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Transient TLR Activation Restores Inflammatory Response and Ability To Control Pulmonary Bacterial Infection in Germfree Mice

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Mammals are colonized by an astronomical number of commensal microorganisms on their environmental exposed surfaces. These symbiotic species build up a complex community that aids their hosts in several physiological activities. We have shown that lack of intestinal microbiota is accompanied by a state of active IL-10–mediated inflammatory hyporesponsiveness. The present study investigated whether the germfree state and its hyporesponsive phenotype alter host resistance to an infectious bacterial insult. Experiments performed in germfree mice infected with Klebsiella pneumoniae showed that these animals are drastically susceptible to bacterial infection in an IL-10–dependent manner. In germfree mice, IL-10 restrains proinflammatory mediator production and neutrophil recruitment and favors pathogen growth and dissemination. Germfree mice were resistant to LPS treatment. However, priming of these animals with several TLR agonists recovered their inflammatory responsiveness to sterile injury. LPS pretreatment also rendered germfree mice resistant to pulmonary K. pneumoniae infection, abrogated IL-10 production, and restored TNF-α and CXCL1 production and neutrophil mobilization into lungs of infected germfree mice. This effective inflammatory response mounted by LPS-treated germfree mice resulted in bacterial clearance and enhanced survival upon infection. Therefore, host colonization by indigenous microbiota alters the way the host reacts to environmental infectious stimuli, probably through activation of TLR-dependent pathways. Symbiotic gut colonization enables proper inflammatory response to harmful insults to the host, and increases resilience of the entire mammal-microbiota consortium to environmental pressures. The Journal of Immunology, 2012, 188: 1411–1420.

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Abbreviations used in this article: ANXA-1, annexin-1; BCG, Bacillus Calmette-Guérin; I/R, ischemia and reperfusion; LTA, lipoteichoic acid; LXA₄, lipoxin A₄; MPO, myeloperoxidase; PRR, pattern recognition receptor; SMA, superior mesenteric artery.

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manent contact of the innate immune system with the intestinal microbiota switches off our innate ability to produce IL-10 and, possibly, other anti-inflammatory molecules in response to various inflammatory insults. However, there is a cost in achieving this greater inflammatory capacity: an exaggerated response to non-infectious stimulation may lead to systemic inflammation and death. The present study investigated whether this primed inflammatory state may be beneficial in terms of responding to an infectious bacterial insult. Experiments were performed in germfree mice to evaluate the relevance of commensal microbiota for the ability of the murine host to control pulmonary infection caused by Klebsiella pneumoniae. Furthermore, we assessed whether priming with LPS reversed the inflammatory hyporesponsive phenotype of germfree mice.

Materials and Methods

Animals

Germfree Swiss/NHI mice were derived from a germfree nucleus (Taconic Farms) and maintained in flexible plastic isolators (Standard Safety Equipment) using classical gnotobiology techniques (12). Conventional Swiss/NHI mice are derived from germfree matrices, and considered conventional only after two generations in the conventional facility. All animals were 8- to 10-wk-old males and females. All experimental procedures in germfree mice were carried out under aseptic conditions to avoid infection of animals.

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. For measuring percentage of surviving mice, reperfusion was re-established, and mice were monitored for indicated time periods. For the other parameters, reperfusion was allowed to occur for 40 min (I/R) when mice were sacrificed. This time of reperfusion (40 min) was chosen based on the presence of significant tissue injury without unduly high mortality rates. Sham-operated animals were used as controls.

Pulmonary infection by Klebsiella pneumoniae

The bacterium used was K. pneumoniae, ATCC 27 736, which has been kept in the Department of Microbiology, Universidade Federal de Minas Gerais, and made pathogenic by 10 passages in BALB/c mice (13). Bacteria were frozen after reaching the logarithmic phase of growth and kept in a −70°C freezer at a concentration of 1 × 10^8 CFU ml⁻¹ 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.

Results

Statistical analysis

Results are shown as means ± SEM. Differences were compared by using ANOVA, followed by Student-Newman-Keuls posthoc analysis. For survival curve comparisons, results were analyzed using the log rank test. Results with a p value <0.05 were considered significantly different.

Results

Germfree mice are more susceptible to pulmonary K. pneumoniae infection

To assess whether the anti-inflammatory phenotype of germfree mice altered the ability of mice to deal with an infectious disease, we first evaluated the response of conventional and germfree mice to pulmonary inoculation with K. pneumoniae. At the inoculum
used (10^6 CFU), all conventional mice injected with bacteria survived until 72 h postinfection (Fig. 1A). Intratracheal inoculation with K. pneumoniae into conventional mice was associated with influx of neutrophils in the lung parenchyma (Fig. 1B) and in bronchoalveolar lavage fluid (data not shown) at 24 h after challenge. There was also significant increase in the levels of TNF-α and CXCL-1 in pulmonary tissue (Fig. 1C, 1D, respectively). At 24 h postinfection, K. pneumoniae could be detected at significant amounts in pulmonary tissue (Fig. 1F), but there was no systemic dissemination of the infection (Fig. 1G). At this time, there was no increase of IL-10 release (Fig. 1E).

In contrast to conventional mice, germfree mice are not capable of circumventing K. pneumoniae infection and died at a much faster rate than conventional mice (Fig. 1A). Indeed, all germfree mice were dead at 72 h postinfection. Moreover, in germfree mice, there was no significant increase in neutrophil influx (Fig. 1B) or enhancement of TNF-α (Fig. 1C) or CXCL-1 (Fig. 1D) concentrations postinfection. Despite the lack of increase in levels of proinflammatory cytokines, there was significant increase of IL-10 production in lungs of infected germfree, in contrast to the situation seen in conventional mice (Fig. 1E). Enhanced lethality was associated with very high number of bacteria in the lungs (Fig. 1F) and marked dissemination to blood (Fig. 1G) of infected germfree mice.

**Exogenous administration of IL-10–induced bacteremia in conventional mice**

As there was enhanced production of IL-10 postinfection of germfree mice with K. pneumoniae, we evaluated whether exogenous administration of murine rIL-10 would be capable of modulating the course of K. pneumoniae infection. In IL-10–treated conventional mice, there was marked increase of bacteria in lung tissue and blood (Table I). Enhanced bacterial load was associated with decreased CXCL-1 production and neutrophil recruitment to pulmonary parenchyma (Table I). Exogenous IL-10 administration did not alter lung TNF-α concentration (Table I).

**Anti–IL-10 reverses the inability of germfree mice to deal with pulmonary infection**

Our previous studies have demonstrated that administration of anti–IL-10 induced inflammation and lethality in germfree mice after I/R or LPS administration, demonstrating that the ability of germfree mice to produce IL-10 was largely responsible for their inflammatory hyporesponsive phenotype (7). As there was significant IL-10 production in the lung of infected mice and administration of IL-10 to conventional mice mirrored the phenotype of germfree mice, we evaluated whether IL-10 played any significant role in the course of K. pneumoniae infection. Treatment of germfree mice with anti–IL-10 was accompanied by significant increase in neutrophil recruitment to the lung, at levels similar to those observed in conventional mice (Fig. 2A). In a similar manner, pulmonary (Fig. 2B, 2C) and serum (data not shown) concentrations of TNF-α and CXCL-1 increased in lungs of anti–IL-10–treated germfree mice to levels similar to those found in conventional mice. Treatment with anti–IL-10 was also associated with better control of infection, as seen by reduction in the counts of bacteria in the lung (Fig. 2E) and reduced bacterial systemic dissemination (Fig. 2F). Overall, our results argue that the reduced acute inflammatory response observed in germfree mice is largely due to their innate ability to produce IL-10 and consequent IL-10–mediated inhibition of the local and systemic inflammatory responses.

**Contact with microbiota reverted anti-inflammatory phenotype after K. pneumoniae infection in germfree mice**

We have previously demonstrated that restoration of microbiota colonization took a long time to reverse the hyporesponsive inflammatory phenotype of germfree mice. Indeed, inflammatory responsiveness in germfree mice was fully regained only 2–3 wk after reposition of microbiota, despite the fact that cultivable bacteria had already been detected 7 d after administration of feces to these animals (7). In this study, we evaluated whether
reposition of microbiota 14 d prior to infection, referred to as conventionalization, restored the inability of germfree mice to deal with *K. pneumoniae* infection. Our data demonstrated that, as assessed by neutrophil influx in lung tissue (Fig. 2A) or in bronchoalveolar lavage (data not shown), conventionalization enabled efficient neutrophil recruitment postinfection to levels similar to those found in conventional mice. In addition, the concentration of CXCL-1 or TNF-\(\alpha\) markedly increased after pulmonary infection in the lung (Fig. 2B, 2C) or serum (data not shown) of infected mice. In conventionalized mice, there was decrease in pulmonary concentration of IL-10 (Fig. 2D). Indeed, levels of IL-10 were similar to those seen in conventional mice. More importantly, microbiota reposition was accompanied by decrease of *K. pneumoniae* concentration in lungs (Fig. 2E) and blood (Fig. 2F), showing that conventionalization of germfree mice for 14 d was sufficient to restore inflammatory responsiveness and ability to deal with *K. pneumoniae* infection.

**Germfree mice are tolerant to systemic LPS administration**

Germfree mice do not possess any live bacteria in the gut and are consequently normally exposed to only minor amounts of bacterial-derived products present in commercial chows. That being the case, it is likely that the innate immune system of these animals has little exposure to bacterial-derived products, such as LPS. To examine whether exposure to LPS would restore the ability of germfree mice to inflame, mice received one single injection of LPS at different doses and were monitored daily. Intraperitoneal administration of LPS to conventional mice induced rapid lethality at dose of 10 mg/kg. Indeed, all animals were dead by 12 h after LPS injection (Fig. 3A). At the dose of 1 mg/kg, 25% of conventional mice were dead.

### Table I. IL-10 treatment leads to reduced inflammatory response and to pathogen dissemination in conventional mice submitted to pulmonary bacterial infection

<table>
<thead>
<tr>
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<th>NI</th>
<th>PBS</th>
<th>IL-10</th>
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<tbody>
<tr>
<td>Neutrophils(^a)</td>
<td>1.3 ± 0.1</td>
<td>9.2 ± 1.3(^*)</td>
<td>3.6 ± 0.5(^*)</td>
</tr>
<tr>
<td>Lung</td>
<td>TNF-(\alpha)</td>
<td>83 ± 7.4</td>
<td>442 ± 39(^*)</td>
</tr>
<tr>
<td>CXCL-1(^b)</td>
<td>272 ± 31</td>
<td>1369 ± 112(^*)</td>
<td>354 ± 41(^*)</td>
</tr>
<tr>
<td>Blood</td>
<td>Bacteria(^c)</td>
<td>—</td>
<td>19 ± 12 × 10(^6)*</td>
</tr>
</tbody>
</table>

Conventional mice were treated (s.c.) with vehicle or rIL-10 (~0.5 \(\mu g/mice\)), and, 45 min later, inoculated with \(3 × 10^6\) CFU *K. pneumoniae* or vehicle (30 \(\mu l\)) and, 24 h later, were culled to evaluation of neutrophil influx, TNF-\(\alpha\), and CXCL1 concentration in lung parenchyma and for bacterial dissemination into lungs and blood.

\(^a\)Neutrophil influx was assessed by measuring the tissue contents of MPO.

\(^b\)The concentrations of TNF-\(\alpha\) and CXCL1 were assessed in the lung by using specific ELISAs.

\(^c\)Bacterial counts were quantified by CFU. Results are shown as number of neutrophils, concentration of cytokine or bacteria number per 100 mg tissue or ml blood, and are the mean ± SEM of 5–6 animals.

\(^*p < 0.05\) when compared with not infected (NI) animals; \(^#p < 0.05\) when compared with vehicle-treated animals (PBS).

ND, Not detected.
after LPS injection. In contrast, at 1 and 10 mg/kg, none of LPS-injected germfree mice were dead until 96 h after LPS injection (Fig. 3A). In fact, there was no lethality even 14 d after LPS injection (data not shown). Only at 30 mg/kg, 30% of injected germfree mice succumbed to LPS administration. Thus, our experiments show that germfree mice present significant resistance to LPS administration; that is, it is necessary for a dose of LPS 30 times greater to induce the same lethality rates observed in presence of microbiota (Fig. 3A).

Germfree mice injected with LPS produced at least 10 times less TNF-α than their conventional counterparts, and this reduced TNF-α production may account for their reduced response to LPS (7). However, it is noticeable that significant amounts of TNF-α were indeed detectable from 6 until 48 h after injection, and declined to undetectable levels by 96 h after LPS administration (Fig. 3B). In contrast to TNF-α, germfree mice produce substantial greater amounts of IL-10 than conventional mice (7). IL-10 release occurred very early and was already maximal at 1.5 h after LPS administration, but it was more transient. As seen in Fig. 3C, elevated levels persisted at 6 h, but dropped to background levels by 24 h after LPS injection.

The hyporesponsiveness of germfree mice to inflammatory stimuli can be transiently switched off by LPS administration

As germfree mice are tolerant to high-dose LPS injection, we investigated whether this component of the microbiota was sufficient to restore the inflammatory responsiveness of these animals, akin to microbiota reposition. To this end, animals were injected with LPS at various times prior to inflammatory stimulation. For this part of the study, the inflammatory stimulus used was induction of intestinal I/R injury. We have previously shown that ischemia of the SMA followed by reperfusion causes inflammation-driven injury to local and remote organs, which is sterile (7), TNF-α dependent (18–20), and modulated negatively by IL-10 (20–23). Conventional mice subjected to intestinal I/R injury die within 90 min of reperfusion, whereas all germfree mice survive to this stimulation (7). Administration of PBS to germfree did not alter their phenotype, and all mice were still alive at 120 min after reperfusion (Fig. 3D). In contrast, administration of LPS (10 mg/kg) to germfree mice greatly altered their responsiveness to reperfusion injury. Indeed, as seen in Fig. 3D, there was no change of responsiveness at 2 h after LPS administration. However, previous treatment with LPS at 6, 24, and 48 h prior to reperfusion injury enhanced the sensitivity of animals to the insult in a time-dependent manner (Fig. 3D). In animals given LPS 48 h prior to reperfusion, results were actually comparable to those seen in conventional mice. Interestingly, the responsiveness of germfree mice to I/R injury was lost at 96 h after LPS administration, at a time when LPS-induced TNF-α production was undetectable in serum, but there was a small recovery in IL-10 production (Fig. 3B, 3C). These results suggest that LPS is capable of switching on the inflammatory phenotype in germfree mice, which is switched off 96 h after administration of this bacterial-derived product. Moreover, both the switching on and off of the inflammatory phenotype correlated with the balance between serum concentrations of TNF-α and IL-10. Other TLR agonists, including...
TLR2 (LTA) or TLR9 (CpG DNA) or both TLR2 and TLR9 agonists (BCG), were also capable of reversing the hyporesponsiveness of germfree mice to reperfusion injury when given 48 h prior to the experiment (Fig. 3E). Hence, germfree mice, pretreated with CpG, LTA, or BCG, presented 100, 60, and 75% lethality after induction of I/R injury, respectively (log rank test: GF + PBS versus GF + CpG, p = 0.011, number of 5 and 8 animals per group, respectively; GF + PBS versus GF + LTA, p = 0.049, number of 5 animals per group; GF + PBS versus GF + BCG, p = 0.022, number of 5 animals per group). At the doses used, the latter agonists did not modify the response of conventional mice to I/R injury (log rank test: CV + PBS versus CV + CpG, p = 0.296, number of 5 per group; CV + PBS versus CV + LTA, p = 0.49, number of 5 and 7 animals per group, respectively; CV + PBS versus CV + BCG, p = 0.10, number of 5 animals per group).

The next series of experiments were carried out to examine whether changes in inflammatory response accounted for reperfusion-associated death of germfree mice after LPS administration. To this end, germfree mice were treated with LPS 48 h before induction of I/R and various parameters of tissue injury and inflammation examined. As we have previously shown, germfree mice have little or no increase in reperfusion-associated increase in vascular permeability, hemorrhage, or neutrophil influx (Fig. 4A–C). However, treatment with LPS (10 mg/kg) 48 h prior to reperfusion was associated with enhancement of tissue damage and inflammation to levels similar to those found in conventional mice subjected to I/R injury (see dotted line in Fig. 4A–C). Similarly, reperfusion-induced elevation in levels of TNF-α and CXCL1 did not occur in germfree mice, but were enhanced to conventional levels after treatment with LPS (Fig. 4D, 4E). We have previously shown that reperfusion injury induced an elevation of IL-10 levels in the intestine of germfree animals, and IL-10 accounted for their hyporesponsive phenotype in the context of reperfusion injury (7). In this study, intestinal I/R of germfree mice was associated with elevation of IL-10, an effect that was prevented by previous treatment with LPS (Fig. 4F). Again, treatment with LPS reversed the phenotype of germfree to levels seen in conventional mice. Similar results were observed when cytokines and inflammation were measured in a remote organ (the lung) or systemically (serum) (data not shown). It must be stressed that sham-operated mice injected with LPS 48 h before the surgical procedure had no significant inflammatory response or alteration in cytokine levels in the intestine, confirming that LPS alone was not sufficient to prime for tissue inflammatory response in germfree mice (Fig. 4).

The enhanced infectivity of germfree mice by K. pneumoniae is reversed by LPS administration

The hyporesponsiveness of germfree mice to LPS was also reversed when LPS was given 48 h previously. Indeed, as seen in Fig. 5A, injection of LPS caused no death in germfree mice treated with PBS 48 h earlier. However, pretreatment with LPS switched on the ability of these animals to respond to a subsequent dose of LPS. As seen in Fig. 5A, germfree mice pretreated 48 h earlier with LPS died in a similar way to conventional mice after a second LPS challenge.

As inflammatory responsiveness to a bacterial component was regained after previous stimulation of the system, we assessed whether pretreatment with LPS could also alter the response of germfree mice to K. pneumoniae infection. Our results demonstrated that the injection of LPS 48 h before pulmonary infection with K. pneumoniae induced significant increase of neutrophil recruitment (Fig. 5B), and release of TNF-α and CXCL-1 (Fig. 5C, 5D). In contrast, previous treatment of germfree mice with LPS was followed by decrease in production of IL-10 in response to the infection (Fig. 5E). Number of bacteria in pulmonary parenchyma (Fig. 5F) and in blood (Fig. 5G) was greatly decreased by pretreatment with LPS, suggesting that better control of in-
infection was achieved. These results culminated with delay of lethality after K. pneumoniae infection of germfree mice treated with LPS (Fig. 5H). Therefore, transient TLR4 activation restores inflammatory responsiveness and host resistance to K. pneumoniae infection in germfree mice.

Discussion

The major findings of the current study can be summarized as follows: 1) Pulmonary infection of germfree animals with K. pneumoniae was associated with greater bacterial growth, dissemination of infection, and greater lethality rates. 2) Germ-free mice responded to infection by producing decreased amounts of proinflammatory cytokines, including TNF-α and CXCL1, and decreased neutrophil influx, and producing large amounts of IL-10. 3) Blockade of IL-10 production reversed the inflammatory hyporesponsiveness of germfree mice and restored the ability of these mice to respond to infection. 4) Colonization of germfree mice with microbiota from conventional mice 14 d prior to infection restored their ability to respond to K. pneumoniae infection. 5) Treatment with LPS or other TLR ligands 48 h prior to stimulation or infection restored transiently the ability of germfree mice to respond to sterile inflammatory stimulation (I/R injury) or to control K. pneumoniae infection.

We have previously demonstrated that germfree mice have greatly decreased inflammatory response and do not die after reperfusion of the ischemic SMA (7). We have also demonstrated that the lack of intestinal microbiota is accompanied by a state of active inflammatory hyporesponsiveness mediated by IL-10 and other anti-inflammatory mediators (7, 11). From the evolutionary point of view, it is unlikely that there is an advantage in inflaming excessively, as after intestinal I/R, when the intestinal microbiota is present. Thus, why would an animal lose its ability to produce anti-inflammatory molecules, such as IL-10, when first faced with a major inflammatory stimulus? Our results showed that germfree mice died much earlier after bacterial infection, whereas conventional animals, which are capable of inflaming in response to the bacterial challenge, survived for >3 d. Our results are in agreement with others, which demonstrated the increase of susceptibility to parasite infection in absence of commensal microbiota. For example, germ-free mice have decreased capacity to deal with Leishmania major (24) and Trypanosoma cruzi infections (25). Altogether, these experiments in germfree mice would suggest that the ability to inflame in response to bacteria, and possibly other parasites, is evolutionarily relevant. 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 an infectious challenge.

The model of pulmonary infection by K. pneumoniae is characterized by a rapid increase in the number of neutrophils, preceded by an increase in the concentrations of TNF-α and CXCL-1 (8, 13, 26–28). As the local influx of neutrophils is determinant in the clearance of bacteria, the inability to recruit neutrophils is associated with increased recovery of bacteria and greater lethality rates. Previous studies have suggested a role for neutrophil-active (CXC) chemokines and chemokine receptors, for the migration of neutrophils into the lungs of mice infected with bacteria (26, 29–31) and others (27) has shown a critical role of TNF-α as part of the pulmonary host defense in a murine model of infection with K. pneumoniae. In germfree mice, production of CXCL1 and TNF-α and recruitment of neutrophils were decreased. In contrast, levels of IL-10 were greatly enhanced postinfection of germfree mice. In our experiments, administration of IL-10 to conventional mice...
decreased lung inflammation and enhanced bacterial load and lethality rates. More importantly, treatment with anti-IL-10 Abs restored levels of CXCL1 and TNF-α and recruitment of neutrophils in infected germfree mice, and this was associated with protection from bacterial dissemination and death. The latter results are consistent with other studies showing that endogenous IL-10 is detrimental for survival and bacterial clearance in a model of peritonitis induced by K. pneumoniae (32). The data are also in agreement with previous studies showing that the phenotype of germfree mice is in great part explained by the greater innate production of IL-10 upon acute inflammatory stimulation. Therefore, the higher innate production of IL-10 by germfree mice explains the decreased inflammatory responsiveness and increased susceptibility to K. pneumoniae infection.

We have previously observed that the greater ability of germfree mice to produce LXA4 and ANXA-1 underlies their greater capacity to produce IL-10 and to prevent acute inflammation during the sterile inflammatory stimuli induced by I/R (11). Lipoxins, such as LXA4, constitute the first recognized class of anti-inflammatory lipid-based autacoids that may function as endogenous "stop signals" that downregulate or counteract the formation and action of proinflammatory mediators and promote resolution (33). ANXA-1 is another mediator of anti-inflammation that was identified originally as responsible for several of the anti-inflammatory actions of glucocorticoids (34). Both LXA4 and ANXA-1 or compounds that mimic their actions have anti-inflammatory effects in several models of acute and chronic inflammation, and in models of inflammation-mediated tissue injury (33, 34). In germfree mice, there was enhanced expression of both LXA4 and ANXA-1 (11). Antagonism of ALX receptors (at which both LXA4 and ANXA-1 act), or simultaneous administration of 5-lipoxygenase inhibitor (blocking LXA4 synthesis) and anti-ANXA-1 Abs, was associated with restoration of neutrophil recruitment and proinflammatory mediator production in germfree mice submitted to reperfusion injury induction (11). Thus, the innate capacity of germfree mice to produce IL-10 is secondary to their endogenous greater ability to produce LXA4 and ANXA-1, and these molecules control their inflammatory hyporesponsiveness. It is likely that these mechanisms are active during response of germfree mice to infectious inflammatory stimulation, such as during K. pneumoniae infection. In addition, the participation of LXA4 and ANXA-1 during response of germfree hosts to inflammatory stimulation suggests that other anti-inflammatory mediators, for example, TGF-β, could play a relevant role in this hyporesponsive phenotype.

Akin to observations in animals subjected to I/R (7) or hypernociception (10), colonization of the gastrointestinal tract of germfree with gut bacteria of conventional mice was capable of reversing the preferential production of IL-10 and restoring inflammatory responsiveness in pulmonary infection model, with consequent clearance of bacterial and decreased lethality. Thus, it appears that the daily contact with the intestinal microbiota switches on a "state of alert" on the cells of the innate immune system, facilitating the ability of these cells to produce cytokines, to inflame, and to deal with an infectious challenge. This ability to inflame is also relevant for the development of an acquired immune response, as inflammatory mediators and activated leukocytes present in the inflammatory milieu provide the necessary costimulation for T cells (35). Therefore, the mammalian host has the innate ability to produce IL-10 and other anti-inflammatory molecules that is lost after colonization by indigenous microbiota after birth or in adult germfree mice through conventionalization. This gain of inflammatory function through conventionalization confers to the host ability to deal with pathogenic microorganisms.

Bacteria and other gut-living microorganisms are recognized by the immune system via pattern recognition receptors (PRRs), including the TLRs (9). Indeed, activation of PRRs by pathogen-associated molecular patterns is essential for adequate inflammatory responses to pathogens and adequate mounting of an adaptive immune response. LPS derived from Gram-negative bacteria induces inflammation, costimulation, and immune priming via activation of TLR4 (9). There was no difference in the expression of TLR4 in splenic leukocytes (CD11b⁺, CD11c⁺, B220⁺, NK1.1⁺, and GR1⁺ leukocytes) from germfree or conventional mice (data not shown), and it has been shown that there is no difference in TLR expression between lung cells of conventional and germfree animals (36). However, contrary to conventional mice, germfree animals produced little TNF-α, did not die, and produced large amounts of IL-10 following exposure to LPS (7) or pulmonary infection with K. pneumoniae (present results). Moreover, our results clearly demonstrate that the systemic administration of LPS is capable of reversing the ability of germfree mice to produce IL-10. Decreased IL-10 production allows the production of TNF-α and other mediators, and adequate mounting of an inflammatory response, characterized by increase of vascular permeability, hemorrhage, and neutrophil recruitment. In the context of intestinal I/R, the inflammatory response after exposure to LPS causes high lethality rates. The effects of LPS were slow in onset (starting at 6 h and peaking at 24–48 h) and transient (over by 96 h), suggesting that mechanisms responsible for switching on inflammatory hyporesponsiveness can potentially be switched off, as soon as the LPS stimulation is lost. Of note, LPS did not induce any inflammatory response in the intestine of sham-operated mice, demonstrating that LPS by itself does not induce inflammation, but prepares germfree mice to respond to a second stimulus. Other TLR agonists were also able to reverse the anti-inflammatory phenotype of germfree mice subjected to I/R. All these findings, in concert, suggest that the ability to mount acute inflammatory responses is largely dependent on the colonization of the host by mutualistic microorganisms, and probably involves continuous activation of PRRs by microbiota-derived products, such as TLR ligands. In this regard, it has been shown that bacterial peptidoglycan from indigenous microbiota constitutively translocates to the circulation and remotely primes leukocyte functions via Nod1 receptor (37). It is conceivable that a similar mechanism may occur in several physiological activities of the host, including its inflammatory responsiveness, and may involve ligands of other PRRs.

There was a close correlation between the reversion of inflammatory hyporesponsiveness in LPS-pretreated animals and the induction of TNF-α and abrogation of IL-10 production. Hence, at 48 h post-LPS injection, at a time when germfree mice responded to secondary stimulation very similarly to conventional mice, TNF-α concentration was maximal, and, reciprocally, IL-10 production was almost vanished. Whether the TNF-α produced or other molecules are necessary for dampening IL-10 production and changing inflammatory responsiveness of germfree mice and the detailed pathways triggered by LPS to restore inflammatory responsiveness clearly deserve further investigation. Nevertheless, these results suggest that continuous activation of TLRs (and probably other PRRs) by the commensal microbiota is sufficient and, perhaps, necessary for priming the innate immune system. This priming is reflected in switching the way the system responds to any inflammatory stimulus: moving from an IL-10–prone producer to being capable of producing proinflammatory mediators and rapidly mobilizing circulating leukocytes.

The administration of LPS, in a dose and schedule that restored the ability of germfree mice to inflame in the sterile model of
reperfusion injury, significantly prolonged the survival of germfree mice after *K. pneumoniae* infection. The delay of lethality induced by priming with LPS was followed by neutrophil recruitment and by production of inflammatory mediators. Interestingly, after pulmonary infection in germfree mice injected with LPS, there was no increase in IL-10 production. Thus, akin to the model of reperfusion injury, a previous treatment with LPS is capable of restoring inflammatory responsiveness. In the context of infection, adequate mounting of an inflammatory response characterized by chemokine and TNF-α production and neutrophil accumulation is sufficient and necessary to control bacterial proliferation and spread. As the infection is controlled in animals given LPS, lethality is greatly delayed and prevented. Therefore, transient (by LPS) or continuous (by microbiota reposition) restoration of inflammatory responsiveness in germfree mice successfully enables the ability of these mice to deal with an infectious insult.

In conclusion, our studies demonstrate that the inability of germfree mice to inflame in response to sterile or infectious stimuli is largely due to the innate capacity of these mice to produce IL-10. The IL-10 produced switches off proinflammatory cytokine production, inflammatory cell influx, and consequent tissue injury and lethality. This IL-10–dependent hyporesponsive state is deleterious for the animal during bacterial infection and can be transiently reversed by systemic injection of LPS, a TLR-4 agonist, or permanently by reposition of the microbiota. In both cases, gain of inflammatory responsiveness is accompanied by effective handling of an infectious insult (*K. pneumoniae* infection). Therefore, altogether these results clearly suggest that prolonged contact with the indigenous microbiota is greatly relevant for the host. In contrast, experiments in animals subjected to intestinal reperfusion injury suggest that the downside of being able to inflame is excessive and systemic inflammation that may cause the death of the host, when severe. Finally, the detailed understanding of the molecular interactions underlying innate IL-10 production seen in the host, when severe. Finally, the detailed understanding of the molecular interactions underlying inflammatory pain seen in the host, when severe. Finally, the detailed understanding of the molecular interactions underlying inflammatory pain seen in the host, when severe. Finally, the detailed understanding of the molecular interactions underlying inflammatory pain seen in the host, when severe. Finally, the detailed understanding of the molecular interactions underlying inflammatory pain seen in the host, when severe. Finally, the detailed understanding of the molecular interactions underlying inflammatory pain seen in the host, when severe. 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