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Microbiota-Induced Antibodies Are Essential for Host Inflammatory Responsiveness to Sterile and Infectious Stimuli

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The indigenous intestinal microbiota is frequently considered an additional major organ of the human body and exerts profound immunomodulating activities. Germ-free (GF) mice display a significantly different inflammatory responsiveness pattern compared with conventional (CV) mice, and this was dubbed a "hyporesponsive phenotype." Taking into account that the deposition of immune complexes is a major event in acute inflammation and that GF mice have a distinct Ig repertoire and B cell activity, we aimed to evaluate whether this altered Ig repertoire interferes with the inflammatory responsiveness of GF mice. We found that serum transfer from CV naive mice was capable of reversing the inflammatory hyporesponsiveness of GF mice in sterile inflammatory injury induced by intestinal ischemia and reperfusion, as well as in a model of lung infection by *Klebsiella pneumoniae*. Transferring serum from Ig-deficient mice to GF animals did not alter their response to inflammatory insult; however, injecting purified Abs from CV animals restored inflammatory responsiveness in GF mice, suggesting that natural Abs present in serum were responsible for altering GF responsiveness. Mechanistically, injection of serum and Ig from CV mice into GF animals restored IgG deposition, leukocyte influx, NF-κB activation, and proinflammatory gene expression in inflamed tissues and concomitantly downregulated annexin-1 and IL-10 production. Thus, our data show that microbiota-induced natural Abs are pivotal for host inflammatory responsiveness to sterile and infectious insults. ***The Journal of Immunology*****, 2017, 198: 4096–4106.**

Inflammation is a major and indispensable physiological phenomenon in mammals. The appropriate development of an inflammatory response is central to the ability of a host to deal with a plethora of, if not all, infectious and sterile insults. Although lethality is the usual outcome of an infectious challenge in the absence of inflammation, excessive or uncontrolled inflammation is commonly the cause of death postinfection. Similarly, inappropriate or unconstrained inflammation in response to biotic or sterile stimuli of known or unknown origin is associated with numerous human diseases, including ischemia and reperfusion (I/R) injury (1). I/R injury is the consequence of the transient interruption of tissue blood supply and subsequent restoration of the blood flow. The reperfusion of the deprived tissues induces a catastrophic cascade of events that causes massive local tissue damage and can also lead to multiple organ damage, depending on the circumstances. In mice, reperfusion of the ischemic mesenteric artery is followed by severe local (intestinal) and remote (pulmonary) tissue pathology, characterized by intense neutrophil influx, edema, hemorrhage, and tissue destruction (2, 3). Together with local and remote tissue damage, I/R injury leads to striking systemic inflammation, as assessed by the increased concentration of proinflammatory cytokines and chemokines in serum (3).

A crucial component that regulates inflammatory responses is the partnership between the microbiota and its mammalian host. Over the past decade, exploration of optimal and deregulated host–microbiota interactions became a centerpiece of immunological research and has led to the rediscovery of a more holistic and physiological view of the mammalian immune response. In all of the modes of dialog between the host and its microbiota, the recognition of conserved microbial-associated molecular patterns is a primary one. Axenic or germ-free (GF) mice display a distinct inflammatory phenotype throughout their lives, and they respond to inflammatory stimuli in a unique way (4, 5). Our group has demonstrated previously that GF mice presented little evidence of local or systemic injury after intestinal I/R (6). The inability of these GF mice to inflame in response to reperfusion injury was largely a result of their innate capacity to produce IL-10. This hyporesponsive or anti-inflammatory state of GF mice is noticeable in the manner in which they respond to other acute inflammatory triggers. Inflammatory hypernociception induced by carrageenan, LPS, TNF-α, IL-1β, and CXCL1 is dramatically reduced in GF

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Abbreviations used in this article: ANXA-1, annexin-1; BAL, bronchoalveolar lavage; CV, conventional; CVN, conventionalized; GF, germ-free; GR, glucocorticoid receptor; I/R, ischemia and reperfusion; LXA_4, lipoxin A_4; MPO, myeloperoxidase; SMA, superior mesenteric artery; WT, wild-type.

mice, and this phenomenon was accompanied by enhanced IL-10 expression upon stimulation (7). Blocking IL-10 with Abs reverses this phenotype and restores inflammation and tissue injury (6, 7). IL-10 production appears to be related to the enhanced production of lipoxin A4 (LXA_4) and annexin-1 (ANXA-1) (8). LXA_4 and ANXA-1 are two effectors of endogenous anti-inflammation that are able to halt leukocyte migration and promote macrophage phagocytosis of infective agents, as well as apoptotic leukocytes (9). In 2007, we reported that the production of LXA_4 and ANXA-1 is enhanced in GF mice, and both mediators appear to cooperatively activate the shared ALX receptor to induce IL-10 production and mediate the inflammatory hyporesponsiveness of GF mice.

GF mice also differ dramatically from CV mice with regard to Ab repertoire and B cell populations. They have almost undetectable levels of circulating IgG and >1000-fold less IgA than CV mice (10–16). Abs are naturally potent inducers of inflammation. They are central components in most autoimmune diseases with self-reactive Abs and activation of the complement cascade, as well as in systemic inflammatory events (17). The deposition of immune complexes is a major event in acute systemic inflammation. Animals deficient in expression of the RAG enzyme, and, as such, free from circulating Abs, show reduced reperfusion injury, and treatment with Abs from wild-type (WT) animals is capable of restoring the inflammatory injury (18). Hence, of the myriad mediators and effectors involved in the inflammatory response to I/R injury, several studies have demonstrated a pivotal role for natural, self-reactive Abs that bind to neoantigens exposed during ischemia, leading to complement activation and amplification of inflammation (18–27). Therefore, the altered response to inflammatory triggers found in GF mice might rely on the blunted production of natural Abs that is characteristic of the noncolonized state.

In the present study, we set out to investigate whether this difference in the circulating Ab repertoire had any impact on the hyporesponsiveness phenotype in GF mice and how it would affect the pathogenesis of acute inflammatory responses. Our data show that injection of Igs from CV mice into GF animals leads to downregulation of ANXA-1 and IL-10 production and restores inflammatory responsiveness to sterile and infectious triggers in these animals. These findings demonstrate that microbiota-induced natural Abs tailor host responses to inflammatory insults and, therefore, are essential for host acute inflammatory responsiveness.

Materials and Methods

Animals

GF Swiss/NIH mice were derived from a GF nucleus (Taconic Farms, Germantown, NY) and maintained in flexible plastic isolators (Standard Safety Equipment, Palatine, IL) using classical gnotobiology techniques (6). CV Swiss/NIH mice are derived from GF matrices and are considered CV only after two generations in the CV facility. $MuT^{-/-}$ mice were kindly donated by J.S. da Silva (Universidade de São Paulo, Ribeirão Preto, Brazil). All animals were 8–10-wk-old males and females. All experimental procedures using GF mice were conducted under aseptic conditions to avoid infection of animals and had prior approval from the local animal ethics committee.

Ischemia and reperfusion

Mice were anesthetized with urethane (1400 mg/kg, i.p.), and laparotomy was performed. The superior mesenteric artery (SMA) was isolated, and ischemia was induced by totally occluding the SMA for 60 min. To measure the percentage of surviving mice, reperfusion was re-established, and mice were monitored for the indicated time periods. For the other parameters, reperfusion was allowed to occur for 40 min when mice were sacrificed. This time of reperfusion (40 min) was chosen based on the presence of significant tissue injury without unduly high mortality. Sham-operated animals were used as controls. In experiments evaluating the survival rates induced by I/R injury, mice were monitored for 180 min after reperfusion and then euthanized for ethical reasons.

Pulmonary infection by Klebsiella pneumoniae

The bacterium used was *K. pneumoniae* (ATCC 27 736), which was kept in the Department of Microbiology, Universidade Federal de Minas Gerais, and made pathogenic by 10 passages in BALB/c mice (28). Bacteria were frozen after reaching the logarithmic phase of growth and kept in a −70°C freezer at a concentration of 1×10^9 CFU/ml in tryptic soy broth (Difco, Detroit, MI) containing 10% glycerol (v/v) until use. The bacteria were cultured for 18 h at 37°C prior to inoculation. The concentration of bacteria in broth was routinely determined by serial 1:10 dilutions. A total of 100 μl of each dilution was placed on MacConkey agar plates and incubated for 24 h at 37°C and then colonies were counted. Each animal was anesthetized i.p. with 0.2 μl of solution containing xylazine (0.02 mg/ml), ketamine (50 mg/ml), and saline in a 1:0.5:3 ratio, respectively. The trachea was exposed, and 30 μl of the suspension containing 3×10^6 *K. pneumoniae* or saline was administered with a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Conventionalization of GF mice

The process of colonizing GF mice with microbiota from CV mice is referred to as conventionalization, and this was performed as previously described (6). Briefly, fecal samples removed from the large intestine of CV mice were homogenized in saline (10%) and administered by oral gavage to GF mice. Fourteen days later, blood was collected from these mice for serum isolation. To assess whether there was adequate conventionalization of GF mice, fecal samples were cultured using a thioglycollate test (6).

Serum and purified Ig injection in mice

Serum (400 μl per animal) aseptically obtained from coagulated blood from CV, GF, C57Bl6j, or $MuT^{-/-}$ mice was injected i.p. 24 h before induction of intestinal I/R or pulmonary infection by *K. pneumoniae*. In some experiments, serum was heat inactivated by incubation at 56°C for 60 min. The efficiency of complement activation by this procedure was evaluated by the reduced ability of inactivated serum to lyse *Escherichia coli* cells in vitro. Total Ig purification was achieved using the T-Gel Purification Kit (Pierce, Rockford, IL), according to the manufacturer's instructions. After purification and concentration using Amicon Ultra Centrifugal filters (100K; Millipore), 400 μg of total Ig in 400 μl of PBS was injected i.p. per animal 24 h before intestinal I/R induction.

Evaluation of changes in vascular permeability

The extravasation of Evans Blue dye into the tissue was used as an index of increased vascular permeability, as previously described (29, 30). Briefly, Evans Blue (20 mg/kg) was administered i.v. (1 ml/kg) via a tail vein 2 min before reperfusion of the ischemic artery. Forty minutes after reperfusion, a segment of the duodenum (3 cm) or the flushed left lung was cut into small pieces, and Evans Blue was extracted using 1 ml of formamide. The amount of Evans Blue in the tissue (micrograms of Evans Blue per 100 mg of tissue) was obtained by comparing the extracted absorbance to that of a standard Evans Blue curve read at 620 nm in an ELISA plate reader.

Myeloperoxidase concentrations

The extent of neutrophil accumulation in the intestine and right lung tissue was measured by assaying myeloperoxidase (MPO) activity, as previously described (30, 31). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone I/R injury were removed and snap-frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for MPO activity by measuring the change in OD at 450 nm using tetramethylbenzidine. Results were expressed as the total number of neutrophils by comparing the OD of tissue supernatant with the OD of casein-elicited murine peritoneal neutrophils processed in the same way.

Histopathology and immunohistochemistry

Intestinal samples from adult euthanized mice were obtained after intestinal I/R. They were immediately fixed in 10% buffered formalin for 48 h, processed, and embedded in paraffin. Tissue sections (5 μm thickness) were stained with H&E using routine histological procedures.

For immunohistochemical evaluation, sections were treated with 3% H_2O_2 diluted in PBS (pH 7.4) for 30 min. Tissue sections were then immersed in citrate buffer (pH 6) for 20 min at 95°C for Ag retrieval. The next step was the blocking of unspecific reactions with 2% BSA for 30 min at room temperature. Fcγ receptors were blocked using purified rat anti-mouse CD16/CD32 (Fc Block; BD Pharmingen) 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. Subsequently, the sections were

rinsed with a solution of PBS and DAB Chromogen and counterstained with hematoxylin. IgG^+ cells present in intestinal vilosities were counted in 20 consecutive microscopic fields (400×) using ImageJ software (National Institutes of Health). Results were expressed as the number of IgG^+ cells per square millimeter of tissue.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed to obtain leukocytes from the alveolar spaces. The trachea was exposed, and a polyethylene catheter (1.7 mm outside diameter) was inserted. BAL was performed by instilling three 1-ml aliquots of PBS, and ~2 ml of fluid was retrieved per mouse. The number of total leukocytes was determined by counting in a modified Neubauer chamber after staining with Turk's solution. Differential counts were obtained from cytospin preparations by evaluating the percentage of each leukocyte on a slide stained with May-Grünwald-Giemsa.

Determination of lung K. pneumoniae *CFU*

At the time of sacrifice, the right ventricle was perfused with 3 ml of sterile saline, and lungs were harvested. Tissues were homogenized with a homogenizer in a vented hood. The homogenates and plasma were placed on ice, and serial 1:10 dilutions were made. One hundred microliters of each dilution was plated on MacConkey agar plates (Difco) and incubated for 24 h at 37°C, and the number of CFU was counted. The detection limit of the assay was 100 bacteria per 100 mg of tissue.

Measurement of mRNA expression by real-time RT-PCR

Total RNA was isolated from intestine using an RNeasy Mini Kit (QIAGEN). The RNA obtained was resuspended in diethylpyrocarbonate-treated water and stored at −70°C until use. Real-time RT-PCR was performed on an ABI PRISM 7900 sequence detection system using SYBR Green PCR Master Mix (both from Applied Biosystems) after a reverse-transcription reaction of 2 μg of RNA using M-MLV Reverse Transcriptase (Promega). The relative level of gene expression was determined by the ΔΔ cycle threshold method, as described by the manufacturer; data for each sample were normalized to 18S rRNA and expressed as a fold change compared with naive animals. The following primer pairs were used: 18S rRNA, 5′-TTGGTTACAGGCCAGACTTTGTTG-3′ (forward) and 5′-GAGGGTAGGCTGGCCTATAGGCT-3′ (reverse); *il-10* 5′-GCTCTTACTGACTGGCATGAG-3′ (forward) and 5′-CGCAGCTCTAGGAGCATGTG-3′ (reverse); *AnxA-1* 5′-ACTCTGCGAAGATGAGGAAAG-3′ (forward) and 5′-AGATGCCAGGGCTTTGTATG-3′ (reverse); *Tnfa* 5′-ACGGCATGGATCTCAAAGAC-3′ (forward) and 5′-AGATAGCAAATCGGCTGACG-3′ (reverse); and *Tsc22d3* 5′-CAGCAGCCACTCAAACGAGC-3′ (forward) and 5′-ACCACATCCCCTCCAAGCAG-3′ (reverse).

Measurement of cytokine/chemokine concentrations in serum, intestine, and lungs and of IgG/IgM in serum

Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at 20°C until further analysis. Serum samples were analyzed at a 1:3 dilution in PBS. One hundred milligrams of jejunum/ileum or lung of animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM Na_3PO_4) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 U of aprotinin A) and 0.05% Tween 20. The samples were centrifuged for 10 min at 3000 × *g*, and the supernatant was immediately used for ELISA at a 1:3 dilution in PBS. The concentration of TNF-α and IL-10 was measured in serum and tissue of animals using commercially available Abs and according to the procedures supplied by the manufacturer (R&D Systems). For measurement of total IgG or IgM in serum, samples were diluted (1:50,000 and 1:2,000, respectively) in PBS containing 0.1% BSA and then assayed by ELISA using commercially available Abs (R&D Systems).

Western blotting

One hundred milligrams of jejunum/ileum from sham-operated and reperfused animals were homogenized in 1 ml of cell lysis buffer (1% Nonidet P-40, 100 mM Tris-HCl [pH 8], 20% glycerol, 0.2 mM EDTA, 1 mM Na_3PO_4, 1 mM DTT, 1 mM PMSF, 200 mM NaCl, leupeptin, and aprotinin). The samples were centrifuged for 10 min at 3000 × *g*, the supernatant was collected, and total protein concentration was determined according to the instructions for the Bio-Rad assay kit. To detect p65 and p–IκB-α, protein extracts (30 μg) were loaded onto a 10% SDS-PAGE gel for electrophoresis, together with the appropriate m.w. markers, and transferred to an ECL Hybond nitrocellulose membrane. Reversible protein staining of the membranes with 0.1% Ponceau S in 5% acetic acid was used to verify even protein transfer. Membranes were incubated for 1 h at room temperature in 5% nonfat dry milk in TBST. The membranes were washed three times for 5 min with TBS and incubated overnight with rabbit hyperimmune serum anti-p65 and p–IκB-α (1:100; Santa Cruz Biotechnology, Dallas, TX) in TBST with 5% BSA. β-Actin was used as a loading control. After additional washing, the membranes were incubated for 60 min at room temperature with peroxidase-conjugated goat anti-rabbit IgG (1:600), and immunoreactive proteins were detected using an ECL Kit (Amersham Biosciences). Relative band intensity was quantified using NIH Image software 1.63.

Statistical analysis

Results are shown as mean ± SEM. Percentage inhibition was calculated by subtracting the background values obtained in sham-operated animals. Data were evaluated according to distribution and variances and then 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. Results with a p value <0.05 were considered significantly different. GraphPad Prism 5.01 software (GraphPad) was used for the analyses.

Results

Serum transfer from CV mice restores the inflammatory response, tissue injury, and lethality induced by I/R injury in the small intestine of GF mice

Several studies have shown that GF mice have a drastically reduced repertoire of Igs compared with CV mice (15, 16). We also observed a major reduction in circulating IgG levels in GF mice compared with CV mice (Supplemental Fig. 1A, $p = 0.0021$), although IgM levels were similar in both groups (Supplemental Fig. 1B, $p = 0.0058$). We first investigated whether the altered response of GF mice to intestinal I/R could be due to the difference in circulating Igs. To address this question, we adopted a serum-transfer strategy and injected serum from CV mice to GF mice 24 h before I/R. The injection of GF serum to CV mice, GF serum to GF mice, and CV serum to CV mice was also performed as controls.

In CV mice that received CV or GF serum, an intense local and remote inflammatory response was observed after I/R injury that was marked by an increase in vascular permeability (Fig. 1A, Supplemental Fig. 2A, $p < 0.001$), neutrophil influx (Fig. 1B, Supplemental Fig. 2B, $p < 0.001$), and TNF-α concentration (Fig. 1C, Supplemental Fig. 2C, $p < 0.001$) in intestines and lungs, respectively. CV mice that received GF serum showed no alterations in any of the investigated parameters (Fig. 1A–C, Supplemental Fig. 2A–C). GF mice that received serum from GF mice displayed no alteration in any of the parameters evaluated upon intestinal reperfusion injury. Remarkably, GF mice that received CV serum and were submitted to I/R induction displayed a complete reversal of plasma extravasation, neutrophil influx, and TNF-α production (Fig. 1A–C, Supplemental Fig. 2A–C, $p < 0.0001$). It is important to point out that injection of serum from CV mice did not induce any alteration in the evaluated parameters compared with sham-operated CV mice or sham-operated GF mice that received serum from GF mice (Fig. 1A–C).

Transfer of CV serum to GF mice is capable of restoring the inflammatory response triggered by I/R injury, as shown above. An interesting aspect of this reversal is the abrogation of IL-10 release. In GF animals that received GF serum, high concentrations of IL-10 were detected in the small intestine and lungs after reperfusion (Fig. 1D, Supplemental Fig. 2D). In GF animals that received CV serum, no IL-10 was detected after reperfusion (Fig. 1D, Supplemental Fig. 2D, $p < 0.0001$), in a similar manner to that observed in both groups of CV mice that were submitted to I/R injury, independently of whether they were previously treated with serum obtained from CV or GF mice.

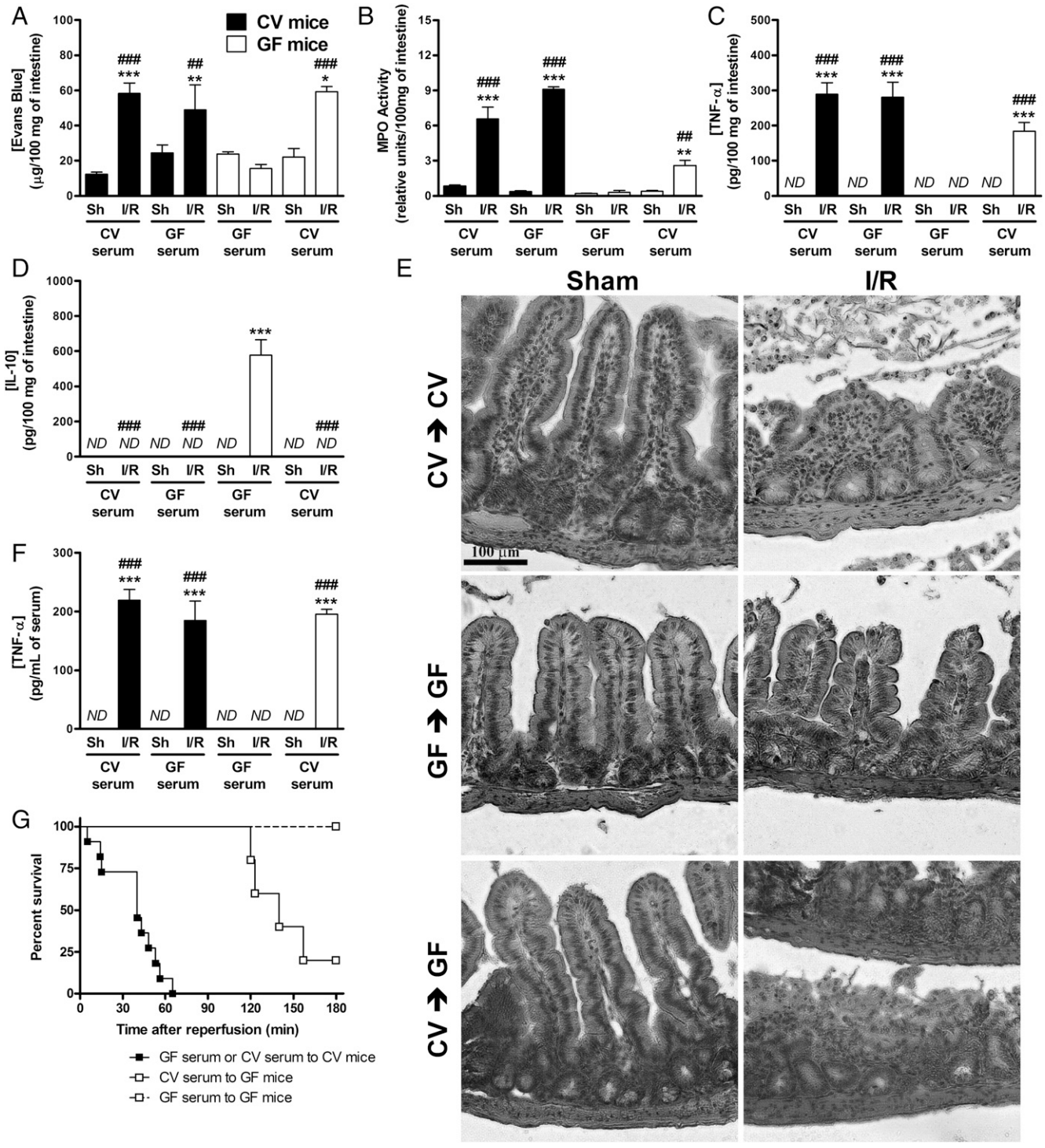

FIGURE 1. Serum transfer from CV mice restores the inflammatory response in the small intestine and lethality in GF mice submitted to intestinal I/R injury. (**A**–**G**) Mice received 400 μl of CV mouse serum or GF mouse serum 24 h before ischemia. After 60 min of ischemia by SMA occlusion, reperfusion was performed, which lasted for 40 min. Subsequently, animals were euthanized, and jejunum/ileum were harvested for the following analyses: plasma leakage (A), MPO activity (B), TNF-α (C) and IL-10 (D) production, and H&E staining (E). (F) TNF-α levels were also assessed in the serum. (G) After 60 min of ischemia by SMA occlusion, the blood flow was restored, and animals were monitored for up to 180 min to evaluate lethality rates. Results are the mean ± SEM of six animals in each group. Sham group comprises the pooled values of CV and GF mice submitted to control surgery. Values were pooled for ease of presentation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the respective sham-operated control group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus GF mice that received GF serum and were submitted to I/R. ND, not detectable.

Reversion of inflammatory parameters in intestines and lungs of GF mice after treatment with serum from CV mice was accompanied by marked tissue injury. I/R induction led to marked injury in the intestines of CV mice that received serum from CV mice (Fig. 1E, top right panel) compared with sham-operated CV mice (Fig. 1E, top left panel), which was characterized by denuded villi and severe distortion of tissue architecture, with several foci of ulceration, hemorrhage, and leukocyte influx. These changes were virtually absent in tissues of GF mice that received serum from GF mice and were submitted to I/R

(Fig. 1E, middle right panel). Intestinal tissue structures of sham-operated GF animals were similarly normal, regardless of the source of the serum transferred to them (Fig. 1E, compare middle and bottom left panels). However, injection of CV serum into GF mice re-established tissue injury in intestines submitted to I/R injury (Fig. 1E, bottom right panel).

Importantly, treatment of GF mice with serum from CV mice led to increased systemic TNF-α production (Fig. 1F, $p < 0.0001$) and increased lethality rates (Fig. 1G, $p < 0.0001$) compared with GF controls that received only GF serum. Again, injection of serum from CV mice did not induce any alteration in the evaluated parameters in sham-operated animals compared with sham-operated CV mice or sham-operated GF mice that received serum from GF mice (Fig. 1F). Therefore, these findings demonstrate that serum transfer from CV mice to GF mice abrogated IL-10 production in the latter, thus favoring re-establishment of the inflammatory response in these mice, as shown by vascular permeability, neutrophil influx, and TNF-α release.

Serum transfer–mediated re-establishment of the inflammatory response in GF mice is associated with Igs

We have previously shown that reposition of microbiota in GF mice (conventionalization) for 14 d re-establishes an inflammatory phenotype in the conventionalized (CVN) mice (6, 32). Conventionalization of mice for this period also led to an increase in serum IgG concentrations to levels that are similar to those observed in CV mice (Supplemental Fig. 1A, $p = 0.0021$). We then conducted experiments involving transfer of serum from CVN mice to GF animals. Injection of serum from CVN mice into GF mice was able to reverse the hyporesponsive phenotype of these animals to reperfusion injury in a similar way that injection of serum from CV mice did. Hence, GF mice that received serum from CV mice or from CVN animals exhibited plasma extravasation (Fig. 2A, $p = 0.0245$), neutrophil influx (Fig. 2B, $p = 0.0010$), and TNF-α production (Fig. 2C, $p < 0.0001$) in intestines upon induction of reperfusion injury. Previous injection of serum from CV or CVN mice into GF animals abrogated IL-10 production during intestinal I/R (Fig. 2D, $p = 0.0196$). Therefore, conventionalization of GF mice leads to IgG production, and serum from CVN mice restores inflammatory responsiveness in GF hosts.

Our next step was to evaluate whether serum transfer would increase levels of circulating IgG in GF mice and whether there would be IgG deposition upon I/R induction in GF mice that received serum from CV animals, an event that was shown to be important for I/R-associated tissue injury. As demonstrated in Fig. 3A, GF mice that received injections of serum from CV mice exhibited higher levels of IgG in serum ($p = 0.0041$). Also, there was increased IgG deposition after I/R induction in intestinal tissue of GF mice that received serum from CV mice (Fig. 3B, $p = 0.0018$ and 3C, lower right panel) but not in the intestines of those that received serum from GF mice (Fig. 3B, 3C, upper right panel). Therefore, transfer of IgG-containing serum to GF mice replenishes IgG circulating levels and re-establishes IgG deposition during I/R injury.

In addition to Igs, several factors, which are important for the development of inflammation, are present in the serum, including complement. Several studies point to a central role for activation of the complement system in the chain of events that leads to reperfusion injury (26, 33–35). To verify whether factors of the complement system present in the transferred serum were involved in reversion of the hyporesponsive phenotype observed in GF mice, heat-inactivated serum was also used in a serum-transfer experiment. GF mice that received the heat-inactivated

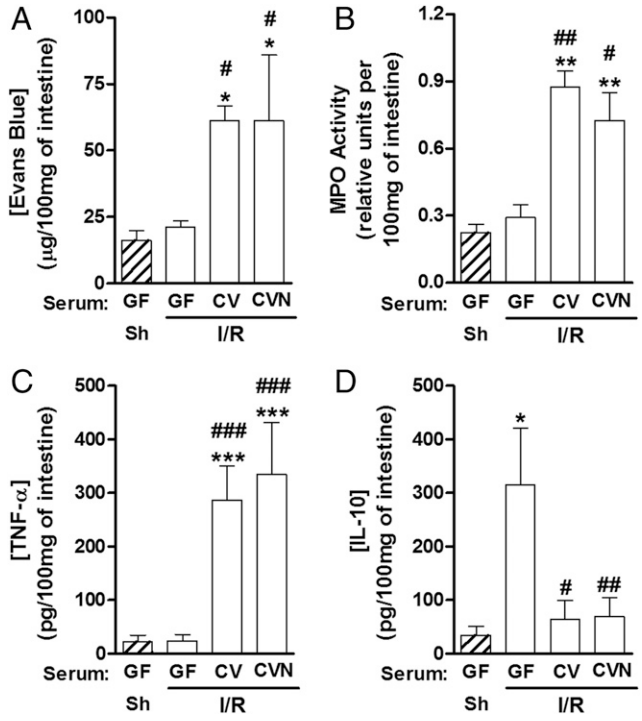

FIGURE 2. Serum from CVN GF mice is also capable of restoring the inflammatory response upon intestinal I/R in GF mice. (**A**–**D**) GF mice received 400 μl of CV mouse serum, GF mouse serum, or CVN GF mouse serum 24 h before ischemia. CVN GF mice were obtained by giving a concoction of CV mice feces to GF mice orally and keeping them under normal specific pathogen–free conditions for 14 d. After 60 min of ischemia by SMA occlusion and 40 min of reperfusion, animals were euthanized, and jejunum/ileum were harvested and assessed for plasma leakage (A), MPO activity (B), and production of TNF-α (C) and IL-10 (D) in tissue. Results are the mean ± SEM of five animals in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the sham-operated control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus GF mice that received GF serum and were submitted to I/R.

serum or noninactivated serum presented the same reversal in inflammatory parameters, as assessed by vascular permeability (Supplemental Fig. 3A, $p = 0.0068$), neutrophil influx (Supplemental Fig. 3B, $p = 0.0002$), and the release of TNF-α in intestine (Supplemental Fig. 3C, $p < 0.0001$) and serum (Supplemental Fig. 3E, $p = 0.0108$). Lethality was also restored (Supplemental Fig. 3F, $p < 0.001$). Notably, IL-10 concentrations were also equally reduced (Supplemental Fig. 3E, $p = 0.0155$) by heat-inactivated and noninactivated sera.

The previous data suggest that the reversion of the anti-inflammatory phenotype in GF mice induced by CV mice serum is related not to complement but to IgG present in the transferred serum. However, these data still cannot determine whether the Abs present in the serum are the key factors to the response re-establishment in GF mice. Hence, the first step taken to determine that was to confirm that circulating Igs are fundamental for the lesion and the response observed in the intestinal I/R model, confirming the findings of other studies (18–27). $MuT^{-/-}$ animals have no circulating Igs, so they were chosen for investigating this aspect of the intestinal I/R model. $MuT^{-/-}$ animals displayed a reduced inflammatory response compared with WT animals, although there was no difference in vascular permeability in the intestines (Supplemental Fig. 4A, $p = 0.0001$), there was reduced neutrophil influx (Supplemental Fig. 4B, $p = 0.0004$), and there was no detectable TNF-α release in intestine (Supplemental Fig. 4C, $p < 0.0001$) or serum (Supplemental Fig. 4D, $p < 0.0001$). Lethality was also

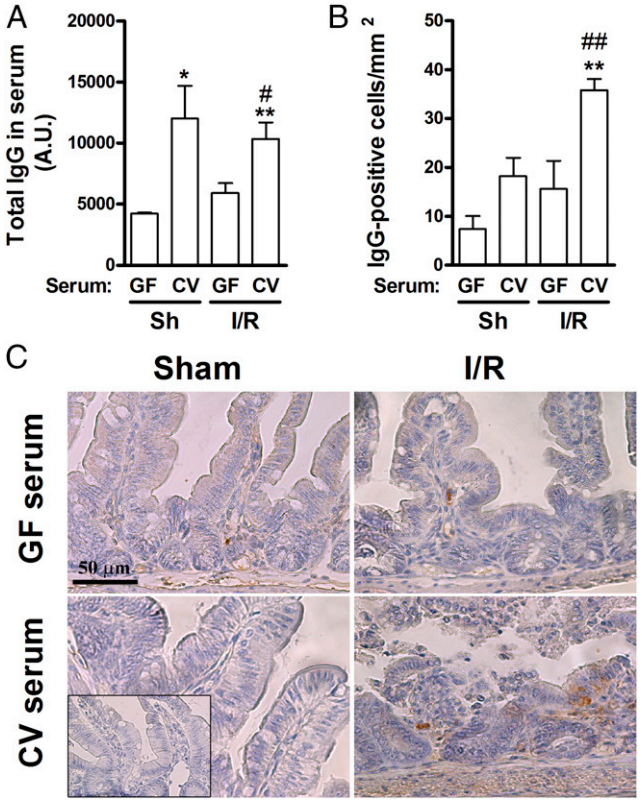

FIGURE 3. Serum transfer from CV mice replenishes IgG circulating levels in GF mice and leads to IgG deposition upon I/R induction. (**A**–**C**) GF mice received 400 μl of CV mouse serum or GF mouse serum 24 h before ischemia. After 60 min of ischemia by SMA occlusion, reperfusion was performed and lasted for 40 min. Subsequently, animals were euthanized, and serum and jejunum/ileum were harvested to assess IgG levels in serum (A) and quantification of IgG^+ cells in intestines (B). (C) Representative images of the tissues after staining for IgG deposition and couterstaining with hematoxylin. The inset in the lower left panel is an image of the staining negative control, original magnification ×400. Results are the mean ± SEM of four animals in each group. $^*p < 0.05$, $^{**}p < 0.01$ versus the sham-operated group that received serum from GF mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus GF mice that received serum from GF mice and were submitted to I/R.

delayed and partially prevented in $MuT^{-/-}$ mice (Supplemental Fig. 4D, $p = 0.0034$). Because these results indicate that the presence of circulating Abs is clearly important for the development of injury in this model, the next step was to investigate whether the serum from $MuT^{-/-}$ would be capable of restoring the inflammatory response in GF mice. Remarkably, $MuT^{-/-}$ serum was not capable of reverting the anti-inflammatory phenotype. GF animals that received $MuT^{-/-}$ serum did not exhibit any significant changes in vascular permeability (Fig. 4A, $p = 0.0199$), neutrophil influx (Fig. 4B, $p < 0.001$), or TNF-α release (Fig. 4C, $p < 0.001$) and were also incapable of abrogating or even diminishing the levels of IL-10 upon reperfusion (Fig. 4D, $p < 0.001$). Together, these results indicate that Igs are pivotal for I/R injury, confirming previous findings, as well as that these molecules are the ones necessary to reverse the anti-inflammatory phenotype in GF mice.

To further clarify the role of Igs in the re-establishment of the inflammatory response in GF mice, treatment included purified total Igs from CV mouse serum instead of whole serum. Igs purified from the serum of CV mice were capable of reverting the inflammatory response to I/R injury in GF mice, as indicated by the increase in vascular permeability (Fig. 5A, $p < 0.001$), neutrophil influx (Fig. 5B, $p < 0.001$), and *Tnfa* mRNA (Fig. 5C, $p < 0.001$), as well as the reduced amounts of *Il10* mRNA (Fig. 5D, $p < 0.001$).

Reversal of the anti-inflammatory phenotype in GF mice by serum transfer is associated with the inhibition of ANXA-1 and GILZ transcription and the activation of NF-κB

The results presented above suggest that the Igs present in the serum of CV mice are necessary for the development of the inflammatory response triggered by reperfusion injury. As demonstrated previously, GF mice exhibit elevated amounts of mRNA for ANXA-1 (Fig. 6A, $p < 0.001$) (8) and the ANXA-1–induced protein GILZ in the small intestine after I/R (Fig. 6B, $p < 0.001$). Serum transfer from CV mice or treatment with purified Igs from CV mice serum is capable of reverting the quantities of mRNA for ANXA-1, GILZ, and IL-10 in mice subjected to I/R (Fig. 6A–C, $p < 0.001$). Consequently, we investigated the activation profile of the transcription factor NF-κB in CV and GF animals subjected to intestinal I/R. In CV mice subjected to I/R, there is a significant quantity of p–IκB-α (Fig. 6D), a key regulatory protein of the NF-κB complex, in the cytoplasm. Accordingly, there is a noticeable increase in the translocation of effector subunit p65/RelA of the NF-κB factor complex to the nucleus of cells in the intestine of CV animals subjected to I/R (Fig. 6D). These hallmark changes in regulatory IκB-α and effector p65/RelA are absent in GF mice subjected to I/R (Fig. 6D). Remarkably, serum transfer from CV animals to GF animals reversed the activation profile in these proteins

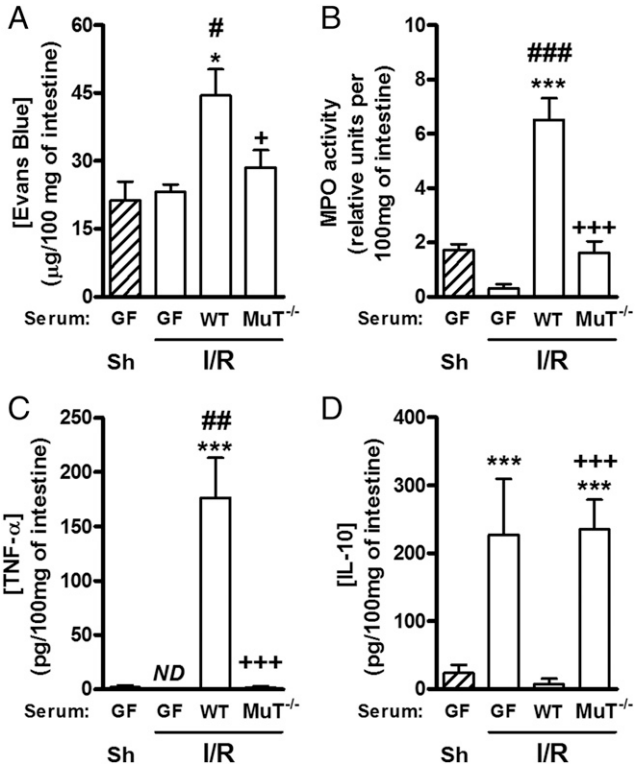

FIGURE 4. Serum from B cell–deficient mice is not capable of reverting the anti-inflammatory phenotype in GF mice. (**A**–**D**) GF mice received 400 μl of GF mouse serum, WT C57BL/6 mouse serum, or $MuT^{-/-}$ serum 24 h before ischemia. After 60 min of ischemia by SMA occlusion and 40 min of reperfusion, animals were euthanized. Jejunum/ileum were harvested to assess plasma leakage (A), MPO activity (B), and production of TNF-α (C) and IL-10 (D) in tissue. Results are the mean ± SEM of six animals in each group. $^*p < 0.05$, $^{***}p < 0.001$ versus the sham-operated control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus GF mice that received GF serum and were submitted to I/R. $^{+}p < 0.05$, $^{+++}p < 0.001$ versus GF mice that received WT serum and were submitted to I/R. Sh, sham-operated animals.

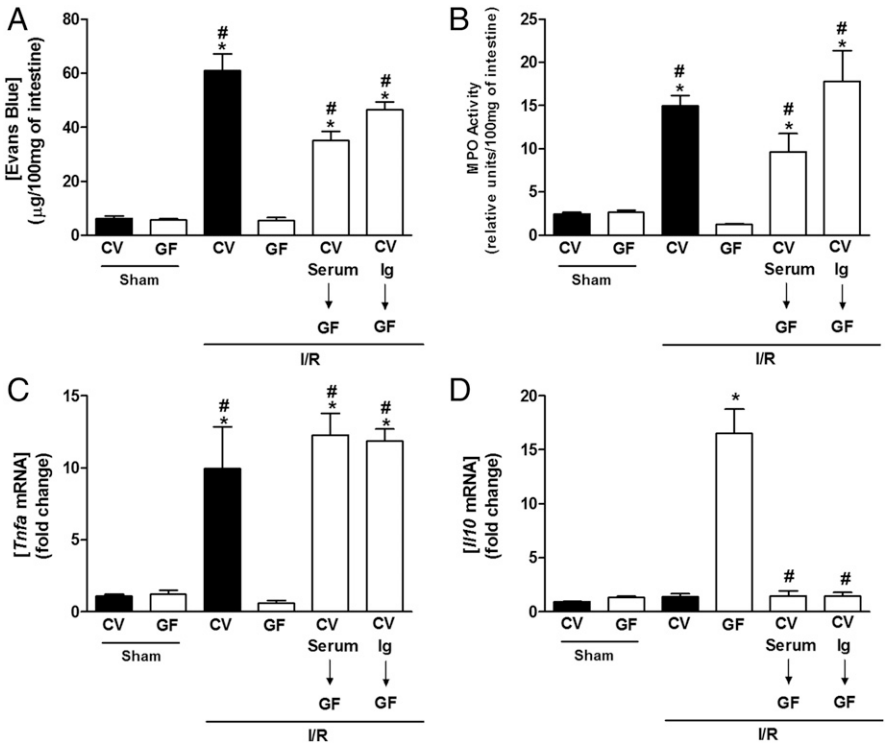

FIGURE 5. Purified Abs from serum of CV mice are also able to restore the inflammatory response in GF mice submitted to intestinal I/R. (**A**–**D**) GF mice received 400 µl of CV mouse serum or 2 mg of purified total Ig (CV Ig) 24 h before ischemia. After 60 min of ischemia by SMA occlusion and 40 min of reperfusion, animals were euthanized, jejunum/ileum were harvested, and plasma leakage (A), MPO activity (B), and transcript amounts (by quantitative RT-PCR) of TNF-α (C) and IL-10 (D) in tissue were assessed. Results are the mean ± SEM of six animals in each group. $^{*}p < 0.05$ versus the sham-operated control group. $^{\#}p < 0.05$ versus GF mice submitted to I/R.

(Fig. 6E). Thus, GF mice receiving serum transfer from CV mice exhibited pronounced p–IκB-α in the cytoplasm and translocation of p65/RelA to the nucleus. With these results, we can conclude that serum transfer from CV mice is capable of restraining expression of anti-inflammatory proteins and re-establishing activation of NF-κB upon reperfusion injury.

Serum transfer reversal of the anti-inflammatory phenotype restores the capacity of GF mice to deal with K. pneumoniae *infection*

Having established that serum transfer is able to reverse the phenotype of GF mice in a model of sterile acute inflammatory injury, we next addressed whether the serum transfer would be capable of restoring the response of GF mice against infections. In

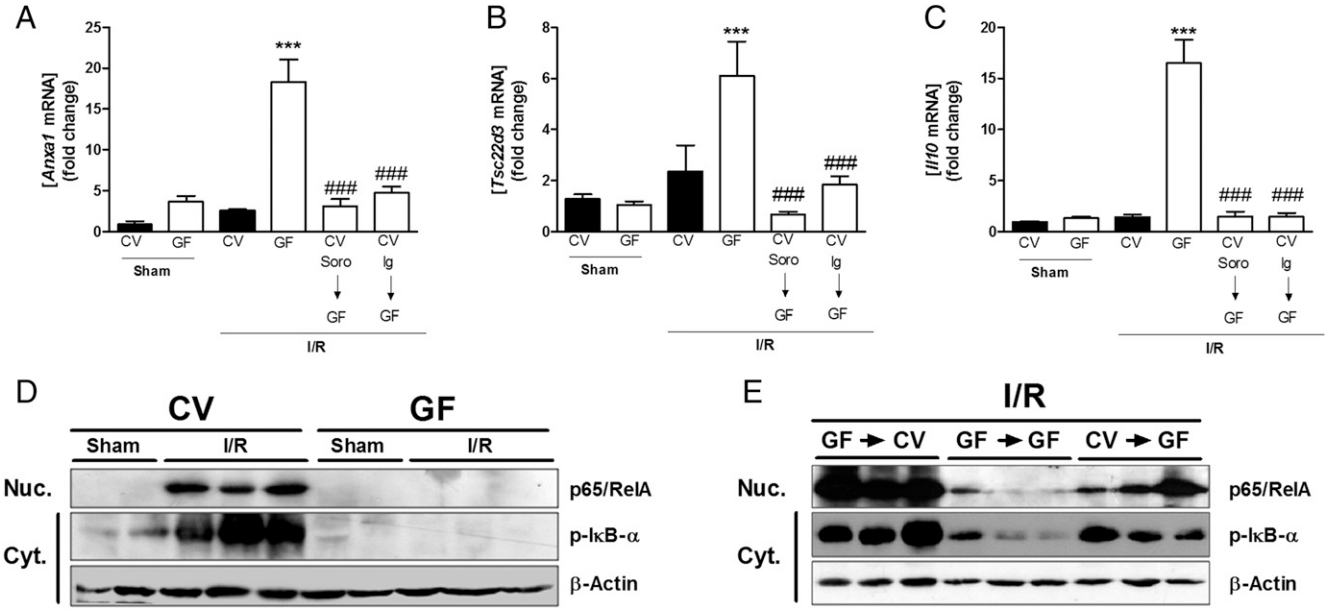

FIGURE 6. Reversal of the anti-inflammatory phenotype in GF mice by Igs of CV mice is associated with inhibition of ANXA-1 and GILZ transcription and the activation of NF-κB. (**A**–**C**) GF mice received 400 µl of CV mouse serum or 2 mg of purified Ig (CV Ig) 24 h before ischemia. After 60 min of ischemia by SMA occlusion and 40 min of reperfusion, animals were euthanized, jejunum/ileum were harvested, and transcript amounts of ANXA-1 (A), GILZ (B), and IL-10 in tissue were evaluated by quantitative RT-PCR. Results are the mean ± SEM of six animals in each group. (**D** and **E**) GF mice received (or not) 400 µl of CV mouse serum or GF mice serum 24 h before ischemia. After 60 min of ischemia by SMA occlusion and 40 min of reperfusion, animals were euthanized, jejunum/ileum were harvested, and Western blot analysis of nuclear and cytoplasmic extracts was conducted for the detection of p65, p–IκB-α and β-actin. (D) Comparison of sham-operated CV and GF animals or after intestinal I/R. (E) The groups that received control (GF to GF) or CV (CV to GF) serum are shown. $^{***}p < 0.001$ versus the respective sham-operated control group. $^{\#\#\#}p < 0.001$ versus GF mice submitted to I/R. Cyt., cytoplasmic extracts; Nuc., nuclear extracts.

this article, we show that the serum transfer was able to render the GF mice resistant to *K. pneumoniae* infection in a similar manner to infected CV mice. GF mice that received CV serum exhibit numbers of CFU in the lungs that are comparable to control CV mice (Fig. 7A, $p = 0.0094$). The numbers of total cells (Fig. 7B, $p = 0.0008$) and polymorphonuclear cells in BAL fluid (Fig. 7C, $p = 0.0022$) are also restored to levels similar to infected CV mice, and the same is observed for MPO activity in the lung tissue (Fig. 7D, $p < 0.0001$). TNF-α production was also re-established to levels close to those of infected CV mice (Fig. 7E, $p < 0.0001$); accordingly, IL-10 levels dropped, closely matching CV levels (Fig. 7F, $p = 0.0078$). Therefore, injection of serum obtained from CV mice into GF mice also restores inflammatory responsiveness during infectious stimuli.

Discussion

In the present study, we demonstrate that a soluble factor present in the serum of conventionally colonized mice is able to restore responsiveness to inflammatory challenges in GF mice; the serum of Ab-deficient mice is unable to reverse inflammatory hyporesponsiveness in GF mice and injection of Abs obtained from serum of CV mice into GF hosts restores inflammatory responsiveness; previous treatment with Abs from CV mice inhibits production of anti-inflammatory mediators in GF hosts, favoring NF-κB activation and expression of proinflammatory mediators upon inflammatory insult; and the reversion of inflammatory responsiveness of GF mice by serum from CV mice confers resistance to *K. pneumoniae* pulmonary infection. Taken together, these data suggest that part of the hyporesponsive phenotype found in GF mice is due to the altered repertoire of Abs found in the noncolonized state and demonstrate that indigenous microbiota-induced natural Abs are necessary for assembly of a "conventional" inflammatory response by the host upon sterile and infectious challenges.

It has been known for decades that, in the absence of indigenous colonization, the Ab repertoire is dramatically changed (10–16). In fact, diminished Ig levels were one of the first documented immune alterations found in GF mice, and we noted a marked reduction in IgG levels in our colony. Ab deposition during ischemic insults and its importance for subsequent inflammatory tissue injury during reperfusion have been extensively documented (18–27). It is well established that natural Abs recognize Ags exposed in ischemic tissues, unleashing the cascade of events that leads to reperfusion injury. Hence, in the model of intestinal reperfusion injury used in this study, animals deficient in circulating Abs ($MuT^{-/-}$ mice) were protected from lethality induced by reperfusion as a result of the reduced production of proinflammatory mediators and diminished leukocyte infiltration into intestinal tissue, confirming the previous observations of other groups. These findings led us to evaluate whether reposition of natural Abs in GF mice would be enough to re-establish inflammation and death upon reperfusion injury. Indeed, in the first strategy used, treatment of mice with serum obtained from specific pathogen–free CV mice led to IgG deposition in tissue and restored inflammatory responses in the reperfused intestine, an effect that was recapitulated by previous treatment with Abs purified from

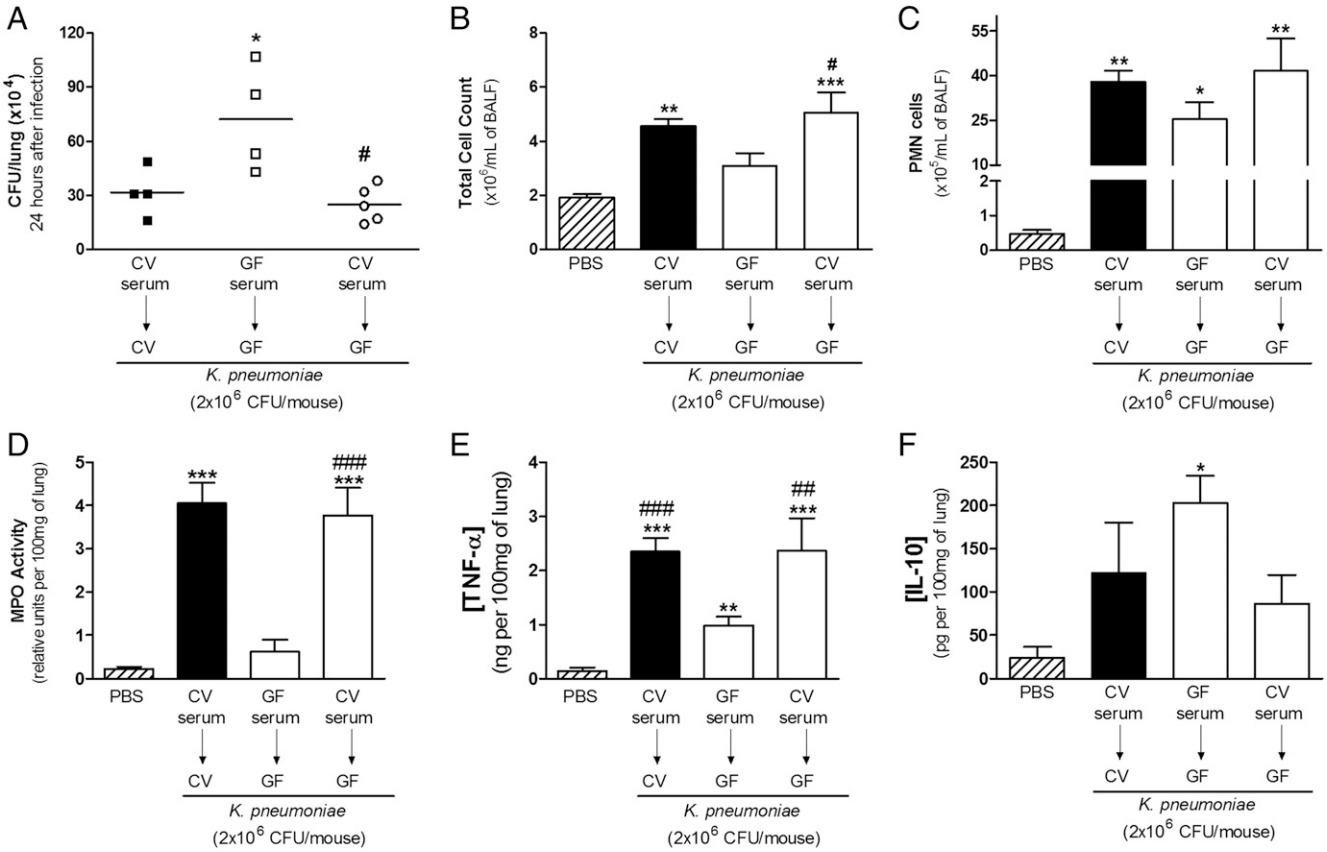

FIGURE 7. Serum transfer from CV mice restores the capacity of GF mice to deal with pulmonary *K. pneumoniae* infection. (**A**–**F**) Mice received 400 μl of CV mouse serum or GF mouse serum 24 h before intratracheal inoculation with 3×10^6 CFU *K. pneumoniae* or vehicle (PBS). Twenty-four hours later, animals were euthanized for evaluation of the number of bacteria in the lungs (A), total cell count (B) and polymorphonuclear cell count (C) in BAL fluid, MPO activity (D), and TNF-α (E) and IL-10 (F) production in the lung parenchyma. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus mock-infected group (PBS). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus GF mice that received GF serum and were submitted to infection.

the serum from CV mice but not from the serum from mice devoid of circulating Abs (the same $MuT^{-/-}$ mice that are resistant to I/R injury). Together, these data demonstrate that natural Abs that are involved in inflammation establishment after reperfusion injury are induced by mutualistic colonization of host intestines by the microbiota.

Because the approaches used in this study involved whole serum or total Ig injection into GF mice, it is not possible to define the nature of the natural Abs and Ags recognized by them during the promotion of inflammatory events associated with reperfusion injury. Natural Abs are usually shown to recognize common bacterial Ags and autoantigens (36–38). In the case of reperfusion injury in the intestines, it has been suggested that nonmuscle myosin H chain type II A and C epitopes are exposed during the ischemic process and then recognized by natural IgMs that trigger complement activation and inflammatory tissue injury (18, 20–22). Also, natural IgGs accumulate during reperfusion of the ischemic intestine and are involved in chemotactic mediator production and neutrophil influx during intestinal reperfusion injury (20), although the nature of the Ags recognized by these natural IgGs remains to be defined. Therefore, in concert with our findings, we might suggest that natural autoreactive Ab production is promoted by indigenous colonization of the intestine, and this is essential for tissue injury upon intestinal I/R. Because mice deficient for complement receptor 2 lack these Abs involved in reperfusion injury progression (20), it is possible that one of the signals involved in the promotion of pathogenic natural Abs by the microbiota involves complement receptor 2 activation. Microbiota-induced systemic IgG Abs were also shown to rely on TLR activation in B cells by Gram-negative bacteria–derived murein lipoprotein (39). However, given the several mechanisms involved in the stimulation of humoral immunity by indigenous microbiota (reviewed in Ref. 40), other mechanisms might be involved.

Reversal of the anti-inflammatory phenotype is not limited to the strong reaction observed in the intestinal I/R model. In a previous study (32), we showed that GF mice are drastically susceptible to *K. pneumoniae* infection in an IL-10–dependent fashion. The present data also show that serum transfer is capable of reversing this phenotype in GF mice when faced with an infectious insult with *K. pneumoniae*. Although GF mice would succumb from infection with an otherwise sublethal inoculum in CV mice, pretreatment with serum from CV mice is capable of rescuing them. These experiments in GF mice suggest that the ability to inflame (conferred by microbiota-induced Abs), although potentially harmful during sterile tissue injury, is evolutionarily relevant for dealing with bacterial and, possibly other, parasitic infections. Therefore, the contact with the microbiota induces a state of inflammatory responsiveness that is necessary for the ability of a host to deal appropriately with environmental inflammatory stimuli, and this is partially dependent on the natural Ab repertoire conferred by colonization.

Although the mechanism by which this reversal occurs in serum-treated GF mice is still unknown, the reversal of the anti-inflammatory state of GF mice by Abs from CV mice is associated with reduction or ablation of IL-10 production. Previous studies demonstrated that blocking IL-10 activity re-establishes inflammation induced by reperfusion injury or the intraplantar injection of carrageenan (6, 7). Blocking IL-10 activity also restores the TNF-α release induced by LPS injection (6) and by *K. pneumoniae* infection (32) in GF mice. Taken together, these studies reveal that modulating the production of IL-10 is central for the reversion of the anti-inflammatory phenotype in GF mice. The expression of IL-10 in GF mice challenged with inflammatory stimuli seems to be controlled by the production of high quantities of ANXA-1 and LXA_4 (8). It is known that ANXA-1 controls the expression of cytokines in vivo through activation of the transcription factor GILZ (41). Furthermore, previous studies showed that GILZ is capable of inducing IL-10 expression (42–44). In our experiments, inflammation in intestines of GF mice that received serum from CV mice during reperfusion injury was associated with reduced ANXA-1 and GILZ expression, suggesting that the hyporesponsive state of GF hosts is lost in the presence of circulating natural Abs. Interestingly, GILZ not only induces the expression of IL-10, it further regulates the inflammatory response by directly interacting with NF-κB and blocking its activation (42, 44). Additionally, in GF mice treated with CV serum, abrogation of GILZ expression was associated with enhanced NF-κB activation in reperfused intestines. NF-κB is a transcription factor that is important for the promotion of inflammation during reperfusion injury (45). Therefore, modulation of host inflammatory responsiveness by microbiota-induced Abs involves inhibition of ANXA-1–induced IL-10 production and the consequent promotion of NF-κB activation and the production of proinflammatory mediators during responses to an inflammatory trigger.

The mechanisms involved in Ab-mediated inhibition of ANXA-1 and IL-10 production in GF mice are still under investigation in our laboratories, but they may involve enhancement of complement activation, as well as FcR activation, during tissue injury. Complement activation is known to be pivotal for reperfusion injury progression and for control of bacterial infection, and it has been shown to downregulate IL-10 expression during brain I/R (46). It is unknown whether downregulation of IL-10 expression by complement activation during reperfusion injury involves modulation of ANXA-1 production. Nevertheless, enhanced complement activation in serum-treated GF animals could favor inflammatory responses in the systems evaluated in this study. FcR activation is also known to promote inflammation in reperfusion injury (C.B. Brito and D.G. Souza, unpublished observations) (47) and in bacterial infection (48), although modulation of IL-10 production and signaling by FcR activation seem to be context dependent, and there is no evidence for whether ANXA-1 is involved in this regulation. ANXA-1 expression is controlled, in part, by the glucocorticoid receptor (GR) (49). GF mice are known to exhibit elevated corticosterone levels (C.T. Fagundes, M.M. Teixeira, and D.G. Souza, unpublished observations) (50), and our unpublished observations show that heightened ANXA-1 expression upon inflammatory challenge is inhibited by pretreatment of GF mice with a GR antagonist. It is possible that IgG deposition during I/R injury in serum-treated GF mice and the consequent complement and/or FcR receptor activation could inhibit GR-mediated ANXA-1 transcription in a manner resembling the phenomenon described as inflammation-induced glucocorticoid resistance by other investigators (51, 52). Therefore, the mechanisms of ANXA-1 and IL-10 downmodulation in serum-treated GF mice remain to be evaluated.

Serum of CVN mice was also capable of restoring inflammatory responsiveness in GF mice. In previous studies, we showed that injection of pattern recognition receptor ligands, such as LPS, rendered GF mice transiently responsive to inflammatory triggers, such as reperfusion injury, LPS-induced endotoxemia, carrageenan-induced hypernociception, and pulmonary infection by *K. pneumoniae* (7, 32). Hence, 48 h after injection of LPS into GF mice, they responded to inflammatory triggers in a way that was similar to CV mice submitted to the same stimuli, but this was not the case for GF animals injected with the TLR4 agonist 96 h earlier. Importantly, serum collected from mice injected with LPS 48 h earlier does not change GF mice resistance to reperfusion injury (D. Cisalpino, C.T. Fagundes, and D.G. Souza, unpublished

observations), suggesting that the promotion of Ab production is not involved in the reversion induced by LPS injection. The fact that stimulation with pattern recognition receptor ligands or natural Abs is able to modulate host responsiveness to inflammatory triggers suggests that components of innate (TLR ligands) and acquired (natural Abs) immunity are able to modulate host inflammatory responsiveness in a redundant manner. It has been shown that innate and acquired immune mechanisms cooperate flexibly to maintain homeostasis of the host–microorganism interaction in such a way that, in the absence of one of these arms, the activity of the other is enhanced to keep host–microorganism mutualism (53). Therefore, based on Slack et al.'s observations (53), as well as our findings discussed above, it is possible to suggest that modulation of host inflammatory responsiveness by the microbiota through promotion of innate and acquired mechanisms is among the mechanisms that favor adequate host–microorganism mutualism.

In conclusion, our data show that natural circulating Abs that are induced upon mutualistic colonization by indigenous microbiota tune host inflammatory responsiveness, favoring proinflammatory mediator production and leukocyte mobilization upon stimulation with an inflammatory trigger. Although these microbiota-induced Abs are involved in the progression of sterile tissue damage, they are pivotal for mounting an acute inflammatory response and controlling an infectious agent. Therefore, promotion of natural humoral immunity through continuous contact with the microbiota is of extreme importance for the host during the response to environmental noxious stimuli.

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Disclosures

The authors have no financial conflicts of interest.

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