Mechanisms of renal injury and repair: role of stem cells, chemokines and the nodosome
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Depletion of microbiota protects the kidney against ischemia/reperfusion injury by reducing granulocyte influx

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In preparation
Role microbiota in renal I/R injury

Abstract

Evidence is accumulating that gut microbiota plays a role in modulating systemic immunity, both innate and adaptive. Recently it was proposed that peptidoglycan, which is turned over from the microbiota and translocated into the circulation, can prime granulocytes. Peptidoglycan signals via its receptors NOD1 and NOD2, which are both members of the nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family. Priming of granulocytes is likely favourable during a bacterial infection as the inflammatory response might be faster. However, during sterile inflammation such as renal ischemia/reperfusion (I/R) injury granulocytes that enter the damaged tissue may exacerbate injury by collateral damage due to the release of mediators and enzymes that potentially damage the surrounding tissue. We hypothesized that the microbiota plays a detrimental role during renal I/R injury.

Depletion of microbiota by broad-spectrum antibiotic-treatment significantly attenuated renal damage and preserved kidney function in WT mice. Tubular epithelial cell (TEC) survival was enhanced in antibiotic-treated mice upon renal I/R as less apoptotic TEC were observed while no difference was seen in proliferation. Although renal KC levels were not significantly lower, granulocyte influx was significantly attenuated in antibiotic-treated WT mice. No evidence was found for bacterial translocation during ischemia and intestinal permeability was not affected by clamping renal pedicles, suggesting a role of the microbiota in priming the immune system. Next we subjected mice deficient for both NOD1 and NOD2 (NOD1/2 DKO) with intact or depleted microbiota to renal I/R injury. NOD1/2 DKO mice showed the same phenotype as WT mice; antibiotic-treated NOD1/2 DKO mice had reduced renal damage and granulocyte influx and preserved renal function as compared to control NOD1/2 DKO mice.

In conclusion, gut microbiota enhances renal damage upon I/R injury. This is probably not regulated via translocation of bacteria or increased intestinal permeability during ischemia. Although others have shown a role for NOD1 in the priming of the immune system by microbiota, we did not observe a role for NOD1/2 in our system.
Introduction

The role of microbiota in gut homeostasis and gut immune development is well established. However, systemic effects of the microbiota are less well investigated. Previous studies have demonstrated a role for microbiota in modulating the adaptive immunity. Mice lacking intestinal microbiota develop less severe symptoms in autoimmune models for arthritis\(^1\) and encephalomyelitis\(^2\) (i.e. a mouse model for multiple sclerosis). In contrast, lack of microbiota increases disease severity in other autoimmune conditions as demonstrated in non-obese diabetes mice deficient for the toll-like receptor adaptor molecule Myd88 where normal intestinal microbiota alleviated spontaneous progression of type 1 diabetes\(^3\). However, not only the adaptive immunity is shaped by microbiota, but evidence is accumulating that there is also a direct effect on innate immunity as shown by Clarke et al\(^4\). In this study granulocytes isolated from antibiotic-treated or germ-free mice were less efficient in killing of bacteria \emph{ex vivo}. Moreover, they show that the bacterial cell wall component peptidoglycan (PGN), which is constantly turned over in the gut and either excreted or translocated across the gut mucosa into the circulation, primes granulocytes. PGN signals via its receptors NOD1 and NOD2, which are both members of the nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family. NOD1 and NOD2 detect specific substructures from bacterial PGN. NOD1 senses Gram\(^{-}\) derived PGN containing diaminopimelic acid (DAP)\(^5,6\), while NOD2 senses Gram\(^{-}\) and Gram\(^{+}\) derived PGN containing muramyl dipeptide (MDP)\(^7,8\). NOD1 is widely expressed in many cell types and organs\(^9-12\), while expression of NOD2 is believed to be more restricted and has been described in leukocytes and various epithelial cells\(^11-15\).

The abovementioned studies have revealed both beneficial and detrimental effects of gut microbiota on many inflammatory cells. Especially during sterile inflammation, e.g. renal ischemia/reperfusion (I/R) injury, the priming effect of microbiota on granulocytes might be unfavourable. Currently it is not known whether gut microbiota plays a role in the innate immune response in the post-ischemic kidney. Upon renal I/R injury, granulocytes are the first inflammatory cells infiltrating the damaged kidney\(^16\). These recruited granulocytes can clear dead cells and debris but also amplify renal damage. Moreover, renal I/R injury is exacerbated\(^17\) or attenuated\(^18-20\) by experimentally increasing or reducing granulocyte infiltration, respectively. Based on the role of granulocytes during renal I/R injury and the priming of granulocytes by microbiota, we hypothesized that lack of microbiota dampens the inflammatory response and thereby inhibits cell damage and preserves renal function. To investigate the effect of microbiota on renal I/R injury, we depleted gut microbiota by broad-spectrum antibiotic treatment in wildtype (WT) mice and compared their response to renal I/R injury with WT control mice that had intact gut microbiota. In a second \emph{in vivo} experiment we subjected mice deficient for both NOD1 and NOD2 (NOD1/2 DKO) with intact or depleted microbiota to renal I/R injury to determine whether priming of the immune system by gut microbiota might be regulated via these receptors.
Materials and methods

**Mice**
C57Bl/6 (WT) mice were purchased from Janvier (Le Genest, France). NOD1 and NOD2 double knockout (NOD1/2 DKO) mice were generated as described before\textsuperscript{21,22} and bred in the animal facility of the Academic Medical Center in Amsterdam. Mutant mice were backcrossed at least 10 generations. Mice were housed under specific pathogen-free conditions receiving food and water *ad libitum*. Age-matched male mice were used in all experiments. The Animal and Use Committee of the University of Amsterdam approved all experiments.

**Microbiota depletion**
Depletion of gut microbiota prior to renal I/R was achieved by administering mice broad-spectrum antibiotics via drinking water (1 g/L ampicillin (Sigma Aldrich, Zwijndrecht, The Netherlands), 1 g/L metronidazole (Pharmachemie, Haarlem, The Netherlands), 1 g/L neomycin (Sigma Aldrich) and 0.5 g/L vancomycin (EuroCept Pharmaceuticals, Ankeveen, The Netherlands)) as described previously\textsuperscript{23,24}. A treatment period of 2 weeks was sufficient to deplete the microbiota as determined by plating faeces on blood agar plates for the growth of anaerobes and aerobes.

**Ischemia/reperfusion injury**
I/R injury was induced by bilateral clamping of the renal pedicles of 8-12 weeks old male mice for 25 minutes under general anesthesia (0.07 mg per 10 g mouse of fentanyl citrate fluanisone midazolam mixture, containing 1.25 mg/ml midazolam (Roche, Mijdrecht, the Netherlands), 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanisone (Janssen Pharmaceuticals, Beerse, Belgium)). After removal of the clamps, the kidneys were inspected for restoration of blood flow. For analgesic purposes, mice received a subcutaneous injection of 50 µg/kg buprenorphin (Temgesic, Schering-Plough, Brussels, Belgium) after closing the abdomen. To maintain fluid balance and volume state, mice were supplemented with 100µl sterile 0.9% NaCl intraperitoneally. Sham-operated animals underwent the same procedure except clamping of the renal pedicles. At the time of sacrifice, blood was collected by heart puncture in heparin-containing tubes. Kidneys were snap frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin o/n prior to further processing.

**Plasma biochemical analysis**
Using standard autoanalyzer methods plasma levels of creatinine, ureum, lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT) were determined by our hospital research facility.

**Histology and immunohistochemistry**
Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-
µm thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid-Schiff reagents after diastase digestion (PasD). Injury to tubules was assessed, by a pathologist in a blinded fashion, semi-quantitatively by determining the percentage of affected tubules per 10 fields (magnification x400) according to the percentage of necrotizing tubules in the corticomedullary region on a scale from 0 to 5 (0 = 0%, 1 = <10%, 2 = 10-25%, 3 = 25-50%, 4 = 50-75%, and 5 = >75%).

For immunohistochemical staining sections were boiled for 10 min in 10 mM sodium citrate buffer (pH 6.0) for apoptosis, and proliferation detection or digested with a solution of 0.25% pepsin (Sigma Aldrich) in 0.01 M HCl for granulocyte detection. Subsequently, sections were incubated for 2 hours with FITC-labelled anti-mouse Ly-6G (Pharmingen, BD Biosciences, Alphen a/d Rijn, The Netherlands), rabbit anti-mouse active caspase-3 (Cell Signaling Technology, Beverly, MA, USA) or rabbit anti-human Ki67 (Neomarkers, Fremont, CA, USA) to detect granulocytes, apoptosis, or proliferation respectively. Next, sections were incubated for 30 min with relevant peroxidase-conjugated secondary antibodies and stained using 3,3′-diaminobenzidine dihydrochloride (DAB).

The number of Ly-6G positive cells and the number of capase-3 and Ki67 positive tubular epithelial cells (TEC) was counted in 10 non-overlapping high power fields (magnification 400x).

**ELISA**

Frozen kidneys were blended in PBS containing 1% Triton X-100, 1 mM EDTA and 1% protease inhibitor cocktail II (Sigma Aldrich). KC DuoSet ELISA kits (R&D Systems, Abingdon, UK) were performed according to the supplied protocol. Cytokine levels were corrected for total protein content per sample using Bio-Rad Protein Assay (Bio-Rad, Veenendaal, The Netherlands).

**Intestinal permeability**

Eight to twelve weeks old WT (n=6) mice were fasted o/n (water ad libitum). The following morning 450 mg/kg FITC-dextran (4kD; Sigma Aldrich, Zwijndrecht, The Netherlands) was administered orally. After 3 hours mice were subjected to renal ischemia for 15, 30 or 45 minutes or sham surgery as described above. Four hours following FITC-dextran administration, mice were sacrificed and blood was collected by heart puncture in heparin-containing tubes. The concentration of FITC in plasma was measured with a Synergy HT Multi-Mode microplate reader (BioTek, Bad Friedrichshall, Germany).

**Bacterial translocation**

To determine bacterial translocation during renal ischemia, both renal pedicles were clamped for 45 minutes after which mice were sacrificed and blood, kidneys, liver, spleen, and mesenteric lymph nodes were removed aseptically. Bacterial cultures
for (an)aerobics from these organs and 16S PCR on blood was performed by the department of medical microbiology from our hospital.

Statistical analyses
All statistical analyses were performed using Graphpad Prism 4 software (San Diego, CA, USA). Data were analysed using the non-parametric Mann-Whitney U-test. Results are expressed as mean ± standard error of the mean (SEM). Values of $P \leq 0.05$ were considered statistically significant.

Results

Depletion of intestinal microbiota preserves renal function
Following depletion of microbiota by means of broad-spectrum antibiotic treatment, a striking and profound protection against renal damage, as assessed by semi-quantitatively scoring of the percentage of necrotic tubules 24 hours after I/R injury, was observed. In control mice on average between 50-75% (score 4.1 ± 0.1) of the tubules were necrotic, while in antibiotic-treated animals tubules were significantly protected against necrosis with on average less than 25% of tubules

![Figure 1](image)

**Figure 1.** Renal damage, renal function and organ damage 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) WT mice. (a) Renal damage was assessed 1 day after I/R by semi-quantitative scoring the percentage of necrotic tubules in PAS-stained sections. Significant less damage was observed in antibiotic-treated mice. Renal function was determined by plasma creatinine (b) and ureum (c) levels. One day after I/R injury antibiotic-treated WT mice had a preserved renal function as shown by lower plasma creatinine and ureum levels. Plasma levels of general organ damage markers LDH (d), ASAT (e), and ALAT (f) were determined. All were increased following I/R in control WT mice, while in antibiotic-treated WT mice these markers did not raise significant upon renal I/R injury. Data are expressed as mean ± sem. *$P < 0.05$
that were affected (score 1.6 ± 0.6; figure 1a). In line with renal damage, renal function was significantly preserved in antibiotic-treated mice as indicated by lower plasma creatinine and ureum as compared with control mice (figure 1b,c). LDH, ASAT, and ALAT plasma levels were determined as general markers of tissue injury that were previously described to be increased upon renal I/R injury. All three enzymes were significantly increased in plasma of control mice 24 hours following renal I/R injury (figure 1d-f). Interestingly, in antibiotic-treated mice LDH, ASAT, and ALAT levels did not raise significantly upon renal I/R and consequently these levels were significantly lower in antibiotic-treated mice as compared with control mice following renal I/R (figure 1d-f). In sham mice antibiotic treatment did not affect plasma LDH or ASAT levels, whereas plasma ALAT levels were significantly lower in sham-operated mice that had received antibiotic treatment as compared with control.

**Increased tubular epithelial cell survival upon antibiotic treatment**

In addition to necrosis, tubular epithelial cells (TEC) can as well undergo apoptosis upon renal I/R injury. Twenty-four hours following renal I/R there was an increase in the number of apoptotic TECs in both control and antibiotic-treated mice compared with sham (figure 2a). Similar to tubular necrosis, we observed significantly less apoptosis in TEC of antibiotic-treated mice as compared with control mice after I/R injury (figure 2a).

Survival of TEC is determined by the balance between apoptosis and proliferation. Next we analyzed proliferation of TEC by means of Ki67 expression to see whether this balance is altered upon antibiotic treatment. Indeed, upon renal I/R injury we observed a trend \( (P=0.06) \) towards more Ki67-positive TEC in antibiotic-treated mice as compared with control mice (figure 2b). This indicates that in antibiotic-treated mice TEC survival is higher as shown by less apoptosis and more proliferation.

![Figure 2](image.png)

**Figure 2.** Survival of TEC 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) WT mice. (a) The amount of apoptotic TEC was determined by scoring caspase-3 + TEC. Following I/R injury there is a significant increase of apoptotic TEC which is lower in antibiotic-treated as compared with control mice. (b) The amount of proliferating TEC was determined by scoring Ki67 + TEC. No significant difference in TEC proliferation was observed between control and antibiotic-treated WT mice in sham or I/R injured kidneys. Data are expressed as mean ± sem. *\( P<0.05 \)
Reduced granulocyte influx upon antibiotic treatment

One day after renal I/R injury the inflammatory milieu is characterized by a vast influx of granulocytes\textsuperscript{16} that are known to play a detrimental role in the post-ischemic kidney\textsuperscript{17-20,26}. To determine whether the protective effect of depletion of gut microbiota on renal I/R injury could be explained by an alteration in the renal inflammatory response, we analysed granulocyte influx in I/R damaged kidneys. Interestingly, we found that antibiotic-treated mice had significant lower influx of granulocytes upon I/R (Figure 3a). In addition, a trend towards lower renal KC levels was observed in antibiotic-treated mice compared with control mice following I/R injury (control 445 ± 39 pg/mg protein; antibiotic-treated 304 ±46 pg/mg protein, $P=0.07$).

![Figure 3](image)

**Figure 3.** Granulocyte influx and renal KC levels 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) WT mice. (a) Influx of granulocytes was determined by scoring Ly-6G+ cells in the corticomedullary region of the kidney. Following I/R there is a vast influx of granulocytes in control WT mice, this was significantly attenuated in antibiotic-treated WT mice. (b) Renal KC levels were significantly increased following I/R injury in both groups. A trend towards lower KC was observed in antibiotic-treated compared with control WT mice upon renal I/R injury. Data are presented as mean ± sem. *$P<0.05$.

Intestinal permeability and bacterial translocation not affected by renal ischemia

Next we investigated whether the above described effects could be ascribed to an increased intestinal permeability and an enhanced translocation of bacteria from the intestines into the blood stream during renal ischemia. We detected low levels of FITC, as a direct measure of intestinal permeability, in plasma of sham-operated animals (Figure 4). Ischemia did not increase plasma FITC, and hence using this method we could not detect an effect of renal ischemia on intestinal permeability (Figure 4). Moreover, no evidence of bacterial translocation was found following renal ischemia (data not shown).
Role of NOD1 and NOD2 in protection against I/R via microbiota depletion

Recently it was shown that NOD1 can prime innate immunity remotely via recognition of PGN from the intestinal microbiota. These results prompted us to investigate whether the detrimental effect of intestinal microbiota on renal inflammation and injury after I/R injury could be dependent on NOD1 and NOD2. For this, we subjected control and antibiotic-treated NOD1/2 DKO mice to renal I/R injury.

Figure 4. Intestinal permeability in WT mice was determined by the plasma concentration of FITC ([FITC]) after oral administration of 4kDa dextran-FITC followed by renal ischemia or sham surgery. Low concentration of FITC was present in plasma of sham-operated mice. Intestinal permeability was not enhanced by clamping renal pedicles for 15, 30 or 45 minutes. Data are presented as mean ± sem.

Figure 5. Renal damage, renal function and organ damage 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) NOD1/2 DKO mice. (a) Renal damage was assessed 1 day after I/R by semi-quantitative scoring the percentage of necrotic tubules in PasD-stained sections. Significant less damage was observed in antibiotic-treated mice. Renal function was determined by plasma creatinine (b) and ureum (c) levels. One day after I/R injury antibiotic-treated WT mice had a preserved renal function as shown by lower plasma creatinine and ureum levels. Plasma levels of general organ damage markers LDH (d), ASAT (e), and ALAT (f) were determined. All three markers were significantly lower in antibiotic-treated NOD1/2 DKO mice following renal I/R injury compared with control NOD1/2 DKO mice. Data are expressed as mean ± sem. *P<0.05
Antibiotic treatment significantly inhibited tubular necrosis and preserved renal function, determined by plasma creatinine and urea levels, in antibiotic-treated NOD1/2 DKO mice as compared to control NOD1/2 DKO mice (figure 5a-c). In addition, we observed a reduction of the general damage markers LDH and ASAT and the liver damage marker ALAT in NOD1/2 DKO mice subjected to renal I/R when microbiota was depleted (figure 5d-f). In line with reduced tubular necrosis, less TEC apoptosis was detected following renal I/R in antibiotic-treated NOD1/2 DKO mice as compared with control NOD1/2 DKO mice (figure 6a), while no difference between the amount of proliferating TEC was observed between control and antibiotic-treated NOD1/2 DKO mice (figure 6b). These results indicate that TEC survival is enhanced once microbiota is depleted. To determine whether the inflammatory response is affected by antibiotic treatment influx of granulocytes and renal KC levels were measured. We observed significant less granulocytes in kidneys of antibiotic-treated NOD1/2 DKO mice as compared with control NOD1/2 DKO mice (figure 7a). Renal KC was significantly elevated following I/R injury in NOD1/2 DKO mice, while no difference was observed between control and antibiotic-treated animals (figure 7b).

Figure 6. Survival of TEC 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) NOD1/2 DKO mice. (a) The amount of apoptotic TEC was determined by scoring caspase-3+ TEC. Following I/R injury there is a significant increase of apoptotic TEC which is lower in antibiotic-treated as compared with control mice. (b) The amount of proliferating TEC was determined by scoring Ki67+ TEC. No significant difference in TEC proliferation was observed between control and antibiotic-treated NOD1/2 DKO mice in sham or I/R injured kidneys. Data are expressed as mean ± sem. *P<0.05
Despite the progresses made in health care, acute kidney injury is still a major clinical problem affecting 5% of hospitalized patients and has a mortality rate of 50-80% in these patients\textsuperscript{27}. I/R injury, caused by a sudden transient drop in blood flow to the kidney frequently occurring in shock, sepsis, and during renal transplantation, is the major initiator of acute kidney injury. Once blood flow is restored, inflammatory cells enter the kidney and, apart from clearing dead cell debris, can additionally amplify renal damage. Therefore dampening the inflammatory response is a promising therapy. Our data clearly show that depletion of the gut microbiota profoundly protects mice against renal I/R injury as shown by less tubular damage, preserved renal function and less granulocyte influx. Recently, the role of microbiota on the immune system has gained a lot of attention. More evidence is accumulating that the gut microbiota is not only involved in development of the local immune system, but also plays an important role in shaping the systemic immune system. Bacterial translocation is a well known phenomenon during intestinal\textsuperscript{28} and liver\textsuperscript{29} ischemia. As far as we know, no study has been published that investigated whether renal I/R injury affects bacterial translocation. In the present study no evidence of bacterial translocation during renal ischemia was found. Moreover, intestinal integrity seemed not affected by renal ischemia as we observed no effect of clamping the renal pedicles on intestinal permeability. These results imply that the microbiota does not seem to play a direct role during renal I/R injury. However, several studies have shown microbial products (e.g. PGN) derived from gut microbiota systemically\textsuperscript{4,30-32}, where they might exert immunomodulatory effects.

**Figure 7.** Granulocyte influx and renal KC levels 1 day after I/R injury in control (white bars) and antibiotic-treated (black bars) NOD1/2 DKO mice. (a) Influx of granulocytes was determined by scoring Ly-6G\textsuperscript{+} cells in the corticomedullary region of the kidney. Following I/R there is a vast influx of granulocytes in control NOD1/2 DKO mice, this was significantly attenuated in antibiotic-treated NOD1/2 DKO mice. (b) Renal KC was significantly increased following I/R injury in both groups, no difference between control and antibiotic-treated NOD1/2 DKO was observed. Data are presented as mean ± sem. *P<0.05
Therefore the protective effect of broad-spectrum antibiotic treatment on renal I/R injury might be ascribed to the immunomodulatory properties of the microbiota. Although we observed a remarkable protection against renal I/R injury upon depleting gut microbiota, others have shown that germ-free mice are more prone to renal I/R injury. Germ-free mice had greater renal dysfunction and more tubular damage compared with control mice. The discrepancy between both studies might be explained by the role of gut microbiota in postnatal development of the immune system. During the early postnatal period, intestinal microbiota stimulate the development of both local and systemic immunity, while later on these components evoke inhibitory mechanisms intended to keep both mucosal and systemic immunity in check. From a clinical perspective it is interesting to see that temporary depletion of gut microbiota has protective effects on renal I/R injury, as shown in the present study.

Recently the priming effect of gut microbiota on granulocytes has been described. They show that granulocytes derived from antibiotic-treated mice were less efficient in killing bacteria, and that the microbiota can directly enhance granulocyte function. We observed a significant reduced influx of granulocytes in antibiotic-treated mice upon renal I/R injury, while renal levels of the chemokine KC were not significantly lower. Since KC is the main chemoattractant for granulocytes, as demonstrated by significantly inhibited granulocyte influx into the ischemic kidney upon neutralization of this chemokine, depletion of microbiota does not impair the response of the kidney to attract granulocytes to the damaged tissue. These results imply that apart from enhancing granulocyte function, microbiota may also influence their migratory capacity. Next to removing cell debris, granulocytes may also contribute to collateral damage through the release of mediators and enzymes that potentially damage the surrounding tissue. Indeed, the detrimental role of granulocyte influx in the post-ischemic kidney has been reported by several groups. Therefore, the reduced influx of granulocytes observed in antibiotic-treated mice might in part explain the preserved renal function and attenuated renal damage.

Clarke et al. reported that the microbiota are a source of PGN that systemically primes the granulocytes, which requires signalling via the receptor NOD1. NOD1 and its family member NOD2, that share high structural homology, contribute in a redundant manner to the immune response following infection. Therefore we questioned whether the protective effect of microbiota depletion during renal I/R injury is mediated via NOD1 and NOD2. We observed the same phenotype in antibiotic-treated NOD1/2 DKO mice compared with control NOD1/2 DKO mice as in WT mice; i.e. tubular necrosis and damage was significantly lower, renal function was preserved and granulocyte influx was significantly impaired in microbiota depleted animals. Together these results suggest that the detrimental effect of intact microbiota on renal I/R is not regulated via the innate immune receptors NOD1 and NOD2.
Overall, the present study shows that the microbiota plays an important role during renal I/R injury. No evidence for increased intestinal permeability was observed during renal ischemia. Moreover, renal I/R injury did not result in bacterial translocation from the gut into the systemic circulation. Interestingly, influx of granulocytes was significantly lower in post-ischemic kidneys of microbiota depleted mice. Together, these results argue for a priming role of the microbiota on the immune system.

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