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TGF-β1 improves mucosal IgA dysfunction and dysbiosis following intestinal ischaemia–reperfusion in mice

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Abstract

Intestinal ischaemia/reperfusion (I/R) severely disrupts gut barriers and leads to high mortality in the critical care setting. Transforming growth factor (TGF)-β1 plays a pivotal role in intestinal cellular and immune regulation. However, the effects of TGF-β1 on intestinal I/R injury remain unclear. Thus, we aimed to investigate the effects of TGF-β1 on gut barriers after intestinal I/R and the molecular mechanisms. Intestinal I/R model was produced in mice by clamping the superior mesenteric artery for 1 hr followed by reperfusion. Recombinant TGF-β1 was intravenously infused at 15 min. before ischaemia. The results showed that within 2 hrs after reperfusion, intestinal I/R disturbed intestinal immunoglobulin A class switch recombination (IgA CSR), the key process of mucosal IgA synthesis, and resulted in IgA dysfunction, as evidenced by decreased production and bacteria-binding capacity of IgA. Meanwhile, the disruptions of intestinal microflora and mucosal structure were exhibited. Transforming growth factor-β1 activated IgA CSR as evidenced by the increased activation molecules and IgA precursors. Strikingly, TGF-β1 improved intestinal mucosal IgA dysfunction, dysbiosis and epithelial damage at the early stage after reperfusion. In addition, SB-431542, a specific inhibitor of activating mothers against decapentaplegic homologue (SMAD) 2/3, totally blocked the inductive effect of TGF-β1 on IgA CSR and almost abrogated the above protective effects on intestinal barriers. Taken together, our study demonstrates that TGF-β1 protects intestinal mucosal IgA immunity, microbiota and epithelial integrity against I/R injury mainly through TGF-β receptor 1/SMAD 2/3 pathway. Induction of IgA CSR may be involved in the protection conferred by TGF-β1.

Keywords: immunoglobulin A • class switching • mucosal immunity • microbiota • ischemia–reperfusion injury

Introduction

Intestinal ischaemia/reperfusion (I/R) is a grave condition during haemorrhagic or septic shock, severe trauma and burn, abdominal aortic surgery and cardiopulmonary bypass, and it usually results in high morbidity and mortality in the critical setting [1]. A latest multicenter study showed that acute mesenteric ischaemia in 780 patients was associated with a 58% death rate [2]. We previously demonstrated that intestinal mucosal structure was severely damaged following intestinal I/R [3, 4]. In addition, several studies showed that intestinal I/R also disrupted mucosal immunity and microflora [5, 6]. The impaired intestinal mechanical, immune and biological barriers lead to bacterial translocation, and subsequently gut-origin sepsis and multiple organ dysfunction syndrome [7, 8]. To date, however, few studies have investigated the mechanism and therapy for the immune disorder and dysbiosis caused by intestinal I/R injury.

Gut mucosal immunoglobulin (Ig) A is critical for not only defending the host against pathogens but also regulating the host-commensal relationship [9, 10]. However, we recently demonstrated that intestinal I/R resulted in impaired class switch recombination (CSR) of IgM B^+ cells, a key biological process involved in mucosal IgA synthesis, in peyer's patches (PPs) and decreased secretory IgA (sIgA) concentration in the gut lumen at 2 hrs after reperfusion [11]. Transforming growth factor (TGF)-β1, a polypeptide member of the TGF-β superfamily, plays a pivotal role in cellular proliferation, differentiation and apoptosis as well as mucosal inflammation and immunity [12, 13]. Importantly, TGF-β1 can potently and independently activate IgA CSR and promote IgA-secreting plasma cells [14]. Therefore, we hypothesized that TGF-β1 administration may increase IgA synthesis by inducing IgA CSR and subsequently improve gut mucosal dysbiosis and histological injury following intestinal I/R.

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Taken together, this study was designed to investigate the effects of TGF-β1 on intestinal mucosal immunity, microflora and histology after intestinal I/R and to explore the potential signalling and mechanisms.

Materials and methods

Animals and operative procedure

The current experiment was approved by the Animal Care Committee of Sun Yat-sen University and was performed in accordance with National Institutes of Health guidelines for the experimental animals. Eight- to ten-week-old SPF male Balb/c mice (28.6–32.3 g) were housed in individual cages and acclimated for 1 week before protocol entry.

Mice were anaesthetized with intraperitoneally pentobarbital (70 mg/kg at induction and 30 mg/kg at 1 hr later) and ketamine (50 mg/kg at induction). The small intestine was exteriorized by midline laparotomy, and the intestinal I/R model was established by occluding the superior mesenteric artery (SMA) with a microvessel clip for 1 hr, as we described previously [3, 4]. After 1 hr of occlusion, the clip was removed and abdominal incision was sutured. During the study period, all procedures were performed on spontaneously breathing animals, and body temperature was maintained at about 36°C with the aid of a heating pad.

Groups and drug administration

The current experimental protocol was determined according to previous literatures [15, 16] and our preliminary results. The mice were randomly allocated into five groups. Sham group (sham): the animals received adjuvants and underwent laparotomy without occlusion of the SMA. I/R group (injury): the animals received adjuvants, and the SMA was clamped for 1 hr. Transforming growth factor-β1 group (TGF): 1 μg (100 μl) recombinant human TGF-β1 (PeproTech Inc, Rocky Hill, NJ, USA) was infused through caudal vein at 15 min. before ischaemia. Transforming growth factor-β1 inhibitor group (SB): 0.5 mg SB-431542 was dissolved in 100 μl 10% dimethyl sulfoxide and injected intraperitoneally at 30 min. before ischaemia. Transforming growth factor-β1 + SB-431542 group (TGF+SB): isometric TGF-β1 and SB-431542 was administrated at corresponding time-point, respectively. The detailed experimental protocol was shown in Figure 1.

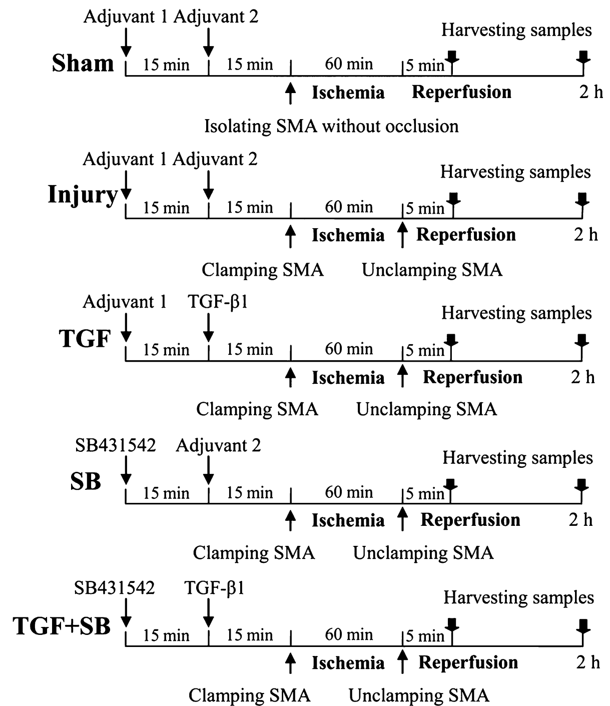

Fig. 1 The experimental protocol of groups and drugs. Sham group: adjuvants were given and SMA was isolated without occlusion; injury group: adjuvants were given and SMA was occluded for 60 min.; TGF group: 1 μg TGF-β1 was dissolved in adjuvant 2 and then injected at 15 min. before ischaemia; SB group: 0.5 mg SB-431542 was dissolved in adjuvant 1 and then administrated at 30 min. before ischaemia; TGF+SB group: the same dose of TGF-β1 and SB-431542 was administrated at corresponding time-point, respectively. Adjuvant 1 = 10% DMSO 100 μl; adjuvant 2 = 100 μl mixture (10 mM citric acid 10 μl and PBS containing 5% trehalose 90 μl); SMA: superior mesenteric artery; DMSO: dimethyl sulfoxide; TGF: transforming growth factor; SB: SB-431542, specific inhibitor of SMAD 2/3 transcription.

Biological samples collection

After euthanizing the mice, a ~0.5-cm segment of intestine was cut from 10 cm to terminal ileum for immunohistochemical analysis. PP samples were carefully excised for isolating lymphocytes [5]. Then, the intestine was opened longitudinally and the faecal contents were collected for detecting the bacteria-binding capacity of IgA. A ~10-cm intestinal fragment next to ileocecal valve was flushed with 3-ml chilled PBS, and the washings were collected for detecting sIgA concentration. Then, this fragment was cut into small pieces for isolating lymphocytes from lamina propria (LP) [5]. Another segment of small intestine (~10 cm) was obtained from 12 cm to terminal ileum, and the intestinal mucosa was scraped off gently and preserved at liquid nitrogen for detecting IgA mRNA expression [17]. Finally, caecal faeces were collected for analysing gut microbiota. For each parameter, 5–6 samples per group were collected and analysed.

Immunohistochemical analysis

The small intestine segment was fixed in paraformaldehyde and embedded in paraffin. The fixed tissues were sectioned and stained with haematoxylin–eosin. Two independent pathologists assessed the histological damage by using Chiu's score [4].

Moreover, the sections were subjected to deparaffinization and rehydration and then stained with FITC-conjugated antimouse IgA diluted 1/100 for 1 hr. Finally, nuclear staining was accomplished with 4′, 6-diamidino-2-phenylindole. The stained slides were examined with a fluorescence microscope (Nikon, Tokyo, Japan).

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Cells isolation from PP and LP

Cells suspension of PP and LP was prepared according to the modified method descried previously [5]. Briefly, PPs were pressed through a steel mesh grid. The PP fragments were incubated with collagenase VIII (40 U/ml) in RPMI 1640. The cell suspensions were passed through 100-μm nylon filters.

The small pieces of intestine were incubated in PBS containing ethylenediaminetetraacetic acid-Na_2, dithiothreitol and foetal bovine serum (FBS). Supernatants containing epithelial and intraepithelial cells were removed. The remaining tissues were incubated with RPMI 1640 containing collagenase VIII (100 U/ml), 5% FBS and a 1% antibiotic mixture. Supernatants were filtered through nylon filters and centrifuged. The cell pellets were resuspended in 40% Percoll and then overlaid with 80% Percoll. After centrifugation, lymphocytes from LP were aspirated from the interface of 40/80% Percoll and diluted with RPMI 1640. Viable cells were counted by using trypan blue dye exclusion.

Quantitative real-time PCR and flow cytometry

By using quantitative real-time PCR (qRT-PCR) and flow cytometric analysis, mRNA expression of biomarkers and percentage of IgA^+/IgM^+B220^+ cells were applied to investigate IgA class switching.

Total RNA from the cells and tissues was isolated by using TRIzol reagent according to the manufacturer's protocol. The qRT-PCR was performed on a C1000 Touch Thermal cycler with SYBR Green. Transforming growth factor-β1 and activation-induced cytidine deaminase are essential to IgA CSR, and germline α transcripts and Iμ-Cα circle transcripts are the representative activation biomarkers for IgA switching [17]. The primers for these four biomarkers and IgA were determined based on the previous data [18–21] (Table 1). The relative amounts of mRNA transcripts were calculated by using the standard curve method and normalized by control GAPDH.

Flow cytometric analysis was performed as previously described [5]. Briefly, the cell pellets obtained from PP and LP were resuspended. The lymphocytes were stained with 1 μg/ml antimouse IgA, antimouse IgM and antimouse B220 to identify IgA^+ and IgM^+B220^+ cells, respectively. The cells were detected with a FACSCalibur flow cytometer. The data were analysed by Flowjo software.

Table 1 Primer sets used for quantitative real-time PCR

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
TGF-β1	ctgtagcccacgtcgtagc	ttgagatccatgccgttg
AID	cgtggtgaagaggagagatagtg	cagtctgagatgtagcgtaggaa
GLTα	caagaaggagaaggtgattcag	gagctggtgggagtgtcagtg
αCTs	ccaggcatggttgagatagagatag	aatggtgctgggcaggaagt
IgA (α-chain)	cgtccaagaattggatgtga	agtgacaggctgggatgg
GADPH	tgtgtccgtcgtggatctga	cctgcttcaccaccttcttgat

Detection of sIgA in intestinal lavage

Secretory IgA concentration was detected by using the ELISA kit (Cusabio, Wuhan, China), as described previously [18]. Briefly, the intestinal washing was centrifuged at 1250 g for 20 min., and the supernatant was harvested to measure the sIgA concentration.

Gut bacterial community analysis

Gut microbiota was analysed by a 16S rRNA gene sequencing on the Illumina MiSeq platform according to the protocol of Miseq system. Briefly, bacteria genomic DNA was extracted from 0.5 g caecal faeces by using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The primers of V4 hypervariable regions were used to amplify templates from genomic DNA. Data analysis was performed by Mothur software (Version 1.28, http://www.mothur.org/, USA).

Evaluation of intestinal bacteria coated with IgA

Flow cytometric analysis of IgA-binding bacteria was performed as previously described [19]. Briefly, the faeces were suspended in PBS and centrifuged. Then, supernatant was centrifuged at 5650 g to remove non-bound Igs. The pellets were resuspended in 1 ml of FBS/PBS (1% w/v). Bacteria were stained with antimouse IgA. Finally, bacteria pellets were resuspended in 4 μg/ml propidium iodide (PI)/PBS and analysed by FACScalibur. All events that stained with PI were regarded as bacteria. The percentage of bacteria coated with IgA was calculated as: the number of PI^+ IgA^+ cells/the number of total PI^+ cells×100.

Survival analysis

The survival analysis was performed in independent mice as we previously described [4]. Briefly, the mice were managed with the same interventions and then transferred to their cages. Each mouse was monitored *via* video recording for 24 hrs.

Statistical analysis

The data were analysed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). Survival time after reperfusion was expressed as median (95% confidence intervals), and results were compared by Kaplan–Meier log-rank test. The other data were expressed as mean ± S.E., and one-way ANOVA (Tukey post-test) was used for comparisons. $P < 0.05$ in two-tailed testing was considered statistically significant.

Results

The effects of TGF-β1 on IgA CSR following intestinal I/R

At 5 min. after reperfusion, the mRNA expression of all molecular biomarkers in PP and LP was lower in the injury group than that in

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the sham group (all $P < 0.01$). At 2 hrs after reperfusion, in the injury group, the expression of all molecules in PP was elevated but the expression in LP was lower (all $P < 0.01$ *versus* sham; Fig. 2A and B). Transforming growth factor-β1 increased the expression of all molecules in LP. Whereas, in PP, TGF-β1 increased the molecular expression at 5 min. after reperfusion (all $P < 0.01$), but decreased the expression at 2 hrs (all $P < 0.01$; Fig. 2A and B).

In the injury group, the percentage of IgA^+B220^+ cells decreased and IgM^+B220^+ cells increased in PP and LP at 2 hrs after reperfusion (all $P < 0.01$ *versus* sham; Fig. 2C). Transforming growth factor-β1 restored the balance of IgA^+ and IgM^+ B cells in both PP and LP (Fig. 2C).

The effects of TGF-β1 on gut mucosal IgA production and bacteria-binding capacity following intestinal I/R

IgA mRNA expression in the intestinal mucosa and sIgA concentration in the lavage were lower in the injury group than that in the sham group at 2 hrs after reperfusion (both $P < 0.001$; Fig. 3A and B). Meanwhile, the percentage of bacteria coated with IgA in the faeces was lower in the injury group than that in the sham group (Fig. 3C). Transforming growth factor-β1 increased IgA production and bacteria-coating percentage (all $P < 0.01$ *versus* injury; Fig. 3).

The effects of TGF-β1 on gut microbiota, histological injury and mortality following intestinal I/R

The species diversity of colonic bacteria was lower in the injury group than that in the sham group ($P < 0.001$; Fig. 4A). In the injury group, *Enterobacteriaceae* increased about 230-fold, but the obligate anaerobes, *Ruminococcaceae* and *Lachnospiraceae*, and the healthy bacteria, *Lactobacillus* and *Bifidobacterium*, significantly decreased (all $P < 0.01$ *versus* sham; Fig. 4C). *Faecalibacterium* did not significantly change, but the *Faecalibacterium prausnitzii/Escherichia coli* ratio, an indicator of intestinal dysbiosis [22], was lower in the injury group (Fig. 4B). Transforming growth factor-β1 significantly alleviated the alterations of microbiota (all $P < 0.05$; Fig. 4).

Chiu's score was higher in the injury group ($P < 0.001$ *versus* sham). Significant decrease of Chiu's score was detected in the TGF group when compared with the injury group (0.43 ± 0.13 *versus* 4.03 ± 0.39, $P < 0.001$; Fig. 5).

The survival time and mortality rate of the mice in the injury group was 3 hrs (0–6.4 hrs) and 83.3%, respectively (Fig. 6). Transforming growth factor-β1 dramatically reduced mortality rate to 20% ($P = 0.004$ *versus* injury).

The effects of SB-431542 on TGF-β1-induced intestinal I/R protection

SB-431542 alone (SB group) produced no active effects on the above-mentioned variables in comparison with the injury group (all $P > 0.05$; Figs. 2–6). On the other hand, SB-431542 eliminated the effects of recombinant TGF-β1 on IgA switching biomarkers and IgA^+ precursor cells (Fig. 2). Moreover, the protective effects of TGF-β1 on the mucosal IgA, microbiota and survival were totally diminished (Figs 3, 4 and 6). Whereas the Chiu's score in the TGF+SB group (2.57 ± 0.23) was still statistically lower than that in the injury group ($P = 0.002$; Fig. 5).

Discussion

This study shows that TGF-β1 provides striking protection for intestinal barriers following intestinal I/R. During perioperative period, manipulation of TGF-β1 expression through several medical interventions can help the clinicians to prevent and treat intestinal I/R-related diseases.

Mucosal IgA is essential to maintain homeostasis in the gut lumen. By specifically and non-specifically binding bacteria, intestinal IgA regulates gut microbiota and maintains mucosal integrity [10, 23]. Whereas the present data showed that intestinal I/R reduced not only gut IgA production but also its bacteria-binding capacity at 2 hrs after reperfusion (Fig. 3). To date, the mechanisms by which intestinal I/R disrupts gut IgA immunity remain unclear. In the organized mucosa-associated lymphoid tissues, the preferential presence of IgM^+B220^+ and IgA^+B220^+ cells belongs to pre- and post-IgA isotype class-switched B cells [20, 24]. Therefore, in this study, IgM^+ and IgA^+B220^+ cells as well as four biomarkers were used to explore the change of IgA class switching during intestinal I/R. In the inductive sites (PP) and effector sites (LP) of gut immunity, decreased expression of biomarkers at 5 min. and imbalance of IgA^+/IgM^+ B cells at 2 hrs after reperfusion indicate that IgA CSR is inhibited at early reperfusion phase (Fig. 2). Subsequently, impaired IgA CSR results in IgA dysfunction (Fig. 3). In addition, the destruction of gut mucosal tissues (Fig. 5) also disturbs IgA synthesis, transport and secretion. Therefore, the disrupted gut IgA immunity caused by I/R injury deteriorates intestinal dysbiosis and epithelial damage.

The gut microflora was regarded as 'a forgotten organ' [25]. Actually, pathogens and virulence factors from gut flora are thought to be integral to the development of multiple organ dysfunction syndrome [26, 27]. Moreover, enteric dysbiosis increases septic complications and mortality in surgical and critically ill patients [28, 29]. Recently, Hayakawa *et al.* reported that the gut flora in critically ill patients altered immediately after a severe insult [30]. Through denaturing gradient gel electrophoresis, Wang *et al.* demonstrated that, after 30-min. occlusion of SMA in rats, colonic *E. coli* dramatically increased within 1 hr and microflora differed significantly at 6 hrs after reperfusion [6]. Whereas, in this study, the sequencing data showed that, at 2 hrs after reperfusion, both the obligate anaerobes and healthy bacteria markedly reduced in colonic faeces. Meanwhile, decreased species diversity and *F. prausnitzii/E. coli* ratio were detected (Fig. 4). We suggest that long-duration ischaemia, dissimilar animals and analytical techniques may be involved in the discrepancies. Breakdown of gut microflora after critical insults increases the risk of pathogens colonization, bacterial translocation, and uncontrolled inflammation

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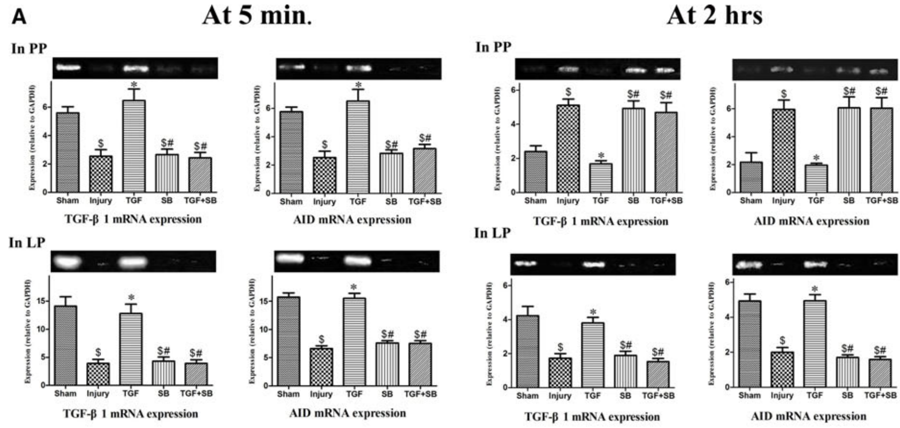

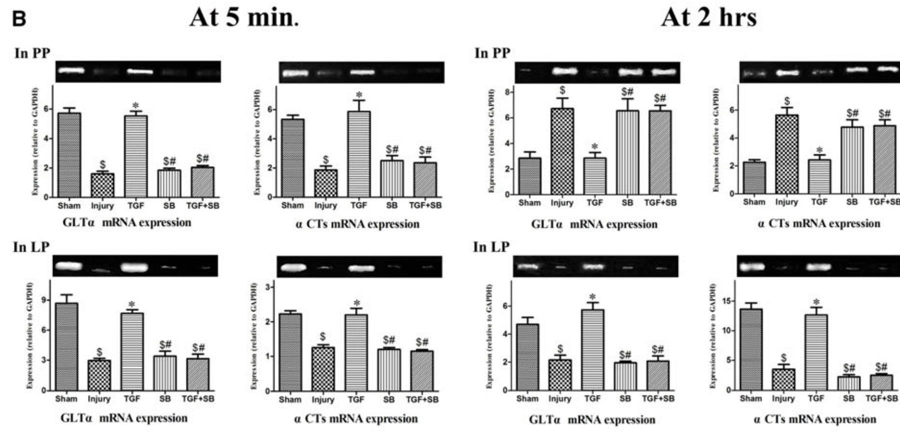

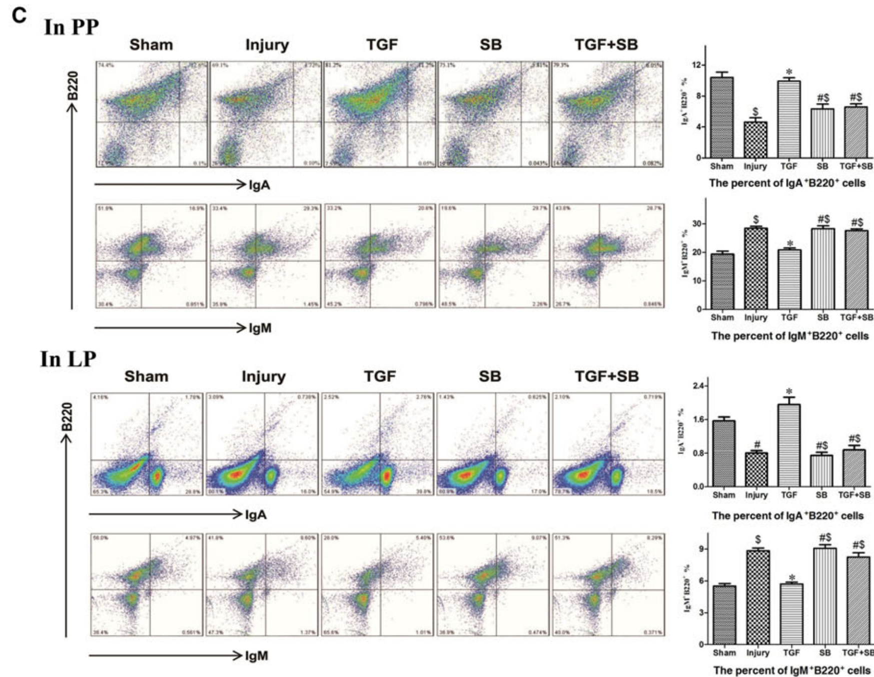

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Fig. 2 Changes of IgA CSR in PP and LP following intestinal I/R. (**A**) Changes of TGF-β1 and AID in PP and LP at 5 min. and 2 hrs after reperfusion. Analysis of relative mRNA expression of these two biomarkers was shown, respectively. And representative blots were presented on the top. (**B**) Changes of GLTα and αCTs in PP and LP at 5 min. and 2 hrs after reperfusion. (**C**) Changes of IgA^+B220^+ and IgM^+B220^+ cells from PP and LP at 2 hrs after reperfusion. Representative flow cytometric profiles were presented in the left panel. Analysis of the percentage of IgA^+ and IgM^+B220^+ cells was shown in the right. Data are expressed as mean ± S.E., $n = 5$ or 6. Results were compared by ANOVA with Tukey post-test. $^{\$}P < 0.01$ *versus* the sham group; $^{*}P < 0.01$ *versus* the injury group; $^{\#}P < 0.01$ *versus* the TGF group. Ig: immunoglobulin; TGF-β1: transforming growth factor-β1; AID: activation-induced cytidine deaminase; CSR: class switch recombination; GLTα: germline α transcripts; αCTs: Iμ-Cα circle transcripts; PP: peyer's patches; LP: lamina propria.

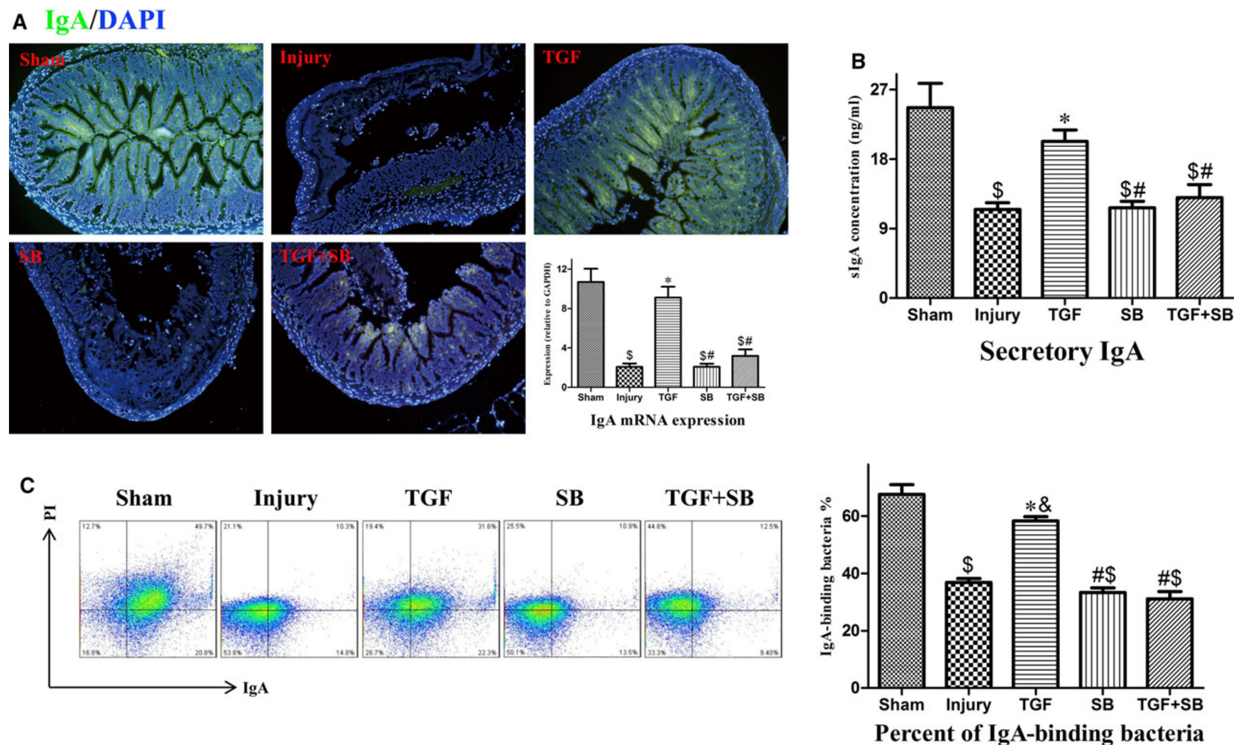

Fig. 3 Changes of mucosal IgA production and bacteria-binding capacity following intestinal I/R. (**A**) Changes of intestinal IgA expression at 2 hrs after reperfusion. Immunofluorescence of mucosal tissues in each group was shown, respectively. The intestinal sections were stained with anti-IgA (green) and DAPI (blue) counterstained nuclei. Analysis of relative mRNA expression of IgA in the intestinal mucosa was also presented. (**B**) Changes of IgA secretion at 2 hrs after reperfusion. Analysis of secretory IgA concentration in the lavage was shown. (**C**) Changes of IgA-binding bacteria in the gut lumen at 2 hrs after reperfusion. Representative flow cytometric profiles of IgA^+PI^+ cells were presented in the left panel. Analysis of the percentage of IgA-binding bacteria in the faeces was presented in the right. Data are expressed as mean ± S.E., $n = 5$ or 6. Results were compared by ANOVA with Tukey post-test. $^{\&}P < 0.05$ *versus* the sham group; $^{\$}P < 0.01$ *versus* the sham group; $^{*}P < 0.01$ *versus* the injury group; $^{\#}P < 0.01$ *versus* the TGF group. IgA: immunoglobulin A; DAPI: 4′,6-diamidino-2-phenylindole; PI: propidium iodide.

and infection [31]. Overall, severe disruption of intestinal homeostasis, as evidenced by IgA dysfunction, dysbiosis and injured epithelia, at the early reperfusion phase may lead to the high mortality (Fig. 6). Indeed, survival analysis in previous literatures also showed that most animals underwent intestinal I/R died within several hours after reperfusion [4, 32].

It has been demonstrated that isoflurane, a volatile anaesthetic, decreased epithelial injury after intestinal I/R by upregulating TGF-β1 expression [33]. However, the mechanisms by which TGF-β1 alleviates intestinal damages under intestinal I/R have not been investigated. Several authors previously demonstrated that induction of IgA CSR by oral clarithromycin could protect against influenza A virus infection in animals and human beings through increasing the production and virus-neutralizing activities of airway sIgA [34, 35], suggesting that activation of IgA CSR is an effective way to reduce mucosal injury. In general terms, development of IgA generation is a long-term process [36]. However, Yoshiya *et al.* reported that intestinal IgA expression significantly increased at 4 hrs after reperfusion [37]. Furthermore, our results also showed that all activation biomarkers for IgA CSR elevated in the inductive sites at 2 hrs after

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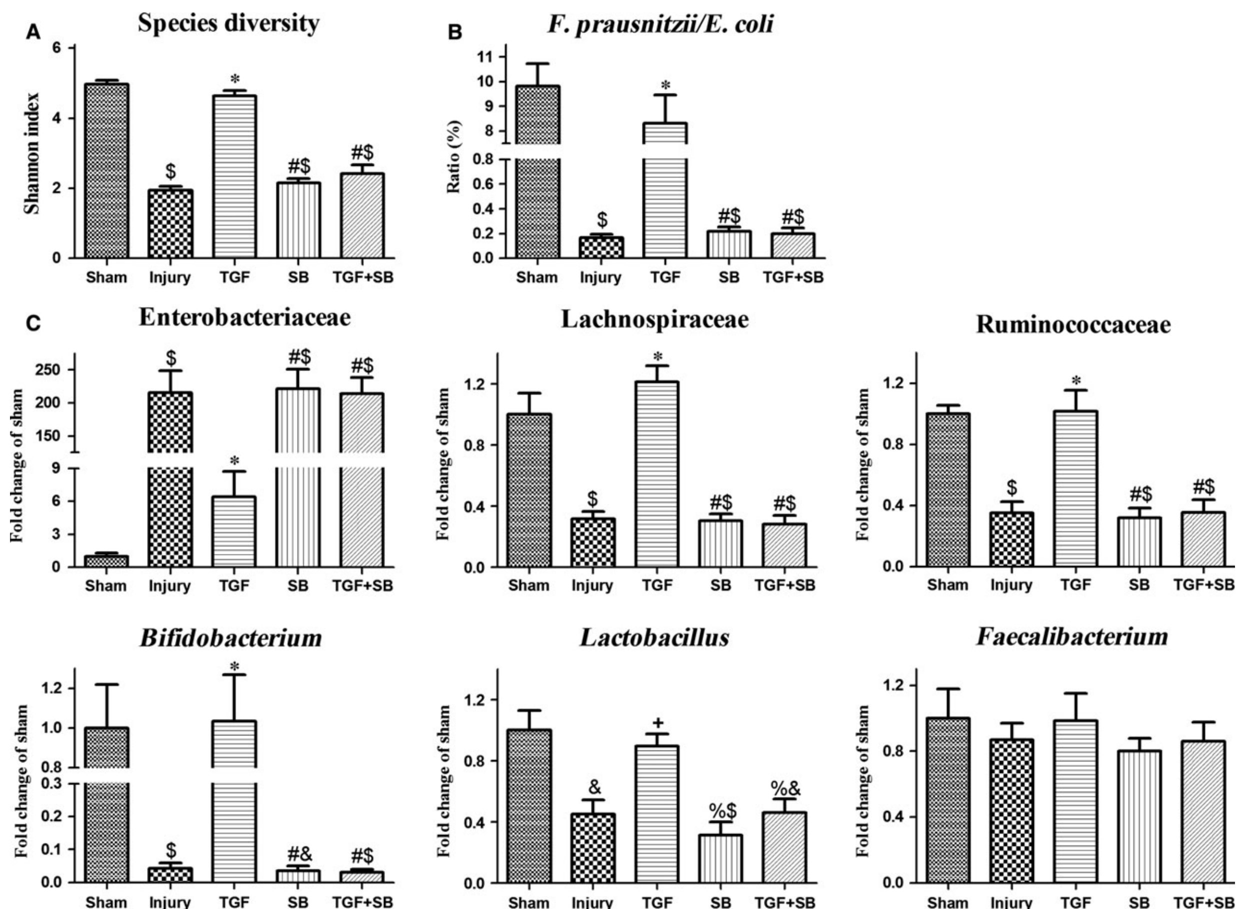

Fig. 4 Changes of gut microbiota following intestinal I/R. Changes of colonic bacterial diversity and compositions at 2 hrs after reperfusion were shown, respectively. (**A**) Species diversity. The Shannon index in each group was analysed. (**B**) *Faecalibacterium prausnitzii*/*Escherichia coli* ratio. This usual indicator for gut dysbiosis in each group was analysed. (**C**) Bacterial compositions. The number of bacteria in specific bacterial taxa in each group was analysed. Changes of bacterial quantity were represented by fold change relative to sham mice. Data were expressed as mean ± S.E., $n = 3$ or 4. Results were compared by ANOVA with Tukey post-test. $^{\$}P < 0.01$ *versus* the sham group; $*P < 0.01$ *versus* the injury group; $^{\#}P < 0.01$ *versus* the TGF group; $^{\&}P < 0.05$ *versus* the sham group; $^{+}P < 0.05$ *versus* the injury group; $^{\%}P < 0.05$ *versus* the TGF group.

reperfusion (Fig. 2A and B), indicating that, under conditions of intestinal I/R, IgA response initiates within several hours following reperfusion. Based on the results of biomarkers at 5 min. after reperfusion (Fig. 2A and B), we suggest that recombinant TGF-β1 accelerates the activation of IgA CSR. Thus, at 2 hrs after I/R, although the biomarkers decreased in PP, the use of TGF-β1 restores the balance of IgA^+/IgM^+ B cells in both inductive and effector sites (Fig. 2C) and subsequently diminished IgA dysfunction (Fig. 3). In different gastrointestinal diseases, robust IgA coating can reduce mucosal invasion through accurately identifying and eliminating pathogenic bacteria [38, 39]. Also, Suzuki *et al.* reported that reconstitution of normal IgA level by anastomosis restored the normal composition of gut microbiota in IgA-deficient mouse [40]. Therefore, we suggest that the restoration of mucosal IgA induced by TGF-β1 *via* inducing IgA CSR may attenuate gut dysbiosis and epithelial injury and ultimately reduce mice' mortality. To the best of our knowledge, this study, for the first time, explores the therapeutic approach for intestinal I/R-induced mucosal damages by enhancing cellular switching and immune molecular activities. Our findings suggest that, in the clinical setting, inhalational anaesthesia may be appropriate for the patients with potential intestinal I/R. In contrast, morphine should be prohibited in these critically ill patients due to its inhibitory effect on gut TGF-β expression [41].

Transforming growth factor-β1 regulates the cellular and immune functions through differential signalling pathways [42, 43]. In this study, SB-431542, a specific inhibitor of TGF beta receptor 1/activating mothers against decapentaplegic homologue (SMAD) 2 and 3 [44], was added to explore the protective signalling of TGF-β1. Because TGF-β1 induces IgA CSR *via* SMAD 2/3 [45], SB-431542 totally eliminated the activation of IgA CSR and the improvement of IgA responses induced by TGF-β1 (Figs 2 and 3). Interestingly, SB-431542 just partly diminished the protective effect of TGF-β1 on

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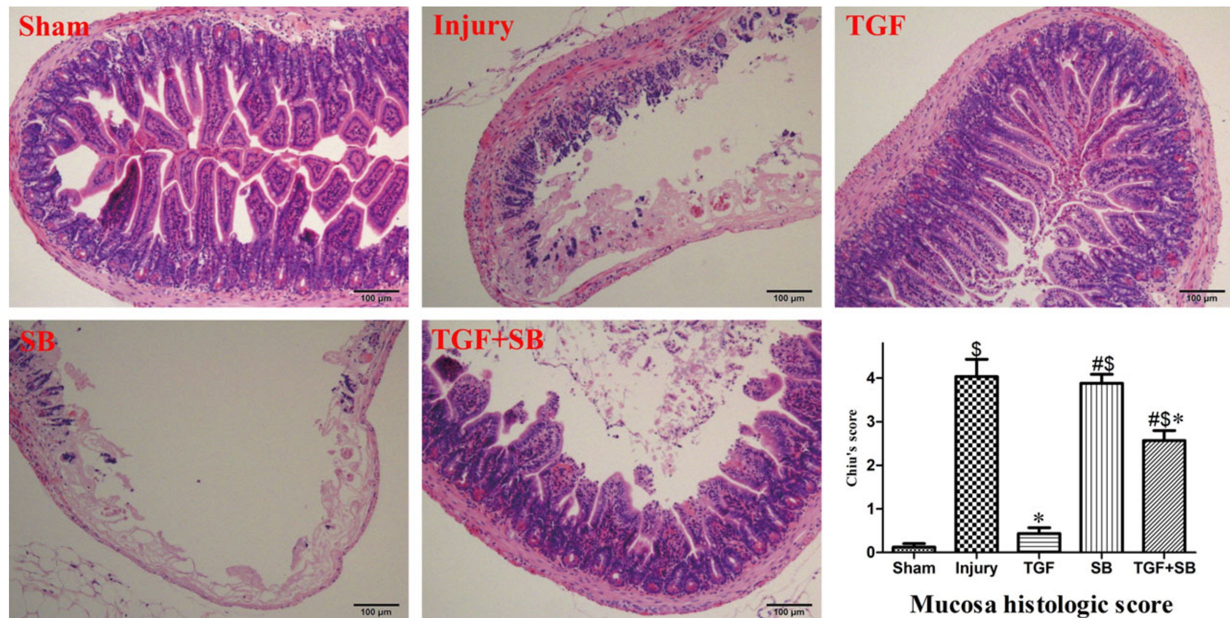

Fig. 5 Changes of intestinal mucosal histology. The intestinal sections (×100) were stained with haematoxylin–eosin. In the sham group, the intestinal villi and glands were normal. Severe intestinal histological damage was observed in the injury and SB groups: normal structure of mucosa disappeared, and severe haemorrhage was present. In the TGF group, the mucosal structures were almost normal, and only very slight mucosal sloughing was seen at villi tips. In the TGF+SB group, disintegrated intestinal villi and increased subepithelial gap were observed. Whereas haemorrhage was rarely detected and the mucosal injury was much better than that seen in the injury group. Analysis of Chiu's score in each group was also presented. Data are expressed as mean ± S.E., n = 5 or 6. Results were compared by ANOVA with Tukey post-test. $^{\$}P < 0.01$ *versus* the sham group; $*P < 0.01$ *versus* the injury group; $^{\#}P < 0.01$ *versus* the TGF group.

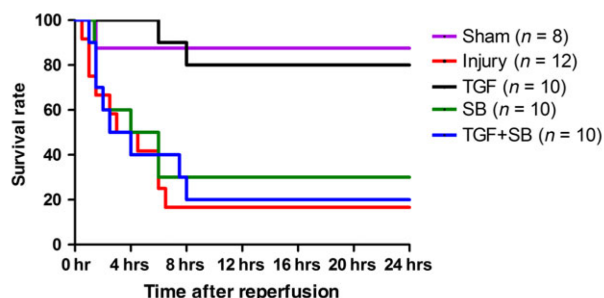

Fig. 6 Survival analysis. The independent animals underwent various interventions based on the experimental protocol. Survival time is calculated from the beginning of reperfusion, n = 8–12. Results were compared by Kaplan–Meier log-rank test.

intestinal epithelia (Fig. 5), indicating that TGF-β1 protected epithelial tissues through SMAD-dependent and SMAD-independent pathways and TGF-β1 improved IgA dysfunction mainly by inducing IgA CSR, but not by preserving mucosal tissues. Therefore, we suggest that, during early reperfusion period, activation of IgA CSR conferred by TGF-β1 is involved in the improvement of gut mucosal immunity and other damages.

There were several limitations in our experimental design. First, because there is no feasible technique, either *in vivo* or *in vitro*, to exclusively block the process of IgA CSR, the role of IgA CSR in TGF-β1-induced intestinal I/R protection was not definitely clarified in this study. Nevertheless, induction of IgA CSR has been proven to be beneficial for mucosal invasion [34, 35]. Second, TGF-β1 has numerous physiological effects. In this study, we focused on investigating its effect of IgA switching in intestinal I/R protection. The exact impacts and mechanisms of TGF-β1 for intestinal I/R injury need to be further clarified. Third, TGF-β1 was administrated before ischaemia in this model. However, in the clinical setting, the vast majority of intestinal I/R is diagnosed after or concurrent to the insult. The effects of TGF-β1 post-treatment on intestinal barriers need to be explored. Finally, severe intestinal damages commonly take place during the early reperfusion stage. Thus, we just detected the variables within 2 hrs after reperfusion. Prolonged observation is needed to evaluate the long-term effect of TGF-β1 on intestinal I/R injury.

In summary, intestinal I/R leads to decreased production and bacteria-binding capacity of gut mucosal IgA, as well as mucosal dysbiosis and histological injury during the early reperfusion phase. Recombinant TGF-β1 pretreatment comprehensively improves the above damages mainly through TGF-β Receptor 1/SMAD 2/3 signalling pathway. The changes of IgA CSR may be involved in the pathogenesis and treatment for intestinal I/R injury. The present study

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provides new insights into the relation of the gut IgA immunity and mucosal barriers under intestinal I/R insult and exhibits a novel avenue for treating the relevant diseases.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Materials and methods.

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