *$p < 0.05$ versus the respective sham-operated control group. #$p < 0.05$ versus WT mice. Experimental N: 3–9. SMA, superior mesenteric artery

IgG repertoire in $Fc_{\gamma}RIIb^{-/-}$ mice are important to reperfusion injury, we adopted a serum-transfer strategy and injected serum from WT or $Fc_{\gamma}RIIb^{-/-}$ mice into $M\mu T^{-/-}$ mice (lacking circulating Igs) 12 hr before I/R induction. Previously, our group demonstrated that $M\mu T^{-/-}$ mice are protected from reperfusion injury [12]. We observed that $M\mu T^{-/-}$ mice that received serum from $Fc_{\gamma}RIIb^{-/-}$ mice presented higher tissue damage than $M\mu T^{-/-}$ mice that received serum from WT mice, leading to a marked increase in the histopathologic score (Figure 3d,e). These data suggest that the IgG repertoire in $Fc_{\gamma}RIIb^{-/-}$ mice was associated with increased damage upon intestinal I/R.

$Fc_{\gamma}RIIb^{-/-}$ mice also presented higher levels of IgG reactive to extracts from faecal microbiota than WT mice (Figure 4a). Previously, our group demonstrated that the microbiota is essential to control the production of antibodies important to reperfusion injury and IgG deposition after I/R [12]. Therefore, our next step was to evaluate the role of the gut microbiota in controlling IgG production through $Fc_{\gamma}RIIb$. We observed that after 6 weeks of administration of an antibiotic cocktail, $Fc_{\gamma}RIIb^{-/-}$ mice presented decreased levels of IgG that were reactive to IECs extracts (Figure 4b). In addition to decreased autoreactive IgG production, $Fc_{\gamma}RIIb^{-/-}$ mice that received a cocktail of antibiotics presented reduced

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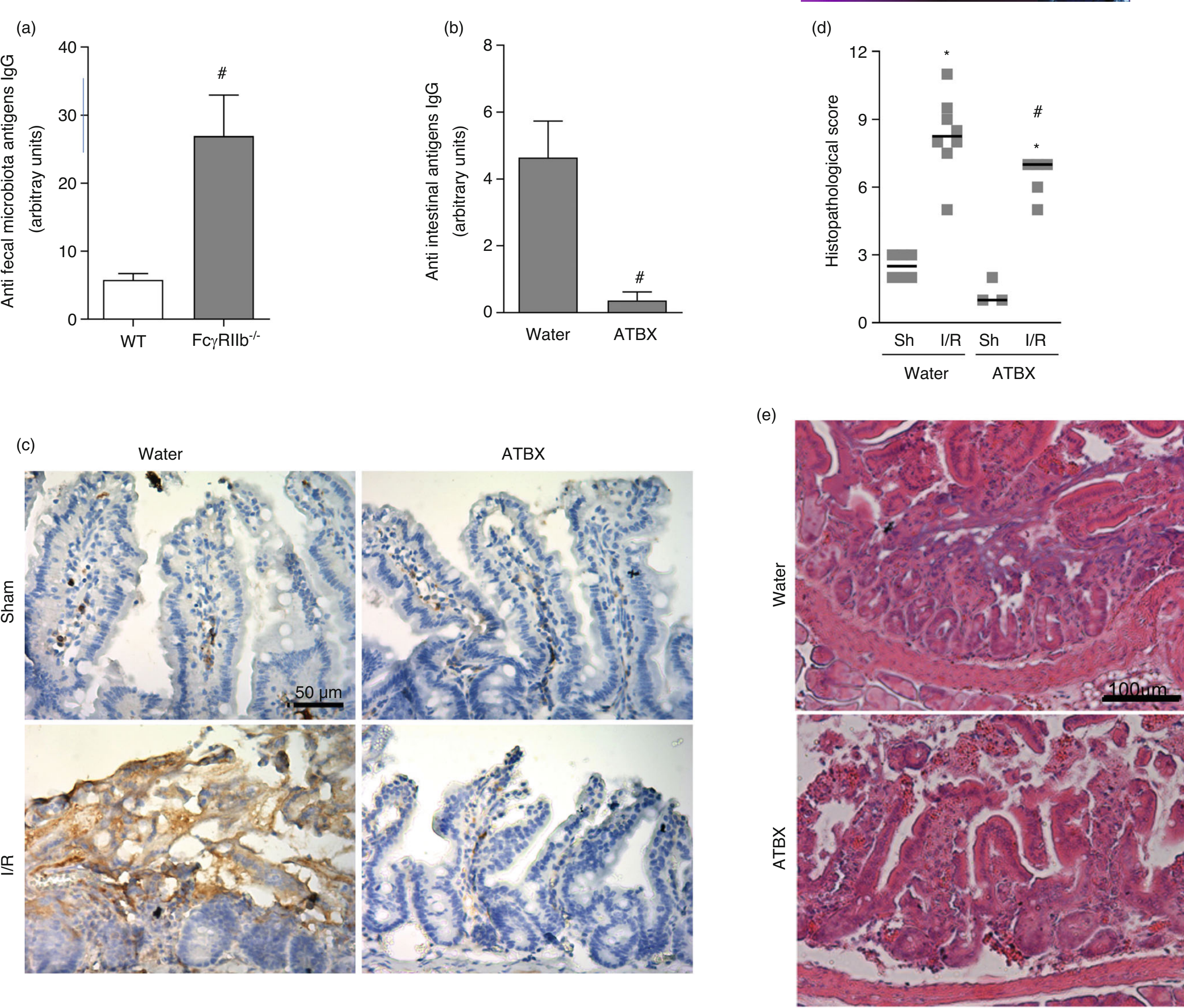

FIGURE 4 Depletion of microbiota alters repertoire of IgG and response to ischemia/reperfusion in Fc$_\gamma$RIIb$^{-/-}$ mice. Serum was collected from 8 weeks old WT and Fc$_\gamma$RIIb$^{-/-}$ mice to evaluate IgG reactivity towards faecal microbiota antigens (a). Fc$_\gamma$RIIb$^{-/-}$ mice received a cocktail of antibiotics for 6 weeks. In the sixth week of treatment serum was collected to evaluate IgG reactivity towards intestinal antigens (b). After the end of depletion, Fc$_\gamma$RIIb$^{-/-}$ mice were submitted to 30 min of ischemia by SMA occlusion and 1 h of reperfusion. After this, animals were euthanized, and small intestines were harvested for IgG deposition by IHQ (c), histopathological score (d) and H&E staining (e). Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. *$p < 0.05$ versus the respective sham-operated control group. *$p < 0.05$ versus the respective sham-operated control group. #$p < 0.05$ versus WT. Experimental *N*: 3–8, except for (b) where *N*: 5–12. ATBX, group that received antibiotic cocktail; IHQ, immunohistochemistry; SMA, superior mesenteric artery

deposition of IgG in the intestine after 1 hr of reperfusion (Figure 4c). Importantly, treatment with antibiotics was also able to decrease reperfusion injury and histopathologic score in Fc$_\gamma$RIIb$^{-/-}$ mice when compared to Fc$_\gamma$RIIb$^{-/-}$ that received water (Figure 4d,e). It is important to emphasize that microbiota depletion by treatment with an antibiotic cocktail in WT mice does not alter susceptibility to reperfusion injury (data not shown) [25]. Together, these data suggest that Fc$_\gamma$RIIb activation regulates the production of microbiota-induced reperfusion-relevant IgG.

Fc$_\gamma$RIIb controls neutrophil activation after intestinal ischemia and reperfusion

After 3 hr of reperfusion WT and Fc$_\gamma$RIIb$^{-/-}$ mice presented similar numbers of neutrophils in the intestines

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FIGURE 5 Fc$_{\gamma}$RIIb is important to control neutrophil activation during reperfusion injury. WT and Fc$_{\gamma}$RIIb$^{-/-}$ mice were submitted to ischemia of the SMA for 30 min, after reperfusion was performed, which lasted for 3 h. subsequently, animals were euthanized and cells from the lamina propria were isolated. Next, cells were incubated with CellROX probe and stained with anti-CD45 and anti-Ly6G antibodies to analyse ROS production by neutrophils. FACS analysis results were expressed as (a) number of neutrophils, (b) the percentage of ROS^{+} neutrophils, (c) number of ROS^{+} neutrophils (D and E) ROS mean fluorescence intensity. (F) After I/R induction the intestines were collected for measuring TNF production. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. *$p < 0.05$ versus the respective sham-operated control group. *$p < 0.05$ versus the respective sham-operated control group. #$p < 0.05$ versus WT. Experimental *N*: 3–5, except for (f) where *N*: 5–8. IHQ, immunohistochemistry; SMA, superior mesenteric artery

(Figure 5a). However, Fc$_{\gamma}$RIIb$^{-/-}$ mice submitted to I/R presented an increased percentage (Figure 5b) and numbers (Figure 5c) of neutrophils producing ROS when compared to sham-operated Fc$_{\gamma}$RIIb$^{-/-}$ mice and WT mice submitted to I/R. Moreover, neutrophils of Fc$_{\gamma}$RIIb$^{-/-}$ mice after I/R presented higher ROS mean fluorescence intensity (Figure 5d,e). Also, Fc$_{\gamma}$RIIb$^{-/-}$ mice presented higher amounts of TNF in the intestines after I/R when compared to WT mice (Figure 5f). These data suggest that despite Fc$_{\gamma}$RIIb$^{-/-}$ and WT mice presenting similar levels of neutrophils influx, neutrophils of Fc$_{\gamma}$RIIb$^{-/-}$ mice are more activated, a fact that may explain the greater intestinal injury in these mice.

The balance between Fc$_{\gamma}$RIIb and Fc$_{\gamma}$RIII activation controls ischemia/reperfusion-induced tissue injury

Our next step was to evaluate the role of Fcγ receptor III activation in reperfusion injury. Interestingly, Fc$_{\gamma}$RIII$^{-/-}$ mice subjected to I/R had a lower lethality rate (Figure 6a) and more discrete tissue injury, as demonstrated by histological analyses (Figure 6b), which resulted in the lower histopathological score (Figure 6c), as compared to WT mice. Moreover, there was no significant increase in MPO activity in Fc$_{\gamma}$RIII$^{-/-}$ mice after reperfusion injury (Figure 6d). Fc$_{\gamma}$RIII$^{-/-}$ mice presented a small increase in anti-intestinal antigens IgG (Supl.1A) but similar levels of anti-faecal microbiota IgG (Supl. 1B). In addition, Fc$_{\gamma}$RIII$^{-/-}$ mice subjected to I/R showed IgG deposition (Figure S1C,D).

To test if the protection from reperfusion injury seen in Fc$_{\gamma}$RIII$^{-/-}$ mice involved Fc$_{\gamma}$RIIb activation, we injected an Fc block, an anti-mouse CD16/CD32 antibody, intraperitoneally into Fc$_{\gamma}$RIII$^{-/-}$ mice 1 hr before ischemia. As observed in Figure 6b, injection of the Fc block in Fc$_{\gamma}$RIII$^{-/-}$ mice was associated with worsening of tissue injury in these mice after I/R, leading to an increase in the histopathological score (Figure 6c). However, injection of the Fc block did not

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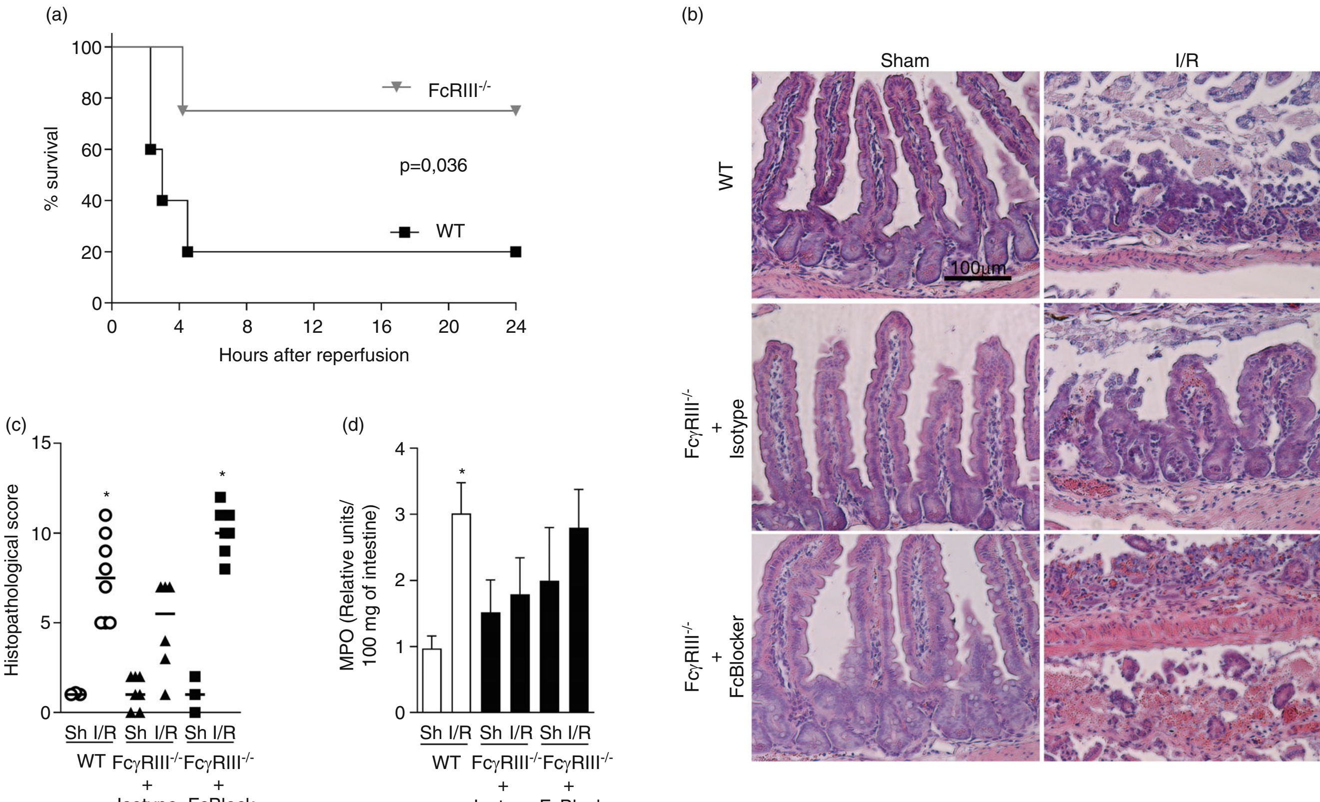

FIGURE 6 The balance between Fc$_\gamma$RIIb and Fc$_\gamma$RIII activation controls ischemia/reperfusion-induced tissue injury. (a) WT and Fc$_\gamma$RIII$^{-/-}$ mice were submitted 30 min of ischemia by SMA occlusion, after that the blood flow was restored, and animals were monitored for up to 24 h to evaluate lethality rates. (b–d) Fc$_\gamma$RIII$^{-/-}$ mice received 100 μg of Fc block (anti-CD16/CD32) 1 h before ischemia. After 30 min of ischemia by SMA occlusion and 3 h of reperfusion, animals were euthanized, and small intestines were harvested for the following analysis: (b) H&E staining, (c) histopathological score and (d) MPO activity. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. *$p < 0.05$ versus the respective sham-operated control group. *$p < 0.05$ versus the respective sham-operated control group. Experimental *N*: 3–13. IHQ, immunohistochemistry; MPO, myeloperoxidase; SMA, superior mesenteric artery

significantly increase MPO activity in Fc$_\gamma$RIII$^{-/-}$ mice after I/R (Figure 6d), demonstrating that blocking of Fc$_\gamma$RIIb in Fc$_\gamma$RIII$^{-/-}$ mice was able to reverse the protective phenotype of Fc$_\gamma$RIII$^{-/-}$ mice in reperfusion injury independently of an increase in neutrophil influx.

IFNβ production is controlled by Fc$_\gamma$RIIb activation, and IFNAR receptor activation promotes reperfusion injury

IFN-Is are inducers of innate and adaptive immunity and have been described as mediators of the inflammatory response associated with I/R injury in the liver, brain, and kidneys [7–9]. First, we assessed the expression of IFN-β at different times of reperfusion and observed a marked increase in IFN-β expression 3 hr post reperfusion compared to sham controls (Figure 7a). On the other hand, there were no significant alterations in IFN-α4 expression at the same time of reperfusion (Figure 7b). We also observed an increase in ISG15 and ISG20 expression after 3 hr of reperfusion (Figure 7b), indicating increased IFN-Is receptor (IFNAR) mediated-gene expression at this time point. Next, we checked IFN-β expression in Fc$_\gamma$RIII$^{-/-}$ mice to understand whether there is a correlation between IFN-I expression and tissue injury in these mice. We observed a marked reduction in IFN-β expression in the small intestine of Fc$_\gamma$RIII$^{-/-}$ mice after reperfusion (Figure 7c). However, treatment of these mice with Fc block restored IFN-β expression after reperfusion (Figure 7d), suggesting that Fc$_\gamma$RIIb receptor activation in Fc$_\gamma$RIII$^{-/-}$ mice might inhibit IFN-β expression. To further assess the role of IFN-Is in intestinal IR injury, we performed 30 min of ischemia on IFNAR$^{-/-}$ mice and observed almost

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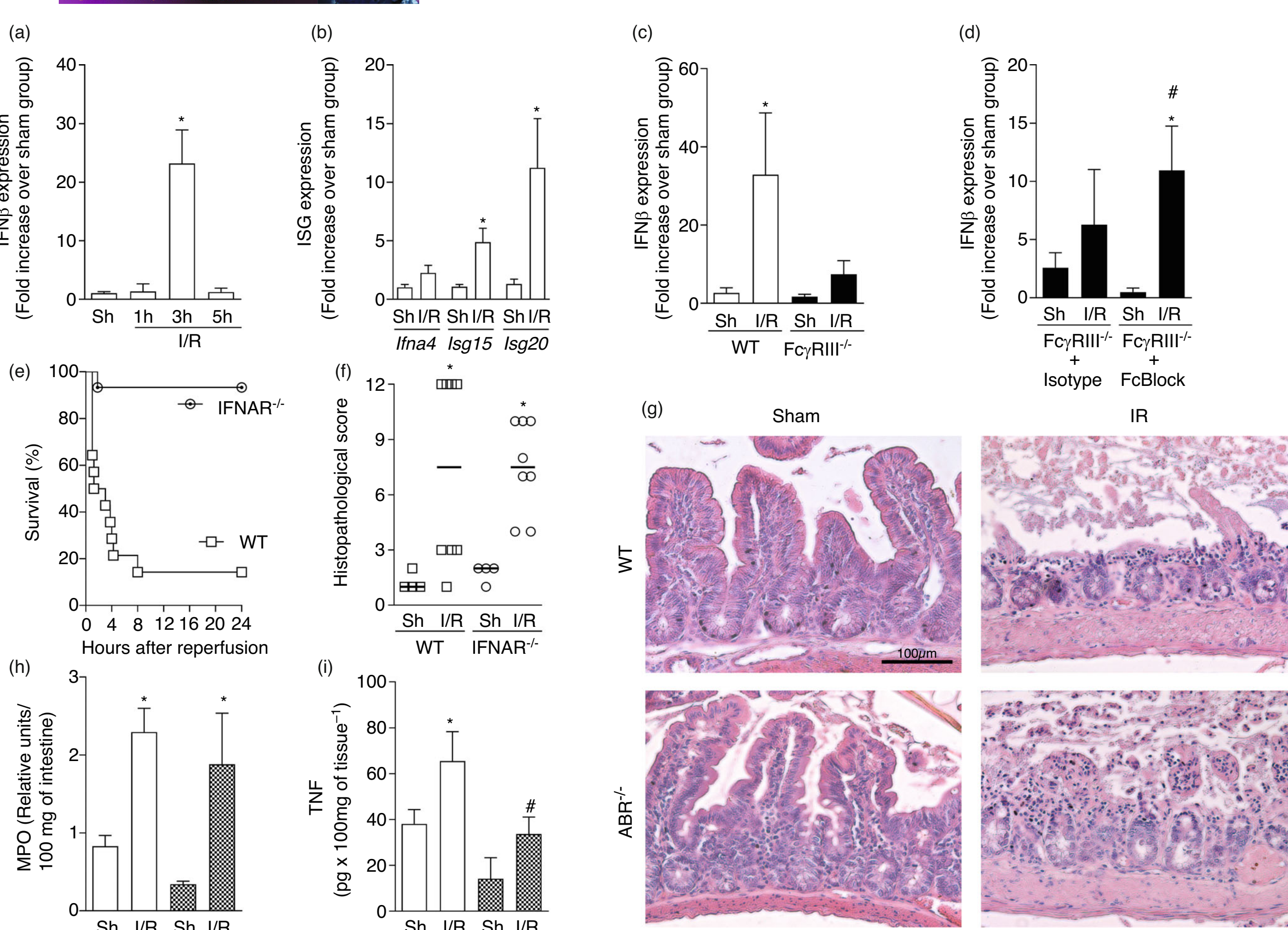

FIGURE 7 Fc_{γ}RIIb regulates IFNβ production during reperfusion injury and IFNAR activation enhances susceptibility to ischemia/reperfusion. WT mice were submitted to 30 min of ischemia by SMA occlusion and 1, 3, or 5 h of reperfusion for analysis of (a) IFNβ and (b) ISG gene expression in intestines. Next, WT $Fc_{\gamma}RIII^{-/-}$ and $Fc_{\gamma}RIII^{-/-}$ mice that received Fc block were submitted to 30 min of ischemia and 3 h of reperfusion for analysis of (c, d) IFNβ gene expression in intestines. (e) WT and $IFNAR^{-/-}$ mice were submitted to ischemia of the SMA for 30 min. After 30 min of ischemia by SMA occlusion, the blood flow was restored, and animals were monitored for up to 24 h to evaluate lethality rates. WT and $IFNAR^{-/-}$ were submitted to 30 min of ischemia and 3 h of reperfusion and intestines was collected for (f) histopathological score, (g) H&E staining, (h) MPO activity and (i) TNF production. Results are the mean ± SEM in each group. Differences were compared using ANOVA, followed by Student–Newman–Keuls post hoc analysis (normal distribution and equal variances). In the case of unequal variances, ANOVA was followed by the Tukey–Kramer post hoc test. Survival curves were compared using the log-rank test. $*p < 0.05$ versus the respective sham-operated control group. $*p < 0.05$ versus the respective sham-operated control group. $\#p < 0.05$ versus WT. Experimental N: 4-8, except for (e), (i), and (j) where N: 5–15. ISG, interferon-stimulated genes; MPO, myeloperoxidase; SMA, superior mesenteric artery

complete survival after 24 hr of reperfusion, whereas its WT control demonstrated less than 20% survival after the same period (Figure 7e). Despite we observed no differences in the histopathological score (Figure 7f,g) or MPO activity (Figure 7h), we have detected diminished TNF production in intestines of $IFNAR^{-/-}$ mice when compared to its WT control group (Figure 7i) 3 hr after reperfusion induction. Therefore, our data suggest that IFN-I expression and release are regulated by $Fc_{\gamma}R$, and IFNAR activation control TNF production and susceptibility to intestinal reperfusion injury.

DISCUSSION

In this study, we demonstrated that antibody deposition followed by IgG receptor activation after intestinal I/R determines the development of tissue injury and that the absence of the Fc_{γ}RIIb receptor aggravates intestinal damage. The phenotype observed in mice lacking Fc_{γ}RIIb is due to alterations in their antibody repertoire, and such alterations are dependent on the intestinal microbiota. We also demonstrated that the balance between Fc_{γ}RIII and Fc_{γ}RIIb activation controls tissue injury after

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I/R. Finally, we show that IFNβ expression is regulated by Fc_{γ}RIIb and that IFNAR activation controls death after intestinal I/R.

We observed an increase in IgG deposition in the small intestines of mice after I/R, as previously reported by our group and others [12, 26, 27]. Interestingly, we observed that $Fc_{\gamma}RIIb^{-/-}$ mice subjected to I/R presented more severe tissue damage and increased lethality rate, despite the lack of an increased neutrophil influx to the small intestine compared to WT mice. However, our findings suggest that worsening of tissue damage induced by the lack of Fc_{γ}RIIb is not dependent on the increase of neutrophil recruitment, but on the neutrophil activation profile. Our previous studies demonstrated an important association between neutrophil influx and reperfusion injury [3–5, 22]. In addition, we showed that the inhibition of neutrophil influx reduces tissue damage and lethality induced by reperfusion injury [28]. The role of neutrophils in intestinal injury after I/R is associated with the capacity of these cells to produce reactive species and cytokines, such as ROS and TNF [24, 29]. Thus, the higher amounts of TNF and neutrophilic ROS production in the small intestine of $Fc_{\gamma}RIIb^{-/-}$ may contribute to the greater tissue damage observed in these animals.

Interestingly, $Fc_{\gamma}RIIb^{-/-}$ mice presented an anticipated reperfusion injury. After 1 hr of reperfusion, $Fc_{\gamma}RIIb^{-/-}$ mice have already shown significant tissue damage despite no neutrophil influx, suggesting Fc_{γ}RIIb tailors early IRI-mediated-tissue injury in a neutrophil-independent manner. $Fc_{\gamma}RIIb^{-/-}$ mice showed higher deposition of antibodies when compared to WT mice. In addition, $M\mu T^{-/-}$ mice, which lack circulating antibodies and are protected from I/R, as previously demonstrated by our group [12], injected with serum from $Fc_{\gamma}RIIb^{-/-}$ mice developed more severe tissue damage than those that received serum from WT mice. Together, these data indicate that, at least in part, the lack of control in tissue injury in the absence of Fc_{γ}RIIb may be caused by the deposition of antibodies. In fact, it was previously demonstrated that autoantibodies play an important role in the initial inflammatory response induced by ischemia and reperfusion [13, 17]. Indeed, antibody deposition allows complement classical pathway activation, ultimately leading to tissue injury [30]. Moreover, previous studies from our group and others have demonstrated that activation of the classic complement pathway is an important event to induce tissue damage after ischemia and reperfusion injury in multiple organs [31–35]. However, it was not yet clear whether $Fc_{\gamma}RIIb^{-/-}$ mice had increased amounts of autoreactive antibodies or increased exposition of antigens during I/R.

Previously, we have demonstrated the essential role of the intestinal microbiota in controlling the production of I/R-inducing antibodies [12]. Here, we observed that in addition to the higher levels of antibodies reactive to IEC extracts, $Fc_{\gamma}RIIb^{-/-}$ mice also present increased levels of faecal microbiota-reactive antibodies when compared to the WT controls. Since $Fc_{\gamma}RIIb^{-/-}$ mice have an altered antibody repertoire, we performed a microbiota depletion protocol with an antibiotic cocktail on $Fc_{\gamma}RIIb^{-/-}$ mice, after which we observed dramatic reduced autoreactive antibody levels, suggesting a shift in antibody production after microbiota depletion. To further investigate this hypothesis, we performed intestinal I/R in $Fc_{\gamma}RIIb^{-/-}$ mice treated with antibiotics and observed reduced IgG deposition and tissue damage after ischemia and reperfusion. Although we have not tested the contribution of specific microbiota groups to IEC-reactive antibody production, it has been demonstrated that mice lacking Toll-like receptor 2 have reduced IgM deposition and tissue damage after ischemia and reperfusion injury [36]. In addition, it has been demonstrated that gut microbiota signals through Toll-like receptor (TLR) 4 to induce IgG with high specificity for conserved bacterial proteins [37]. In this regard, we have previously demonstrated that microbiota-induced antibodies are extremely important for the host inflammatory response. Briefly, treatment with purified antibodies from conventional naive mice (CV) restored the germfree mouse inflammatory responsiveness in both sterile and infectious insults [12]. Taken together, these data suggest that the increase of antibody deposition is a consequence of enhanced I/R-related autoantibody production in $Fc_{\gamma}RIIb^{-/-}$ mice induced by indigenous microbiota. In opposition to what occurs in WT mice [25], antibiotic treatment induces protection from tissue damage in $Fc_{\gamma}RIIb^{-/-}$ mice, probably due to the altered antibody repertoire and elevated concentration of I/R-related autoantibodies found in these mice.

In addition to controlling autoantibody production, Fc_{γ}RIIb has been shown to regulate Fc_{γ}RIII activation in several inflammatory contexts [38–40]. To gain insights into additional potential effects of Fc_{γ}RIIb activation during IR injury, we conducted experiments in mice lacking Fc_{γ}RIII treated with an anti-CD16/CD32 antibody [41]. As our data show, Fc_{γ}RIIb activation partially protects mice from tissue injury in the absence of Fc_{γ}RIII activation. This phenotype was reversed when mice were treated with Fc block, as we observed in these mice a similar histopathological score to the one we have found in WT mice. However, treatment with Fc block did not restore neutrophil influx to tissue on $Fc_{\gamma}RIII^{-/-}$ mice subjected to I/R. As $Fc_{\gamma}RIII^{-/-}$ mice that received Fc-blocker are not protected from I/R-induced tissue damage despite reduced neutrophil influx to the small intestine, our data suggest that Fc_{γ}RIII activation is important for

neutrophil recruitment after I/R, while also suggesting that neutrophil recruitment is a secondary event to I/R-induced tissue damage.

The association of Fc_{γ}RIIb deficiency with the spontaneous development of systemic lupus erythematosus (SLE) has been widely documented [21, 40, 42]. In both SLE patients and SLE-like disease-bearing mice, the disease is associated with a strong IFN-I driven response, with increased concentrations of IFN-I compared to healthy patients and mice [43–46]. It has also been described that IFN-I is involved in hepatic, renal, and cerebral I/R experimental models. Briefly, IFNAR deficiency protects mice from hepatic and renal I/R, whereas IFNAR signalling is protective in cerebral I/R [7–10]. Indeed, we observed an increase in IFN-β and of the IFN-induced genes ISG15 and ISG20, but not IFN-α4, expression after our model of I/R in WT mice. Interestingly, $Fc_{\gamma}RIII^{-/-}$ mice that are protected from I/R injury failed to increase IFN-β post-I/R, and this phenotype was reversed after treatment with Fc block, which also reversed the protection observed in $Fc_{\gamma}RIII^{-/-}$ mice. As triggering of Fc_{γ}RIIb by antibody deposition and subsequent recruitment of SHIP phosphatases to its ITIM-bearing cytoplasmic domain have been shown to impair IFN-I release [47, 48], these data suggest that Fc_{γ}RIIb inhibits IFN-β production, and therefore we hypothesized that IFNAR activation could have a deleterious role during intestinal I/R. In fact, IFNAR-deficient mice showed complete protection from death when compared to their WT counterparts. Surprisingly, $IFNAR^{-/-}$ mice were not protected from tissue damage and had comparable levels of neutrophil recruitment to the intestines as WT mice. We also observed reduced TNF release in $IFNAR^{-/-}$ mice after I/R. TNF has been shown to mediate reperfusion-induced death in previous studies from our and other groups [4, 5, 7–9, 49], suggesting that the lethality prevention in $IFNAR^{-/-}$ mice is, at least in part, secondary to inability to induce TNF production or release upon I/R induction. Thus, these data suggest that while the absence of IFNAR signalling promotes survival to I/R injury, it does not prevent injury itself.

Taken together, our data allow us to propose that Fc_{γ}RIIb controls the production of microbiota-induced intestinal I/R-relevant antibodies. Furthermore, the deposition of these antibodies triggers Fc_{γ}RIII to promote neutrophil recruitment to the intestines. Deposition of antibodies also triggers Fc_{γ}RIIb to inhibit type IFN-I production, controlling TNF release and susceptibility to death after intestinal IRI.

AUTHOR CONTRIBUTIONS

Camila Bernardo de Brito, Fernando Roque Ascenção, Mauro Martins Teixeira, Caio Tavares Fagundes, and Daniele Glória Souza created the study design and prepared the manuscript. Milton Adriano Pelli Oliveira made and supplied the anti-CD16/CD32 antibody. Camila Bernardo de Brito, Fernando Roque Ascenção, Raquel Duque Nascimento Arifa, Renata Lacerda Lima, Zélia Menezes Garcia, Micheli Fagundes, Brenda Gonçalves Resende, Bezerra, Rafael Oliveira Bezerra, Anna Clara Paiva Menezes dos Santos and Celso Martins Queiroz-Junior performed data acquisition. Camila Bernardo de Brito, Fernando Roque Ascenção, Mauro Martins Teixeira, Caio Tavares Fagundes, and Daniele Glória Souza analysed and interpreted data and performed statistical analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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