A leukotriene-dependent spleen-liver axis drives TNF production in systemic inflammation

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Production of the proinflammatory cytokine tumor necrosis factor (TNF) must be precisely regulated for effective host immunity without the induction of collateral tissue damage. Here, we showed that TNF production was driven by a spleen-liver axis in a rat model of systemic inflammation induced by bacterial lipopolysaccharide (LPS). Analysis of cytokine expression and secretion in combination with splenectomy and hepatectomy revealed that the spleen generated not only TNF but also factors that enhanced TNF production by the liver, the latter of which accounted for nearly half of the TNF secreted into the circulation. Using mass spectrometry–based lipidomics, we identified leukotriene B4 (LTB4) as a candidate blood-borne messenger in this spleen-liver axis. LTB4 was essential for spleen-liver communication in vivo, as well as for humoral signaling between splenic macrophages and Kupffer cells in vitro. LPS stimulated the splenic macrophages to secrete LTB4, which primed Kupffer cells to secrete more TNF in response to LPS in a manner dependent on LTB4 receptors. These findings provide a framework to understand how systemic inflammation can be regulated at the level of interorgan communication.

INTRODUCTION

Sepsis and other systemic inflammatory syndromes are leading causes of mortality and morbidity worldwide (1). With more than 100 failed attempts to use anti-inflammatory drugs as adjunct therapy in sepsis, it is becoming clear that the immune response is a double-edged sword in these syndromes (2–4). On the one hand, it seems that an immune response aimed at pathogen elimination or tissue repair is fundamental for survival even when antibiotics are being used. On the other hand, an excessively strong immune response can promote life-threatening collateral damage. Hence, it is important to comprehend the mechanisms that regulate the strength of systemic inflammation.

At center stage in this regulatory network is tumor necrosis factor (TNF), an early, pleiotropic proinflammatory cytokine that must be precisely regulated for a mammalian host to mount effective antimicrobial defense with minimal or tolerable collateral damage (5, 6). Expectedly, TNF has been the most studied cytokine in experimental models of systemic inflammation. However, there are still fundamental questions about its biology that remain unanswered. One such question is whether there is one dominant site of TNF production and, if so, which site that is. Another fundamental question is whether there is multilateral communication across different sites of TNF production.

More often than not, the spleen is regarded as the main source of TNF in systemic inflammation, as highlighted by its appearance in the core of contemporary theories of TNF regulation, such as immunomodulation through autonomic nerves (7, 8). But a critical analysis of the literature reveals that the link between spleen and TNF production is far from firmly established. The chief piece of evidence linking spleen and TNF is that splenectomy drastically impairs the rise of TNF in the plasma of animals with systemic inflammation induced by bacterial lipopolysaccharide (LPS) or cecal perforation (9–11). This fact, however, does not necessarily imply that the spleen is the main source of circulating TNF, because it does not rule out the possibility that splenectomy could render other TNF-producing organs less responsive to inflammatory stimuli. In this scenario, it ought to be considered that, during the course of LPS-induced systemic inflammation, substantial expression of the Tnf gene has been reported not only in the spleen (9, 12) but also in the liver and lungs (13, 14). The interplay among these TNF-producing sites warrants investigation.

In the present study, we assessed the relative contributions of spleen and liver for TNF production in a rat model of LPS-induced systemic inflammation, as well as whether or not there is interdependency between these organs in the context of TNF production. On the basis of partial hepatectomy experiments, we estimated that the liver accounts for nearly 50% of the surge in plasma TNF in this model. These experiments also revealed that TNF production by the spleen occurs independently of the liver. On the other hand, experiments involving splenectomy and cultures of resident macrophages revealed that TNF production in the liver depends on humoral signals originating in the spleen, thus uncovering a mechanism for the regulation of TNF at the level of interorgan communication. In this mechanism, the spleen and its resident macrophages produce humoral, TNF-enhancing signals that are conveyed to the liver and its resident macrophages. With the aid of mass spectrometry–based lipidomics, we identified leukotriene B4 (LTB4) as a key factor in this spleen-liver axis that drives TNF production.

RESULTS

Gene expression profiling suggests that the spleen is not the main source of early cytokines in systemic inflammation

LPS from Escherichia coli O55:B5 or vehicle (saline) was injected intravenously into unanesthetized male Wistar rats. At 30 or 80 min

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after injection, samples of spleen, liver, and lungs were harvested for analysis of cytokine gene expression focused on the genes encoding the proinflammatory cytokines TNF and interleukin-1β (IL-1β) and the anti-inflammatory cytokine IL-10. Because timing is important when evaluating cytokine responses, the fact that we evaluated three cytokines concurrently at an early time window deserves comment. This was feasible because time-course studies have shown that, after a bolus injection of LPS, the surge of IL-1β in the plasma is almost as early as the surge of TNF, although the IL-1β response may last longer and is usually less pronounced than the TNF response in this model (15, 16). Regarding IL-10, its response in the LPS model is biphasic: The first peak is matched in time to the early rises in TNF and IL-1β, presumably serving as a negative regulator of the early inflammatory response; the second peak takes place about 30 hours after LPS administration, is more pronounced, and plays a key role in the resolution of inflammation (16). The latter peak in IL-10 was out of the scope of the present study.

Gene expression was evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR) (table S1). The accuracy of the PCR was verified by the yield of a single amplion with the expected size for each target gene (fig. S1). In the saline-injected rats, the lungs had the highest TNf expression in comparison with the spleen and liver, but LPS enhanced the expression of this cytokine to a lesser extent in the lungs compared with the other organs (Fig. 1A). As a result, TNf expression was similar across all organs evaluated at 30 min after LPS. At 80 min after LPS, there continued to be no difference in TNf expression between liver and spleen, and although the expression of TNf in the lungs was again higher than in the liver, this interorgan difference was not as pronounced as that observed in the absence of LPS (Fig. 1A). Regarding the other cytokines we evaluated, Il1b and Il10 expression in the liver and lungs were greater than in the spleen in at least one of the time points after LPS (Fig. 1A). This was the case, although there was no interorgan difference in the expression of these cytokines in the saline-injected controls (Fig. 1A).

Because an increase in gene expression is a key step for the production and secretion of cytokines, even in the case of the inflammasome-dependent secretion of IL-1β (17), these results favor the notion that the spleen is unlikely to stand alone as the main producer of early cytokines in LPS-induced systemic inflammation. The liver and lungs ought to be taken into consideration as sources for inflammation-associated cytokines. When conjecturing about the relative contributions of these organs, it should be taken into account that they greatly differ in size and mass. In the rats used in the present study, the liver and lungs outweighed the spleen by 16.6 ± 2.1-fold and 2.4 ± 0.2-fold, respectively. To account for this factor, we calculated what we refer to as the expression-mass index (EMI): the product of relative gene expression and relative organ mass, expressed as a logarithm to the base 10. The resulting EMIs placed the liver well above the spleen and lungs in the dimensions of all three cytokines measured at 30 min after LPS (Fig. 1B and movie S1). The lung caught up with the liver at 80 min after LPS, but the spleen maintained lower cytokine EMIs (Fig. 1C and movie S2).

**Resident macrophages from the spleen produce less TNF than those from the liver or lungs**

In search of a cellular correlate of the organ-level analysis described above, we compared cytokine expression and secretion in cultures of resident macrophages freshly isolated from spleen, liver, or lungs. The purity of macrophages in these cultures was higher than 95%, as determined by flow cytometry analysis of adherent cells stained for CD68, a well-validated macrophage marker in the rat (fig. S2) (18). Stimulation with LPS resulted in a statistically significant increase in Tnf expression in all macrophage subpopulations studied, but the expression of Tnf relative to Actb (β-actin) was the highest in the liver macrophages (Kupffer cells), in both the presence and absence of LPS (Fig. 2A). The lung (alveolar) macrophages were second to Kupffer cells in terms of Tnf expression (Fig. 2A). Tnf expression in splenic macrophages was at least 250-fold lower than that in Kupffer cells and at least 50-fold lower than that in alveolar macrophages (Fig. 2A).

To verify whether these differences in gene expression correlated with differences in TNF secretion, cytokine concentrations were measured in the medium of the macrophage cultures. This was an important verification, because although an increase in gene expression is a limiting step for TNF production, its secretion also relies on cleavage of membrane-bound TNF by a converting enzyme that typically requires activation of Toll-like receptor 4 (TLR4) by LPS for its production (19).

To achieve detectable cytokine concentrations in all cultures, the number of splenic macrophages in a culture had to be greater than the numbers of liver and lung macrophages in their respective cultures (fig. S3). For that reason, cytokine concentrations were normalized to the number of cells in culture to yield an index of cytokine secretion that could be compared across the macrophages from different tissues. The secretion index for TNF was significantly higher in the LPS-stimulated Kupffer cells and alveolar macrophages than it was in splenic macrophages (Fig. 2B). There was also a difference in TNF secretion between Kupffer cells and alveolar macrophages, but this difference was not as straightforward as the differences observed in relation to splenic macrophages. More specifically, although alveolar macrophages secreted more TNF than did Kupffer cells in response to LPS stimulation, their baseline secretion (saline group) was similarly higher (Fig. 2B). Consequently, the LPS-induced response (assessed as the LPS-saline group difference) was virtually identical in these two groups of macrophages (Fig. 2C). The reason for the relatively high secretion of TNF by unstimulated alveolar macrophages is presently unknown.

With respect to IL-1β, we observed that its transcript was more abundant in Kupffer cells and alveolar macrophages than in splenic macrophages, and, as in the case of Tnf, this difference persisted after Il1b expression was increased by LPS (Fig. 2A). Despite that, however, LPS failed to promote the release of IL-1β by any of the macrophages investigated in culture (fig. 2, B and C), which presumably reflects insufficient inflammasome activation in the absence of other in vivo factors, such as damage-associated molecular patterns. Regarding IL-10, none of the macrophage subpopulations responded to LPS with changes in Il10 expression, and the corresponding protein was not detectable in culture supernatants (fig. S4).

**Partial hepatectomy attenuates the LPS-induced surge in plasma TNF without affecting Tnf expression in the spleen or lungs**

To directly assess the contribution of the liver to LPS-induced cytokine production, a partial hepatectomy was performed immediately before LPS stimulation. The median and the left lateral lobes of the liver, which account for ~70% of the liver mass (20), were removed, whereas the right lateral and the caudate lobes were retained along
with their vascular connections. To avoid pitfalls associated with the loss of vital liver functions, the experiment was conducted under anesthesia immediately after liver resection or sham operation.

The LPS-induced surge in plasma TNF (at 80 min) was reduced by 32% in the hepatectomized animals, as compared with the sham-operated controls (Fig. 3A). This reduction was not linked to the suppression of Tnf expression in the remaining LPS-processing organs: spleen and lungs (Fig. 3B). Hence, it appears that the hepatectomy-suppressed LPS-induced surge in plasma TNF cannot be solely attributed to the ability of the spleen to produce and secrete TNF. What is more, this finding uncovers a previously unrecognized role for the spleen as a regulator of TNF production at the level of interorgan communication, specifically by boosting Tnf expression in the liver.

Kupffer cells mount stronger TNF responses when primed with conditioned medium from splenic macrophages

A cell culture experiment was designed to test the hypothesis that splenic macrophages are capable of generating humoral, TNF-enhancing signals that act on Kupffer cells but not on alveolar macrophages. We cultured Kupffer cells or alveolar macrophages in splenic macrophage-conditioned medium (SMCM) or unconditioned medium, stimulated them with LPS or vehicle, and measured the amount of TNF secreted by them (Fig. 4C). The amount of TNF present in SMCM before its addition to the Kupffer cell or alveolar macrophage cultures, however small, was deducted from the amount present in these cultures after LPS stimulation.

Using this approach, we observed that SMCM exerted no effect on TNF secretion by Kupffer cells in the absence of LPS stimulation, but it nearly tripled the amount of TNF secreted by this macrophage subpopulation in response to LPS stimulation (Fig. 4D). Contrary to TNF secretion by Kupffer cells in the absence of LPS stimulation, but it nearly tripled the amount of TNF secreted by this macrophage subpopulation in response to LPS stimulation (Fig. 4D). Contrary
to the working hypothesis, though, SMCM was similarly effective in priming alveolar macrophages to secrete TNF in response to LPS (Fig. 4E). Therefore, the lack of an effect of splenectomy on the lung’s Tnf expression cannot be explained by alveolar macrophages being unresponsive to humoral signals from splenic macrophages. Thus, differences between the compartmentalization of Kupffer cells and alveolar macrophages in vivo pose as a more plausible explanation for the observation that splenectomy suppressed Tnf expression in the liver but not in the lungs. Unlike populations of resident macrophages in other organs and tissues, Kupffer cells reside on the lumenal side of blood vessels (21) and are therefore more accessible to blood-borne factors derived from the spleen than are macrophages that reside in the parenchyma of other organs.

**LTB₄ is a spleen-derived, blood-borne factor in LPS-induced systemic inflammation**

We hypothesized that spleen-liver communication involves a lipid-derived messenger because a preliminary investigation on the identity of a spleen-derived factor that modulates the liver uptake of LPS suggests that this factor is both lipophilic and heat resistant (22). Therefore, we used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to conduct a targeted lipidomics and metabolomics analysis (23), screening for spleen-derived messengers among...
Samples were collected at 80 min after LPS injection to measure TNF concentration in rats that underwent total splenectomy or sham operation. Liver and Kupffer cells were stimulated with LPS in the presence of splenic macrophage-conditioned medium (SMCM). The data are shown as median values ± SEM. *P < 0.05 compared with sham-operated rats (Mann-Whitney test).

**Fig. 4.** The spleen and splenic macrophages influence the TNF responses of liver and Kupffer cells to LPS. (A) Effects of intravenous LPS on the circulating concentration of TNF in rats that underwent total splenectomy or sham operation. (B) Effects of splenectomy or sham operation on the relative expression of Tnf in samples of liver and lungs collected at 80 min after LPS injection. Relative gene expression was calculated using Actb as the reference. Data are shown as means ± SEM. **(C)** Schematic representation of the two-step protocol designed to study the effects of splenic macrophage-conditioned medium (SMCM) on Kupffer cells (KC) and alveolar macrophages (AM). (D) and (E) Effects of SMCM on the secretion of TNF by KC (D) and AM (E). Data are shown as means ± SEM. **a**, statistically different from the group exposed to neither LPS nor SMCM; **b**, statistically different from the group stimulated with LPS in the absence of SMCM (ANOVA followed by the Fisher test). n = 7 to 12 (D) and 6 to 11 (E) cultures; cells from a single rat typically yielded two to four cultures, randomly distributed across the groups.

LPS promotes LTB₄ secretion by splenic macrophages through a posttranscriptional mechanism

Splenectomy reduced plasma LTB₄ by 69% despite the fact that the amounts of LTB₄ were not diminished in parenchymal extracts of liver (Fig. 5B) or lungs (Fig. 5C), which were also assessed by LC-MS/MS. On the contrary, the content of LTB₄ in the liver parenchyma was actually increased by splenectomy (Fig. 5B). Because the parenchymal extracts were prepared with livers that had been cleared of blood by transcardial perfusion, the lack of correspondence between LTB₄ concentrations in blood versus liver indicated that the liver itself contributed little or nothing to blood-borne LTB₄, an inference that finds additional support in the fact that hepatectomy had no impact on the LPS-induced surge in plasma LTB₄ (Fig. 5D). In other words, even if the liver produced more LTB₄ to compensate for the loss of spleen-derived LTB₄, this increase was not sufficient to prevent plasma LTB₄ from falling.

These observations point to the spleen as an irreplaceable source of blood-borne LTB₄ during early systemic inflammation. Given the fact that the blood output from the spleen flows directly to the liver through the portal vein, it can be presumed that this blood-borne form of LTB₄ would be highly bioavailable to the liver, particularly to the Kupffer cells that are attached to the lumen of the liver sinusoidal vessels (21).

**Fig. 5.** LTB₄ concentrations in blood versus liver indicated that the liver itself contributed little or nothing to blood-borne LTB₄. (A) Plasma LTB₄ concentrations in nontreated rats 80 min after the injection of LPS. In sham-operated rats, LTB₄ concentrations were significantly higher than those in saline-injected control animals, but the majority of them (11 of 13) did not yield two to four cultures, randomly distributed across the groups.
effects of splenectomy or sham operation on the concentrations of LTB₄ in parenchymal extracts of liver (B) or plasma of the LPS-challenged rats (fig. S5). At doses ranging from 0.1 to 5 mg/kg, pretreatment with MK-886 dose-dependently reduced the concentration of LTB₄ at 80 min after LPS by 42%, as compared with the vehicle-pretreated group (Fig. 6A). This was sufficient to attenuate the surge in plasma TNF by 32% (Fig. 6B). The effect of MK-886 on plasma TNF was associated with a 54% reduction in liver Tnf expression, whereas there was no change in Tnf expression in the spleen or lungs (Fig. 6C). These findings are consistent with the liver being the target of the TNF-enhancing effect of LTB₄.

To evaluate whether MK-886 could be exerting these effects by acting on the spleen, the same experiment was repeated in rats that had undergone splenectomy. Consistent with the hypothesis that spleen-derived LTB₄ enhanced circulating TNF by increasing Tnf expression in the liver, splenectomy abolished the effects of MK-886 on plasma LTB₄ (Fig. 6D), as well as the associated effects on plasma TNF (Fig. 6E) and on the liver’s Tnf expression (Fig. 6F).

LTB₄ mediates humoral communication between splenic and hepatic macrophages during the response to LPS
To evaluate whether LTB₄ could account for the priming of cultured Kupffer cells by SMCM, we tested whether treating splenic macrophages with the FLAP inhibitor MK-886 affected the capacity of SMCM to enhance the response of Kupffer cells to LPS. Compared with the vehicle-treated group, MK-886 significantly reduced the concentration of LTB₄ in SMCM and rendered SMCM less effective at priming Kupffer cells to secrete TNF in response to LPS (Fig. 7A).

We also assessed whether and how the TNF response of Kupffer cells was affected by the presence of either of two LTB₄ receptor antagonists: LY293111 (26) and LY255283 (27), which were individually added to the Kupffer cell culture. The addition of either antagonist completely blocked the enhancing effect of SMCM on TNF secretion by LPS-stimulated Kupffer cells without interfering with TNF secretion by Kupffer cells in unconditioned medium (Fig. 7B).

We then evaluated the effects of adding LTB₄ directly to the Kupffer cell cultures at the beginning of the LPS stimulation. LTB₄ inhibited the LPS-induced surge in plasma TNF in intact (nonsplenectomized) rats (Fig. S6). The highest dose, however, interfered with the biosynthesis of several PGs in addition to inhibiting the biosynthesis of LTB₄ (fig. S7). Therefore, an intermediate dose (1 mg/kg) with confirmed selectivity toward LTB₄ (fig. S7) was chosen for subsequent experiments. At this dose, MK-886 reduced the plasma concentration of LTB₄ at 80 min after LPS by 42%, as compared with the vehicle-pretreated group (Fig. 6A). This was sufficient to attenuate the surge in plasma TNF by 32% (Fig. 6B). The effect of MK-886 on plasma TNF was associated with a 54% reduction in liver Tnf expression, whereas there was no change in Tnf expression in the spleen or lungs (Fig. 6C). These findings are consistent with the liver being the target of the TNF-enhancing effect of LTB₄.

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be adequate to reveal established biological effects of LTB₄, such as neutrophil chemotaxis (28). LTB₄ dose-dependently enhanced LPS-induced TNF secretion by the Kupffer cells, an effect that was strongly \((r^2 = 0.993)\) and significantly \((P < 0.01)\) fitted to an exponential growth function over the entire dose range (Fig. 7C). The effect had not yet reached a saturation maximum at the highest dose tested.

In contrast to its pronounced effect on Kupffer cells, though, not even the highest dose of LTB₄ exerted any effect on LPS-induced TNF secretion by the splenic macrophages (Fig. 7D). In agreement, the expression of the gene encoding the best known LTB₄ receptor, Ltb4r1 (also known as Blt1), was ~35-fold lower in splenic macrophages than in Kupffer cells, in both the presence and absence of LPS (Fig. 7E). LPS itself exerted no effect on Ltb4r1 expression (Fig. 7E). The transcript of the other known LTB₄ receptor, Ltb4r2 (also called Blt2), was not reliably detected in any of the macrophage subpopulations investigated.

**DISCUSSION**

Being a determinant of outcome in infectious and noninfectious systemic inflammatory disorders, the early proinflammatory cytokine TNF has been attracting the attention of scientists and clinicians for more than 30 years. Although it is well established that macrophages are the main source of TNF in systemic inflammation (13, 29, 30), the relative contributions of macrophages residing in different organs remain an unsettled matter. The prevailing view about the spleen being the main source of TNF in systemic inflammation is contested by a few studies showing that, like splenectomy (9, 11), hepatectomy can be effective at reducing the LPS-induced surge in plasma TNF (31–33). A caveat of these studies, however, is that they have not addressed whether the effects of hepatectomy or splenectomy on plasma TNF might have been indirectly influenced by altered TNF production in one or more of the nonexcised organs. This aspect was accounted for in the present study.

In the case of hepatectomy, we found that it reduced the LPS-induced rise in plasma TNF without affecting Tnf expression in the spleen or lungs. The hepatectomy was partial, with the two lobes removed accounting for ~70% of liver mass. This was sufficient to reduce plasma TNF by 32%, thus leading to the estimate that the whole liver accounts for ~46% of the TNF secreted into the circulation. Consistent with such a role, the liver was unmatched by the spleen when the relative expression of Tnf was considered relative to organ mass. The liver’s EMI for Tnf also surpassed that of the lungs at 30 min after LPS, although this difference was no longer evident at 80 min after LPS. This in vivo evidence was further strengthened by the finding that the resident macrophages of the liver, Kupffer cells, secreted more TNF in response to LPS than did their splenic counterparts, being similar to alveolar macrophages in this regard. Together, these findings shed new light on the importance of the liver and its resident macrophages for TNF production in systemic inflammation.

In contrast to hepatectomy, splenectomy reduced plasma TNF while suppressing Tnf expression in a distant organ, more specifically, the liver. Therefore, the effect of splenectomy on plasma TNF cannot be solely attributed to the TNF-producing capacity of the spleen and its macrophages. This finding uncovered a regulatory mechanism for TNF production that extends beyond organ borders, operating at the level of interorgan communication. In vivo and ex vivo experiments further revealed that LTB₄, released by the spleen and its macrophages, which are the main source of TNF in systemic inflammation (13–15), can enhance the secretion of some proinflammatory cytokines in cultured murine macrophages, including those that infiltrate the peritoneum in response to thioglycolate (34) and those that are differentiated in vitro from bone marrow precursors (35). It should be pointed out, however, that these commonly used murine macrophage preparations do not necessarily behave as resident macrophages. In the present study, the use of a larger animal (the rat) permitted harvesting of actual resident macrophages in sufficient numbers for cell cultures that produced detectable amounts of cytokines. This approach revealed differences in the responsiveness of macrophage subpopulations to LTB₄, with Kupffer cells, but not splenic macrophages, responding to it with enhanced TNF secretion. The fact that inhibition of LT biosynthesis with MK-886 reduced Tnf expression in the liver, but not in the spleen, provides additional support for this latter observation.

This role of LTB₄ in spleen-liver communication suggests that, in addition to signaling autocrinally and paracrinally, LTB₄ may function as an endocrine messenger. A similar paradigm has been described for prostaglandin E₂ (PGE₂), a lipid-derived mediator that...
acts locally in tissues, but that is also capable of conveying inflammatory signals from the periphery to the brain in its blood-borne, albumin-bound form (36, 37). Like PGE₂, LTB₄ binds to albumin with high affinity (38) and travels in the blood at biologically relevant concentrations (39). Not only does binding to albumin protect LTB₄ from degradation, but it has also been shown to be reversible (40), thus providing a platform for concentration-dependent delivery, dissemination, and uptake. Another piece of the puzzle that cannot be ignored is that because the splenic vein drains directly into the portal vein, humoral signals from the spleen reach the liver before—and presumably at higher concentrations than—any other organ, where they encounter Kupffer cells residing in the lumen of blood vessels (21). To our knowledge, no other subpopulation of resident macrophages in the body has this unique intravascular location. Perhaps for this reason, the induction of Tnf in the liver was associated with simplification of what happens in actual infections. Hence, future studies are warranted to confirm the relevance of the spleen-liver axis described herein in models of infections and in a clinical setting. One clinical aspect that deserves attention is the vulnerability to infections and lethal sepsis that afflicts splenectomized patients (46, 47). Theories to explain this vulnerability have revolved around the potential importance of the spleen as a phagocytic filter (48) or as a site for the activation and proliferation of antibody-secreting B cells (49). Nevertheless, it is also plausible to propose that splenectomy impairs immunity at a more systemic level, perhaps by affecting interorgan communication through LTB₄ and TNF. Understanding the role of spleen-liver communication in immunity may also lead to new therapeutic approaches for the relatively large number of infectious diseases that are associated with hepatosplenomegaly, such as viral hepatitis, mononucleosis, toxoplasmosis, visceral leishmaniasis, blood-borne LTB₄ rather than with parenchymal LTB₄ in our experiments. The unique location of the Kupffer cells may also underscore the fact that spleen-derived LTB₄ modulated TNF production in the liver but not in the lungs.

Of the two LTB₄ receptor antagonists used in this study, LY293111 is regarded as a selective antagonist of LTB₄R1 (41, 42), whereas LY255283 is considered a selective antagonist of LTB₄R2 (27). Therefore, the fact that either antagonist was capable of blocking the TNF-enhancing effect of LTB₄ on Kupffer cells can be suggestive of a joint involvement of these receptors. Accordingly, there is evidence that other LTB₄-dependent effects such as chemotaxis can be blocked by either LTB₄R1 or LTB₄R2 antagonism (43–45). However, interdependency between LTB₄R1 and LTB₄R2 receptors must be met with a critical eye. One point to consider is that we are unaware of any study that has definitively proven the selectivity of LY293111 toward LTB₄R1 receptors. Regarding LY255283, although its selectivity toward LTB₄R2 has been demonstrated in Chinese hamster ovary cells (27), this selectivity has yet to be confirmed in other studies, and there is no guarantee that the same degree of selectivity is applicable to rat Kupffer cells. In this setting, although our data support a receptor-mediated action of LTB₄ on the TNF response of Kupffer cells, the receptor subtype involved remains to be clarified. It should be appreciated, though, that only the transcript of the LTB₄R1 could be detected in the macrophage populations used in this study.

As a final consideration, it is important to recognize that although the LPS model of systemic inflammation is useful for mechanistic studies, it is an oversimplification of what happens in actual infections. Hence, future studies are warranted to confirm the relevance of the spleen-liver axis described herein in models of infections and in a clinical setting. One clinical aspect that deserves attention is the vulnerability to infections and lethal sepsis that afflicts splenectomized patients (46, 47). Theories to explain this vulnerability have revolved around the potential importance of the spleen as a phagocytic filter (48) or as a site for the activation and proliferation of antibody-secreting B cells (49). Nevertheless, it is also plausible to propose that splenectomy impairs immunity at a more systemic level, perhaps by affecting interorgan communication through LTB₄ and TNF. Understanding the role of spleen-liver communication in immunity may also lead to new therapeutic approaches for the relatively large number of infectious diseases that are associated with hepatosplenomegaly, such as viral hepatitis, mononucleosis, toxoplasmosis, visceral leishmaniasis,
and even bacterial sepsis. Last, but not least, it should be considered that the clinical outreach of an immunomodulatory spleen-liver axis is likely to extend beyond infectious diseases. The spleen has been shown to play a major role in the progression of liver cirrhosis by releasing vasoactive substances (50) and profibrotic mediators (51). The question then arises as to whether and how LTB4 and TNF may interface with these mechanisms of spleen-liver communication in the context of liver cirrhosis and other chronic inflammatory disorders.

In conclusion, we provide a mechanistic framework to understand how the strength of acute systemic inflammation can be regulated at the level of interorgan communication. In this framework, the spleen does not stand alone as a relevant site of TNF production, with the liver accounting for nearly 50% of the TNF released into the circulation of LPS-challenged rats. What is more, the spleen and its macrophages disseminate TNF-enhancing signals to the liver and its Kupffer cells. LTB4 acts as a key messenger in this humoral axis, with its secretion from splenic macrophages being triggered by LPS through posttranscriptional mechanisms, and then priming, in a receptor-dependent manner, Kupffer cells to secrete more TNF in response to LPS. The direct flow of blood from the spleen to the liver through the portal vein is probably important for this mechanism, as is the intravascular (luminal) location of Kupffer cells.

MATERIALS AND METHODS

Animals
The study was conducted in male Wistar rats originated from the specific pathogen–free animal facility of the University of Sao Paulo. The rats were caged in groups of three or four and had free access to standard chow and water. With the goal of providing a thermally comfortable environment for the grouped rats (52), the ambient temperature was maintained at 24° to 27°C. The animal room was on a 12:12-hour light-dark cycle, with lights on at 7:00 a.m. The experiments were conducted when the rats weighed 250 to 400 g. All protocols were approved by the Animal Care and Use Committee at the Institute of Biomedical Sciences of the University of Sao Paulo.

Intraoperative and perioperative care
One or more of the following surgical procedures was performed: implantation of an intravenous (jugular) catheter, partial hepatectomy, and total splenectomy. The rats were allowed to recover from surgery for 1 week before an experiment, except for the rats subjected to hepatectomy, which were studied under anesthesia shortly after the procedure. The surgical procedures were performed aseptically under anesthesia with isoflurane (1.5 to 2.5% in 50% O2). Body temperature (monitored by a thermocouple inserted into the colon, 8 to 10 cm from the anal sphincter) was maintained between 36.5° and 37.0°C with the help of an isothermal pad. When adequate (survival surgeries for experimentation 1 week later), the rats were provided with antibiotic prophylaxis (enrofloxacin, 5 mg/kg, subcutaneously) before surgery and initiated on a 2-day pain management protocol (ketoprofen, 5 mg/kg, subcutaneously, one to two doses per day) at the end of surgery.

Surgical procedures
For the intravenous catheterization, the left external jugular was isolated and ligated. A three-French polyurethane catheter was inserted into the vein caudally to the ligation site and then advanced until its tip reached the superior vena cava. The distal end of the catheter was passed under the skin and exteriorized at the nape, after which the catheter was locked with heparinized glycerol (500 U/ml).

Partial hepatectomy was performed as detailed by Waynforth and Flecknell (20). In brief, after a midline laparotomy, a rat was placed in an arched-up supine position with the help of a bolster. This position facilitated mobilization of the liver. The ligaments of the median and left lateral lobes were cut as close as possible to the diaphragm or blood vessels. Then, the two lobes were tied together at their base with surgical thread, making sure the blood vessels serving them were ligated in the process. The isolated lobes were then excised.

In preparation for splenectomy, a rat was positioned laterally, with its left side facing up. The spleen was accessed via a dorsoventral incision made near the costal margin of the thorax. The organ was pulled through the incision, and the splenic vessels were ligated before organ excision.

Control rats for hepatectomy or splenectomy were subjected to sham operation, which consisted of accessing the organs but not executing ligation or excisions. All surgical wounds were closed in layers with suture.

In vivo experimentation
All in vivo experiments, with the exception of those involving hepatectomy, were conducted in unanesthetized, freely moving rats. On the day of the experiment, each rat was housed and individually caged in an environmental chamber (Environmental Growth Chambers) set to an ambient temperature of 22.0°C. Although this ambient temperature is lower than that preferred by healthy rats, it is preferred by rats challenged with relatively high doses of LPS (53, 54). Each of the freely moving rats was equipped with an infusion harness, which protected a PE-50 extension of the preimplanted intravenous catheter. LPS (1 mg/kg) or its vehicle (saline) was administered via the intravenous catheter extension without disturbing the rat.

When an experiment was conducted under anesthesia (hepatectomy experiment), ventilator support was provided (12 ml per breath per kilogram, 65 to 75 breaths per minute) in addition to the intraoperative care described in the previous section. LPS (1 mg/kg) or saline was administered via the intravenous catheter after a stabilization period of 20 min, during which no surgical manipulation was performed.

Tissue harvesting
At the time points of interest for tissue harvesting, the rats, if unanesthetized, were promptly anesthetized with thiopental (10 mg), administered via the preimplanted venous catheter. Immediately thereafter, the thoracic cavity was opened, and blood was collected from the inferior vena cava into heparinized tubes. The rats were then perfused for 5 min with cold phosphate-buffered saline (PBS) transcardially, and tissue samples were collected and snap-frozen in liquid nitrogen. When a sample was designated for LC-MS/MS–based lipidomics, indomethacin (50 μM) and butylated hydroxytoluene (BHT) (0.005%) were added to the perfusate and to the blood samples.

Spleenic macrophage culture
Under isoflurane anesthesia, rats were perfused transcardially with cold PBS, after which the whole spleen was collected and dissociated in cold, phenol red–free Hanks’ balanced salt solution (HBSS). After centrifugation of the cell extract (600g, 10 min, 4°C), the pellet was
resuspended and incubated with ammonium-chloride-potassium (ACK) lysis buffer (Thermo Fisher Scientific) for 3 min at room temperature. Next, the cell extract was subjected to purification in a four-phase Percoll (Sigma-Aldrich) gradient: 0, 30, 40, and 50% in HBSS. The macrophase-enriched fraction was collected at the interface of the 30 and 40% phases after centrifugation at 400g for 20 min at 4°C. The cells were subjected to two rounds of washing with HBSS and then resuspended in AIM V medium (Thermo Fisher Scientific). Cells in this medium were cultured (37°C, 5% CO2) in six-well, flat-bottom polystyrene plates; 1.0 × 10⁷ live cells were loaded into each well in a final volume of 2 ml. Nonadherent cells were discarded after overnight incubation.

Kupffer cell culture
Under isoflurane anesthesia, the liver was perfused through the portal vein with PBS at 37°C; the use of warm PBS in this step avoids detachment of Kupffer cells from the inner walls of the liver sinusoids. The perfused liver was digested at 37°C for 15 min in type IV collagenase (1 mg/ml) supplemented with fetal bovine serum (5%) and deoxyribonuclease (DNase) (0.05%). The resulting homogenate was passed through a 100-μm mesh cell strainer with the help of cold HBSS supplemented with fetal bovine serum and DNase. The cell extract was subsequently washed three times with the same solution. Next, the cell extract was subjected to purification in a two-phase gradient of HBSS and Ficoll-Paque PLUS (GE Healthcare), with centrifugation at 400g (20 min, 4°C). The macrophase-enriched fraction was collected at the interface of the two phases. After two rounds of washing with HBSS, the cells were suspended in AIM V medium. Cells in this medium were cultured (37°C, 5% CO2) in 12-well, flat-bottom polystyrene plates; 2.0 × 10⁶ live cells were loaded into each well in a final volume of 1 ml. Nonadherent cells were discarded after a 2-hour incubation.

Alveolar macrophage culture
The lungs along with the airways and trachea were excised from rats anesthetized with isoflurane. Bronchoalveolar lavage was performed with cold PBS, and the harvested cells were pelleted by centrifugation (600g, 10 min, 4°C). The pellet was resuspended and incubated with ACK lysis buffer for 3 min at room temperature. Next, the cells were pelleted again and then resuspended in AIM V medium. Cells in this medium were cultured (37°C, 5% CO2) in 12-well, flat-bottom polystyrene plates; 1.0 × 10⁶ live cells were loaded into each well in a final volume of 1 ml. Nonadherent cells were discarded after 2 hours of incubation.

Flow cytometry
A 10-min incubation with ice-cold PBS was used to detach splenic, hepatic, and alveolar cells from Cultureware. At the end of this period, cells were scrapped out of the wells with the help of a syringe barrel and transferred to cytometry tubes (5 × 10⁵ cells per tube). The extracted cells were sequentially blocked with Stain Buffer (BD Biosciences) (30 min, 4°C) and permeabilized with Phosflow Perm Buffer III (BD Biosciences) (20 min, 4°C, in the dark). Immediately thereafter, the cells were stained for 30 min (4°C, in the dark) with a CD68 monoclonal antibody conjugated with phycoerythrin (Novus Biologicals) at a 1:100 dilution. The cells were then washed with cold PBS and fixed with Cytofix buffer (BD Biosciences). Stained and unstained cells were analyzed by flow cytometry in a FACS-Canto II instrument (BD Biosciences) and FlowJo software.

Quantitative RT-PCR
A liquid-liquid sequential extraction protocol was used to isolate total RNA from organ samples. The protocol consisted of tissue homogenization in TRIzol (Invitrogen), phase separation after addition of chloroform, precipitation of RNA from the upper phase with isopropanol, double washing with ethanol, evaporation of ethanol, and dissolution of RNA in water. In the case of cultured macrophages, RNA was extracted and purified by the RNeasy method (Qiagen). The purity of the isolated RNA was confirmed in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) using as quality criteria an A₂₆₀/A₂₈₀ ratio of 1.8 or higher.

The isolated RNA was reverse-transcribed to complementary DNA (cDNA) using Oligo(dT)15 primer and Moloney murine leukemia virus reverse transcriptase (both from Promega), according to the manufacturer’s instructions. In the next step, amplification of the cDNAs of interest was monitored by quantitative real-time PCR in a Stratagene MX3005P system (Agilent). The reactions were conducted in SYBR Green PCR Master Mix (Thermo Fisher Scientific), with the primers shown in table S1. The annealing temperature was 55°C. Quality control of the PCRs was achieved by dissociation curve analysis in combination with agarose gel electrophoresis of the amplicons obtained.

LC-MS/MS–based lipidomics
Plasma was obtained by centrifugation (4,500g, 5 min, 4°C) of the heparinized blood samples. Organ extracts were prepared by (i) homogenization in ice-cold methanol containing indomethacin and BHT, (ii) removal of insoluble remains by centrifugation (4500g, 4°C, 10 min), (iii) evaporation of methanol under a stream of nitrogen at room temperature, and (iv) dissolution of the residue in water.

After the internal standard (PGE₁) was added to plasma, organ extracts, or culture medium, the samples were acidified with 0.1% formic acid. The samples were then subjected to solid-phase extraction in an Oasis mixed-mode anion exchange μElution plate (Waters) in three sequential steps: (i) The plate cartridge was preconditioned with methanol and then with water; (ii) samples were loaded into the cartridge, and unwanted compounds were eluted in water containing 5% of methanol; and (iii) the compounds of interest were eluted in methanol containing 0.1% of formic acid. The prepared samples were then passed through an Acquity UPLC system equipped with a BEH C18 column (1.7-μm particles, 2.1 mm by 50 mm), which was coupled to an Acquity TQD MS/MS detector. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both with 0.1% formic acid, and it was pumped at a flow rate of 0.4 ml/min. The gradient elution program was performed in four stages: (i) solvent B at 35% for 6 min, (ii) solvent B at 90% for 1.5 min, (iii) solvent B at 99% for 2.5 min, and (iv) solvent B at 30% for 3 min. The injection mode was full loop, with injection volume of 10 μl. The MS tune parameters were as follows: 0.16 liter/hour for the flow of the collision gas (argon), 3.02 × 10⁻³ mbar for the pressure in the collision cell, and 1000 liters/hour for the flow of the desolvation gas (N₂). The source and desolvation temperatures were 150° and 400°C, respectively. A multiple reaction monitoring mode was used for the quantification of the analytes in negative ionization mode, according to the parameters shown in table S2. The standards were purchased from Cayman and analyzed in concentrations ranging from 0.1 to 50 ng/ml.

Enzyme-linked immunosorbent assay
The concentrations of TNF-α, IL-1β, and IL-10 in samples of plasma and culture media were quantified by sandwich ELISA, whereas the
concentrations of LTB₄ in culture media were quantified by competitive ELISA. All reagents were from R&D Systems. Detection ranges were 31 to 2000 pg/ml for the cytokines assayed and 13 to 500 pg/ml for LTB₄.

Drugs
LPS from *E. coli* O55:B5 (Sigma-Aldrich) was prepared as a suspension in saline. The suspension was sonicated in bath for 30 min immediately before use. In the whole animal experiments, LPS was administered intravenously at the dose of 1 mg/kg. In the cell culture experiments, LPS was used at the final concentration of 500 ng/ml in the medium.

MK-886 was injected intraperitoneally in its sodium salt form (Cayman Chemical), which was dissolved in a vehicle consisting of propylene glycol, ethanol, and water. The injection was performed in a volume of 1 ml/kg. For MK-866 doses of 0.1 or 1 mg/kg, the ratios of the solvents in the vehicle were 24, 16, and 60%, respectively. For the higher dose of 5 mg/kg, the ratios were 60, 40, and 0%. In the cell culture experiments, a free base of MK-886 (Cayman) was used at the dose of 10 μM. The free base was dissolved in dimethyl sulfoxide (DMSO) and added to the culture so that the end concentration of DMSO in the medium never exceeded 0.3%.

LY293111, LY255283, and LTB₄ (Cayman) were used in cell culture experiments at the following doses: 20 μM for LY293111, 20 μM for LY255283, and 0.8 to 500 nM for LTB₄. LY293111 and LY255283 were dissolved in DMSO, whereas LTB₄ was dissolved in ethanol. After their addition to the cultures, the end concentrations of the solvents in the medium did not exceed 0.3 and 0.1%, respectively.

Data processing and statistical analyses
Relative gene expression was estimated by the 2−ΔΔCt method, using Actb as the reference (housekeeping) gene. EMI was calculated as the logarithm (base 10) of the product of relative gene expression and Actb.

RESULTS AND NOTES

SUPPLEMENTARY MATERIALS
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Fig. S1. RT-PCR amplification products.
Fig. S2. The purity of resident macrophages in culture was higher than 95%.

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inflammatory injury is negatively associated with N. hepatitis.


Bases A. E. on liquid chromatography-mass spectrometry.


A leukotriene-dependent spleen-liver axis drives TNF production in systemic inflammation


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Inflamed from afar

The spleen is thought to be the major source of the proinflammatory cytokine TNF during systemic inflammation. However, Fonseca et al. found that the liver and lungs produced more TNF than did the spleen in response to LPS-induced systemic inflammation in rats. Experiments with splenectomized and hepatectomized animals and isolated tissue-resident macrophages showed that much of the spleen-dependent, LPS-induced increase in circulating TNF also depended on the production of TNF by Kupffer cells, the resident macrophages of the liver. Liver TNF production was enhanced in vivo and in vitro by leukotriene B4 (LTB4) released by the spleen. Together, these findings implicate LTB4 as a spleen-derived endocrine signal that promotes the hepatic production of TNF during systemic inflammation.