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Access to the Spleen Microenvironment through Lymph Shows Local Cytokine Production, Increased Cell Flux, and Altered Signaling of Immune Cells during Lipopolysaccharide-Induced Acute Inflammation

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The spleen is involved in fluid volume regulation, immune responses, and hematopoiesis. Yet, the composition of the fluid phase within the spleen microenvironment, the migratory routes of lymphocytes as well as the splenic response to bacterial endotoxin is incomplete. To address these issues, we isolated postnodal lymph in rats by cannulating an efferent lymphatic draining the spleen, and assessed the secretion of signaling substances during a septic response induced by LPS. Spleen lymph flow increased 8-fold after LPS exposure. The spleen exhibited a permeable microvasculature with low sieving of macromolecules that was absent after exposure to LPS. Furthermore, after LPS exposure the spleen contributed significantly to the production of pro- and anti-inflammatory cytokines, and experiments in splenectomized rats suggested it may induce a protracted inflammation because of a dominant role in IL-6 production. A significant amount of lymphocytes exited via lymphatics draining the spleen in control rats. LPS-induced inflammation resulted in increased T cell and reduced B cell subset fractions, and gave a significant increase in CD4+ and CD8+ subset T cell efflux and a reduced B cell efflux in spleen lymph. Exposure of leukocytes to the spleen microenvironment affected their signaling status, and by phosphorylation specific flow cytometry we could identify STAT3 and CREB as important mediators in the cellular signaling occurring during endotoxemia. We conclude that analysis of spleen lymph may unravel immune cell migration patterns and local signaling, and immune cells exit via lymph having acquired specific activation signatures after exposure to the spleen microenvironment. The Journal of Immunology, 2010, 184: 4547–4556.

The mammalian spleen plays important roles in fluid volume regulation as well as in modulation of immune responses and hematopoiesis. It has been shown that the spleen has a high blood flow, that it filters off cell-free fluid from the blood into the lymphatic system and that the rate of filtration is modulated by neuronal and humoral factors for example (1–3).

With regard to immune function, the spleen combines the role of the innate and adaptive immune system. Its distinctive location in the circulatory system as well as its special structure makes the spleen a unique lymphoid organ and a crucial site for early exposure to encapsulated bacteria (4). At the same time, splenectomy is a frequently used procedure in trauma surgery and in chronic diseases of cell sequestration in the spleen, and the main risk for the splenectomized is asplenic overwhelming sepsis, most frequently because of encapsulated bacteria (5). In addition to the immune function, hematopoietic stem cells may initiate extramedullar hematopoiesis in severe bone marrow failure (6) and thus act as a specialized hematopoietic stem cell-supportive microenvironment.

Generally, prenodal lymph is representative for true interstitial fluid (7). Spleen lymph from rats has been shown to have a protein concentration slightly lower than plasma (2), supporting the common notion that splenic blood vessels are discontinuous and unselective to macromolecules (4). Data on lymph in the context of the spleen microenvironment are, however, scarce, calling for additional experiments to reveal the inherent regulatory mechanisms. We hypothesized that it would be possible to unravel extracellular signaling mechanisms occurring in the fluid surrounding the blood and lymphoid cells by studying the secretion of cytokines and chemokines to the spleen lymph fluid phase. Moreover, analysis of the lymph cell fractions during development of inflammation could give important new information on cell modifications occurring in the splenic lymphatic bed in these conditions.
By isolation of spleen lymph, we show that there is a significant efflux of lymphocytes and that the spleen produces significant amounts of pro- and anti-inflammatory agents that are secreted into the general circulation during an LPS-induced inflammation, thus demonstrating in a quantitative manner the importance of the spleen in a systemic immune reaction. LPS stimulation resulted in a specific lymphatic secretion of IL-6 that was reflected in systemic serum levels, whereas the rise in serum IL-6 was strongly attenuated in the splenectomized animals. Furthermore, IL-6 induced STAT3 and CREB phosphorylation in lymphatic vessel lymphocytes in vivo, demonstrating an integration of extracellular and intracellular signaling that may explain the complex cytokine pattern observed in septicemia.

Materials and Methods

Experimental animals and housing

The experiments were performed in Long-Evans rats of either sex (range 350–520 g, median 436 g) that were fed a standard laboratory diet. The numbers of rats used in the various experiments are specified in Results. All rats were exposed to light on a 12:12 h cycle in humidity- and temperature-controlled environment. Before the experiment the rats were fasted overnight and had free access to water only.

Anesthesia and surgery

Anesthesia was induced with pentobarbital sodium, 50 mg/kg body weight, given i.p. When anesthetized, the body temperature was maintained at 37 °C ± 1 °C using a heating pad and lamp. Polyethylene (PE-50) catheters were placed in the femoral vein for injection of tracers and substances (see below) and in one femoral artery for blood sampling and monitoring blood pressure. On termination of the experiment, the rat was killed by cardiac arrest induced under anesthesia with an i.v. injection of saturated potassium chloride. All animal experiments were conducted in accordance to the regulation of the Norwegian State Commission and with approval from the Local Ethical Committee at University of Bergen.

Lymphatic vessel cannulation

To sample lymph from the spleen, we used the approach described by Kaufman et al. (2). After anesthesia, the rat was laparotomized, and the spleen was carefully cleared from its attachments to the stomach. The intestine was deflected out of the abdominal cavity and wrapped in parafilm. The spleen was carefully laid against the stomach, exposing its dorsal side and vessels. We could then observe multiple small effferent lymphatic tributaries originating in the spleen traversing the spleen dome of the diaphragm and respiratory movements. After optimization of the technique, cannulation of such effferent tributaries (afferents to the splenic nodes) was deemed impracticable. In some cases, it was difficult to identify one main lymph vessel that with certainty was of splenic origin, but usually (in 70–80% of the rats) a large common lymphatic efferent vessel draining one or more splenic lymph nodes could be identified and cannulated along the splenic artery. To reduce the trauma involved during sampling, we did not perform extensive surgery to ligate vessels in the vicinity of the spleen to avoid contamination by extraspinal lymph as described before, during spleen lymph sampling in dogs (8). After ligation using an ethicon 7-0 chirurgical nonabsorbable suture, the lymph vessel swelled, and after incision, a polypropylene tubing pulled out to an outer diameter of ~0.2 mm and filled with undiluted heparin could be inserted into the vessel lumen. The cannulation procedure was technically demanding due to a difficult access to the lymph vessels below the left dome of the diaphragm and respiratory movements. After optimization of the technique, cannulation was successful in ~75% of the rats. After successful lymph vessel cannulation, the vessel could usually be kept open for the 3–4 h duration of the experimental period (see below). Postnodal lymph was thus sampled from a major efferent lymphatic vessel collecting from several prenodal lymphatics (diameter 40–80 μm) that could be followed from their origin and deriving exclusively from the spleen. That the lymph originated from the spleen was verified by visual examination in every experiment. Furthermore, to verify the origin of the sampled lymph, we injected Evans blue bound to human serum albumin in the spleen at the end of the experiment and found that the blue dye appeared in sampled lymph 4–6 min after injection, in agreement with observations in spleen of mice (9). Dye injected in the stomach wall did not appear in the lymph.

Lymph was collected into heparinized microhematocrit tubes allowing quantification of lymph flow. Sampled lymph was either processed for analysis directly or stored immediately at ~80 °C for later analysis.

HPLC of plasma and lymph

The distribution of macromolecules in spleen lymph and serum was determined by HPLC. We used two 4.6 mm ID × 30.0 cm TSKgel Super SW3000 columns coupled in series ( Tosoh Biosciences, Stuttgart, Germany) with an optimal separation range for globular proteins of 10–500 kDa. The protein concentration in the elution fluid was measured by UV detection at 220 nm on an Etta LC System (GE Healthcare Europe, Hillerød, Denmark) and the buffer/mobile phase was 0.1 M Na2SO4 in 0.1 M phosphate buffer pH 6.7–7.0.

Identification of plasma proteins by HPLC-mass spectrometry

After HPLC separation as described previously, macromolecular fractions from plasma and lymph were identified by mass spectrometry (MS) as described in detail elsewhere (10). Briefly, samples were exchanged into 100 mM ammonium bicarbonate and concentrated to a final volume of ~25 μl and denatured by addition of 25 μl trifluoroethanol (T-8132; Sigma-Aldrich, St. Louis, MO), 2.5 μl DTT (D-5545; Sigma-Aldrich) at 60 °C for 45 min, and digested at 37 °C over night by trypsin (T6763; Sigma-Aldrich). Digested proteins from plasma and lymph were analyzed using an Agilent 1100 LC/MSD Trap XCT Plus system consisting of a nanoflow pump, well-plate sampler, capillary pump, HPLC-Chip/MS interface, and a Trap XCT Plus mass spectrometer. Processing of the MS/MS data were performed with Spectrum Mill MS proteomics workbench software (Rev A.03.02.060) using identity mode and default settings.

Colloid osmotic pressure

Colloid osmotic pressure (COP) was measured in a colloid osmometer designed for submicroliter samples (11) using membranes with a cutoff size of 30 kDa. Usually, samples of 0.5–1 μl were applied to the osmometer membrane.

Analysis of inflammatory mediators in serum and lymph

The proinflammatory cytokines IL-1β, IL-6, TNF-α, and the anti-inflammatory cytokine IL-10 were measured simultaneously in serum and lymph samples (n = 10) using Lincop kit (Linco research, St. Charles, MO) according to the manufacturer’s instructions and as described in detail elsewhere (10). A broad range of standards (4.8–20000 pg/ml) was provided in the multiplex kit and the multiplexed assay was analyzed on a flow cytometer (Luminex100; Luminex, Austin, TX) with STarStation 2.0 software (Applied Cytoometry System, Sheffield, U.K.). The minimum detection level ranged from 2.3 (IL-1β) to 5.4 (IL-10) pg/ml.

Flow cytometric analysis

Spleen lymph and blood were stained with the following mAbs: PE Cy5 conjugated mouse anti-rat CD 4, allophycocyanin-conjugated mouse anti-rat CD 3, FITC-conjugated mouse anti-rat CD 8a, and rat T/B/NK cell mixture (BD Biosciences, San Jose, CA).

Analyses were performed on a FACSARia flow cytometer (BD Bio- sciences), equipped with a 488 nm 100 mW, 532 nm 150 mW, 638 nm 30 mW, and 405 nm 50 mW laser. Labeled cells were acquired and immunophenotyping performed using BD FACSDiva (BD Biosciences) software version 6.0. T cells, B cells, NK cells, and CD4/CD8 subsets of CD3 positive T cells were analyzed.

In one series of experiments (n = 10), mAbs were put into 5 ml flow cytometry tubes and 50 μl lymph was added. Tubes were incubated for 30 min at room temperature. Then 1 ml BD Pharm Lyse Lysing Buffer was added and samples were incubated for another 8 min. Washing was performed three times with 2 ml PBS containing with 0.5% BSA. Samples were centrifugated at 350g for 5 min between each washing. Then 250 μl 2% paraformaldehyde was added into each tube before analysis.

To determine absolute counts of leukocytes in spleen lymph in control animals and after LPS treatment, mAbs and 50 μl lymph samples were added to bead-containing tubes (BD TruCount tubes, BD Biosciences, Becton Dickinson, San Jose, CA). Tubes were incubated for 15 min at room temperature before 450 μl BD Pharm Lyse Lysing Buffer was added. Samples were analyzed the same day and the absolute count using TruCount tubes was calculated from the appropriate dot plot values entered into a spreadsheet that was formatted to use the formula [(no. of events in quadrant containing cell population/no. of events in absolute-count bead region)] × [(total no. of absolute-count beads/test volume [50 μl])].

Determination of single-cell phosphosignaling

Single-cell phosphoflow cytometry of blood and spleen lymph cells was determined as described previously (12). Briefly explained, 150 μl spleen lymph was collected and immediately fixed in 850 μl 2% paraformaldehyde...
for 5 min at room temperature before centrifuged at 750g for 5 min. Collected blood cells were immediately added to BD-Lysis/fix buffer for 10 min at 37°C. For in vitro experiments, collected blood was split in aliquots and stimulated with IL-6 (100 ng/ml) or LPS (1 μg/ml) at 37°C for indicated periods (5–120 min). Stimulation was immediately stopped with BD-Lysis/fix buffer at indicated time points. One milliliter 100% ice-cold methanol was added to the preprepared pellet before samples were stored at −80°C for later analysis.

Paraformaldehyde-fixed, methanol-permeabilized cells were rehydrated by adding 4 ml PBS containing 0.5% BSA and washed (centrifugation at 750g for 5 min) twice. Directly conjugated Abs were added at optimized concentrations and incubated protected against light and at room temperature for 30 min. Cells from the spleen lymph were stained with the following Abs: p-p38 Alexa 488 (BD Biosciences, Cat. 612595, 6 μl per sample), p-STAT3 Alexa 488 (BD Biosciences, Cat. 557418, 10 μl per sample), and p-CREB PE (BD Biosciences, Cat. 558436, 5 μl per sample). Data were collected using BD FACSDiva software and analyzed using FlowJo (TreeStar, Ashland, OR) and Cytobank (www.cytobank.org), an open-source flow cytometry storage and analysis application. For all samples, a tight gate was drawn around the lymphocyte populations that were gated using forward light scatter versus side light scatter (SSC) and were deemed viable and used for analysis.

The normalized p-STAT3 (or p-p38 or p-CREB) response was calculated by transforming the raw data using the inverse hyperbolic sine part of the biexponential class of functions used for digital flow cytometry data (13), and calculating a Log_{10} change in the 95th percentile of p-STAT3 response between a LPS-stimulated sample and its unstimulated/basal state.

**Experimental interventions**

**LPS-induced acute inflammation.** We mimicked early sepsis by infusion of LPS from *Escherichia coli* (serotype 0127:88, Sigma-Aldrich). The LPS was diluted in PBS containing 0.1% BSA, and the stock solution had a concentration 0.25 mg/ml. The rats received a dose of 3.0 mg/kg LPS by infusion of stock solution i.v. and were observed for 3 h. In these experiments, the femoral artery was catheterized for monitoring arterial pressure, after the injection of LPS. One subgroup of these rats was used for lymph sampling by lymph vessel cannulation, another subgroup treated identically was used for sampling of blood for flow cytometric analysis and yet another subgroup was subjected to splenectomy (see below). Samples from these were analyzed the same day or stored at −80°C for later analysis.

**Splenectomy**

To assess the quantitative role of the spleen in the cytokine response after an acute LPS-induced inflammation we added a series of experiments using splenectomized rats. After anesthesia, laparotomy was made; the spleen was carefully cleared from its attachments to the stomach. The spleen was isolated, appropriate blood vessels ligated and the spleen removed. Control animals were treated identically, except the spleen was not ligated or removed. After a postoperative stabilization period of 30 min, LPS was injected as described previously. Rats were observed for 180 min either in control or after LPS treatment. Serum was stored immediately at −80°C for later analysis.

**Statistical methods**

All values are means ± SEM unless otherwise stated. Differences were tested with two-tailed t test using paired comparison when appropriate or ANOVA, followed by Dunn’s or Bonferroni’s test. p < 0.05 was considered statistically significant.

**Results**

Lymph was clear without any macroscopic traces of erythrocytes. The flow rate during control conditions ranged from 1.8–3.8 μl/min, averaging 2.3 ± 0.5 μl/min when assessed in n = 10 rats.

**Protein distribution pattern and COP in control situation**

The spleen exchange vessels are assumed to be highly permeable to plasma proteins. To address the sieving properties of the spleen microvessels and some of the factors determining transcapillary fluid flux in this organ, we assessed the protein distribution pattern of spleen lymph. These pherograms were used for a quantification of the amount of proteins in the various fractions as described previously (10, 14). Furthermore, we normalized all pherograms with respect to albumin and plotted the elution curves for lymph together with that of plasma to compare the relative distribution of differently sized macromolecules. As evident from Fig. 1, *upper panel*, the protein distribution patterns for the two fluids were almost similar. Plasma and lymph from seven rats were also analyzed by online reversed-phase nano liquid chromatography coupled with ion trap MS to establish the identity of the most abundant proteins in lymph. This analysis showed that the 20–30 of the most abundant plasma proteins were also found in lymph. In Table I, we have summarized the relative fraction of some abundant plasma proteins identified by MS. All concentrations are based on peak area relative to that of rat serum albumin (RSA). Representative patterns for plasma (red line) and spleen lymph (blue line) in control situation (*upper panel*) and 180 min after exposure to LPS (*lower panel*) are shown. Elution time at 0.2 ml/min, UV signal at 220 nm in arbitrary units. αM, α2-macroglobulin; C, complement; Fib, fibrinogen; Hapt, haptoglobin; MUG, muroglobulin.
Effect on the splenic lymphatic bed by LPS

Based on the central role of the spleen in a systemic inflammatory response, we asked if and how a systemic inflammation induced by LPS affected lymph flow and vascular sieving properties. In this series, collecting lymph into heparinized microhematocrit tubes also allowed quantification of lymph flow. Lymph was sampled during a control period of 30 min and then for 3 h after i.v. injection of LPS (n = 6). As evident from Fig. 2A, LPS injection had a significant effect on spleen lymph flow. Flow increased gradually from 2.9 ± 0.3 μl/min during the first hour after injection to 16 ± 1.6 μl/min 3 h after injection. In control rats, there was a slight gradual increase in lymph flow during the experiment, from 1.8 ± 0.2 during the first hour to 3.8 ± 0.5 μl/min at 180 min (Fig. 2A). These experiments suggest that there is a substantial increase in transcapillary fluid filtration during LPS exposure.

Protein distribution pattern and COP during LPS

LPS is known to affect the microvascular barrier, for example (15). The control experiments suggested that some sieving took place in spleen microvessels, and we asked whether LPS affected the spleen vascular barrier by assessing protein distribution pattern, protein quantity in the various m.w. fractions and COP. The total protein concentration in lymph fell as an effect of LPS injection, whereas the relative distribution of proteins in the peaks was similar to that in control situation. There was, however, a relative increase in large proteins, in particular a notable increase in the IgM fraction, as exemplified in Fig. 1, lower panel.

The COP in spleen lymph in control situation (n = 5) and after LPS treatment (n = 5) are shown in Fig. 2B. Pressure in control lymph was 17.0 ± 0.4 mm Hg in lymph sampled during the first 30 min after cannulation and was stable throughout the duration of the experiment. The corresponding control pressure in plasma was 19.7 ± 0.4 mm Hg (Fig. 2B). Endotoxemia induced by infusion of LPS resulted in reduction in plasma and spleen lymph COP, from 19.0 ± 0.8 mm Hg to 17.0 ± 0.4 mm Hg in plasma and from 17.0 ± 0.4 mm Hg to 15.4 ± 0.2 mm Hg in lymph. These experiments suggest that although small, some sieving properties are maintained in the spleen microvasculature during an inflammation induced by LPS.

Cytokine concentrations in serum and spleen lymph

We next asked whether a systemic inflammation produces detectable changes in inflammatory mediators in spleen lymph and whether there was a local production of cytokines in spleen. To be able to monitor whether a systemic inflammatory process was reflected in the splenic lymphatic bed, the proinflammatory cytokines IL-1β, IL-6, TNF-α, and the anti-inflammatory cytokine IL-10 were measured simultaneously in serum and lymph samples (n = 10) using a multiplex technique. In control animals, we found very low levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in samples from serum and spleen lymph, whereas the anti-inflammatory cytokine IL-10 was not detectable (Fig. 3).

Induction of a systemic inflammatory response produced significant changes in all measured cytokines. In LPS-treated animals the levels of IL-1β, IL-6, IL-10, and TNF-α were increased in both serum and spleen lymph. The concentrations of IL-6 were very high in spleen lymph, whereas the corresponding values in serum were very low. Generally, the concentration of IL-10 in lymph was higher compared with serum, whereas the concentration of IL-1β in serum was slightly higher compared with lymph. The concentration of TNF-α was not detectable in serum and was low in lymph (Fig. 3).

**Table 1. Concentration of plasma proteins relative to albumin in control situation and after exposure to LPS**

<table>
<thead>
<tr>
<th></th>
<th>IgM</th>
<th>αM</th>
<th>Fib</th>
<th>MUG</th>
<th>Hapt</th>
<th>IgG</th>
<th>C</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Plasma</td>
<td>0.006 ± 0.002</td>
<td>0.150 ± 0.036</td>
<td>0.015 ± 0.003</td>
<td>0.271 ± 0.062</td>
<td>0.048 ± 0.008</td>
<td>0.104 ± 0.017</td>
<td>0.110 ± 0.026</td>
</tr>
<tr>
<td>Lymph</td>
<td>0.004 ± 0.001*</td>
<td>0.103 ± 0.017*</td>
<td>0.016 ± 0.004</td>
<td>0.234 ± 0.035</td>
<td>0.053 ± 0.007</td>
<td>0.109 ± 0.014</td>
<td>0.120 ± 0.018</td>
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<td>LPS 30–60 min</td>
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<tr>
<td>Plasma</td>
<td>0.006 ± 0.002</td>
<td>0.144 ± 0.039</td>
<td>0.016 ± 0.004</td>
<td>0.251 ± 0.064</td>
<td>0.043 ± 0.008</td>
<td>0.095 ± 0.017</td>
<td>0.104 ± 0.026</td>
</tr>
<tr>
<td>Lymph</td>
<td>0.005 ± 0.001</td>
<td>0.115 ± 0.026</td>
<td>0.015 ± 0.002</td>
<td>0.225 ± 0.043</td>
<td>0.046 ± 0.007</td>
<td>0.099 ± 0.012</td>
<td>0.110 ± 0.023</td>
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<tr>
<td>LPS 60–90 min</td>
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<tr>
<td>Plasma</td>
<td>0.007 ± 0.003</td>
<td>0.117 ± 0.037</td>
<td>0.015 ± 0.005</td>
<td>0.210 ± 0.072</td>
<td>0.038 ± 0.010</td>
<td>0.079 ± 0.021</td>
<td>0.088 ± 0.027</td>
</tr>
<tr>
<td>Lymph</td>
<td>0.005 ± 0.002</td>
<td>0.088 ± 0.024</td>
<td>0.012 ± 0.003</td>
<td>0.185 ± 0.058</td>
<td>0.035 ± 0.008</td>
<td>0.074 ± 0.017</td>
<td>0.091 ± 0.029</td>
</tr>
</tbody>
</table>

Concentrations expressed as plasma protein peak area relative to that of RSA, set to 1.0, having an average concentration of 35 mg/ml in plasma.

*αM, α2- macroglobulin; C, complement; Fib, fibrinogen; Hapt, haptoglobin; MUG, muroglobulin.

**FIGURE 2.** Spleen lymph flow and COP in experimental endotoxemia. A, Spleen lymph flow after experimental endotoxemia induced by i.v. injection of LPS (3.0 mg/kg) (o). (n = 6). Experiment ended 180 min after LPS administration. Controls (n = 5) (●) received vehicle alone and were kept for 180 min. Values are means ± SEM. ANOVA, *p < 0.05 compared with beginning of experiment; *p < 0.05 when compared with respective values in control animals. B, COP in plasma (circles) and spleen lymph (triangles) in control situation (n = 5) (filled symbols) and in experimental endotoxemia induced by i.v. injection of LPS (3.0 mg/kg) (open symbols) (n = 5). Values are means ± SEM. ANOVA, *p < 0.05 compared with corresponding plasma COP; *p < 0.05 when compared with lymph control value; #p < 0.05 when compared with plasma control value.
lymph were increased compared with control values (Fig. 3). Interestingly, we found that the average concentration of IL-6 was significantly higher in spleen lymph compared with that in serum during the last part of the experiment (Fig. 3B). LPS also gave an increase in TNF-α concentration from 60 min duration of LPS exposure. Although the concentration in lymph tended to be higher than in serum at all times, this difference was significant at 75 min only (Fig. 3C).

Clearly, LPS treatment also resulted in secretion of the anti-inflammatory cytokine IL-10 (Fig. 3D). Thus, at 180 min, lymph IL-10 averaged 6593 pg/ml, as compared to 308 pg/ml in the control situation. The level of IL-10 in spleen lymph samples was markedly higher than the concentration of this cytokine found in serum (Fig. 3D).

Taken together, these observations suggest that there was a local production of inflammatory as well as anti-inflammatory cytokines in spleen after LPS exposure, and that the spleen may provide the systemic circulation with cytokines that can modify the ensuing inflammatory response.

### Cytokine concentrations in serum in splenectomized rats treated with LPS

To investigate more quantitatively the role of spleen as a provider of cytokines to the general circulation in a systemic inflammation, we studied cytokine production in splenectomized rats (n = 10). As for animals with intact spleen, induction of systemic inflammatory response resulted in changes in all of cytokines that were measured (Fig. 4). In the group with intact spleens, the mean concentration of the proinflammatory cytokine IL-6 in control rats was markedly higher (70,764 pg/ml, 183,580 pg/ml, 201,046 pg/ml at 60 min, 120 min, and 180 min, respectively) than the respective concentration in splenectomized rats (1060 pg/ml, 22,980 pg/ml, 46,232 pg/ml, respectively) (Fig. 4A). Although the difference was less pronounced, the average TNF-α levels at 60 min and 120 min (1236 pg/ml and 944 pg/ml, respectively) were higher than that in splenectomized rats (669 pg/ml and 692 pg/ml), whereas at 180 min the level of TNF-α in serum in control animals was less (299 pg/ml) than that in splenectomized animals (333 pg/ml) (Fig. 4B). The levels of anti-inflammatory cytokine IL-10 were slightly but not significantly higher in the control group (3765 pg/ml, 3433 pg/ml, 1913 pg/ml at 60 min, 120 min, and 180 min, respectively) than that in splenectomized animals (3591 pg/ml, 2846 pg/ml, 1717 pg/ml at corresponding time points). Similarly, the average IL-1β concentrations were slightly but not significantly higher in the group with intact spleen (315 pg/ml, 1495 pg/ml, 943 pg/ml at 60 min, 120 min, and 180 min, respectively) than in the splenectomy group (181 pg/ml, 811pg/ml, 643 pg/ml at corresponding time points). These data show the quantitative role of spleen in cytokine production, especially IL-6.

### Flow cytometric analysis of spleen lymph

To characterize the cellular elements in spleen lymph and to study whether this pattern was changed during systemic inflammation, we performed flow cytometry analysis of spleen lymph and compared these with corresponding fractions in blood. Gating of lymphocytes was based on flow cytometric forward light scatter and SSC and expression of the T lymphocyte markers CD3+, CD4+, CD8+, and the B lymphocyte marker CD45RA. The lymphocyte population could be identified as a separate population in the FSC-SSC diagram at the identical place as human lymphocytes (Fig. 5). Throughout our data presentation, we describe the results for both blood and lymph samples in control (n = 5) and LPS study (n = 6). A total of 30,000 events were acquired on average. The absolute number of T and B cells (determined using Trucount tubes) were 7.7 ± 2.3 and 1.7 ± 1.0 × 10^6 cells/ml in the control situation (n = 6).

We found that expression of all T cell subsets monitored was significantly higher in spleen lymph than in blood in control as well as LPS-treated animals (data not shown). Moreover, LPS administration resulted in an increase in the percentage of CD3^+T cells in spleen lymph, from 70.1 ± 4.6% in control situation to 82.8 ± 4.5% after LPS (Fig. 6A). Corresponding numbers for blood were the same.
immune response.

from the splenic lymphatic bed that may be of importance for the induced inflammation results in an early mobilization of T cells 120 min after injection. These experiments showed that a LPS-

serum from splenectomized rats. Values are mean alone and were kept for 180 min. ANOVA, ended 180 min after LPS administration. Intact animals received vehicle toxemia induced by i.v. injection of LPS (3.0 mg/kg). The experiment (Fig. 7D) increased significantly after LPS exposure, the increase was most pronounced for the latter that was 220% of control at (Fig. 7A, B) (n = 5) and splenectomized (o) (n = 5) rats in experimental endotoxemia induced by i.v. injection of LPS (3.0 mg/kg). The experiment ended 180 min after LPS administration. Intact animals received vehicle alone and were kept for 180 min. ANOVA, *p < 0.05 compared with serum from splenectomized rats. Values are mean ± SEM.

48.2 ± 3.5% and 50.6 ± 2.0%. In control situation, the expression of B cells did not differ in spleen lymph and blood, averaging 34.2 ± 6.0 and 33.5 ± 1.9 of the total number of cells in the respective fluids (Fig. 6B). After LPS, however, there was a rapid significant drop in B cells in lymph 60 min after LPS exposure to 21.0 ± 3.2%, with a concomitant rise in B cell fraction in blood to 44.5 ± 4.4%. The flow cytometric analysis suggests that the systemic inflammation affected the distribution of lymphocytes exiting through lymphatics draining the spleen.

We wanted to determine the flux of cells exiting via spleen lymphatics and thus entering the systemic circulation during the initial phase of an inflammation because the mechanism and quantity of this transport is unknown (4). Because of a variable lymph flow and concentration of cells between animals, that may at least partly be due to variable anatomy of the lymph vessels, we chose to normalize relative to the corresponding control flux. As evident from Fig. 7A, there was a significant increase of 60% in the flux of T cells already after 60 min of LPS exposure, increasing slightly further the following hour. The opposite pattern was observed for B cells (Fig. 7B), having ~40% of its initial flux in the same period. Whereas both CD4+ (Fig.7C) and CD8+ flux (Fig. 7D) increased significantly after LPS exposure, the increase was most pronounced for the latter that was 220% of control at 120 min after injection. These experiments showed that a LPS-induced inflammation results in an early mobilization of T cells from the splenic lymphatic bed that may be of importance for the immune response.

In vivo perturbation of phosphosignaling in spleen lymphocytes after LPS treatment

LPS induced a specific cytokine secretion pattern in spleen lymph and resulted in an increase in lymphocyte cell flux. We therefore examined if the LPS response resulted in specific perturbations in lymphocyte signaling compared with peripheral blood and LPS treated full blood. Cells were harvested from isolated spleen lymph before and 60 and 120 min after LPS treatment in eight rats. We determined the level of phosphorylated STAT3 (Y705), CREB (S133), ERK1/2 (T202/Y204), and p38 (T180/Y182) by protein-modification specific Abs and flow cytometry (see Supplemental Fig. 1 for verification of Abs in human and rat leukocytes). The phosphorylation levels of STAT3 and CREB were significantly upregulated after 60 and 120 min of LPS treatment, respectively, whereas ERK1/2 and p38 demonstrated a weaker increase in phosphorylation (Fig. 8A, 8B). Peripheral lymphocytes in LPS-treated animals showed an increased phosphorylation of STAT3 and a weaker CREB phosphorylation compared with spleen lymph leukocytes (Fig. 8C). In addition, peripheral lymphocytes demonstrated a more striking phosphorylation of ERK1/2 and p38. To exclude some of the systemic effects of LPS, we compared with in vitro full blood leukocytes treated with LPS or IL-6. In vitro treated lymphocytes demonstrated a striking STAT3 phosphorylation induced by IL-6 only (Fig. 8E). Based on these experiments, we conclude that the signaling profile in lymphocytes harvested from lymph reflect the cytokine stimulation, and not LPS itself. As judged from Fig. 8B and 8C, it may appear as if the STAT3 phosphorylation in spleen lymphocytes was weaker compared with PBLs, and the CREB phosphorylation more prolonged. The results from the stimulated samples were therefore compared with its own control. When comparing basal/constitutive phosphorylation of STAT3 and CREB we observed a significantly higher phosphorylation level in spleen lymph lymphocytes compared with PBLs (Fig. 8D). This may be explained by the cytokine concentration in the spleen microenvironment from
where lymphocytes are recruited, but a signaling signature reflecting differences in lymphocyte subsets cannot be ruled out. Collectively, these experiments suggest that lymphocytes in spleen lymph acquire a unique signature as response to a systemic LPS-induced cytokine increase.

Discussion

Lymph as a representative for the fluid part of the microenvironment

In this study, we have isolated lymph from the spleen and thereby achieved access to the extracellular fluid phase of an organ of major importance for whole body fluid exchange as well as immunological function of the organism. A central question in our study is whether the lymph sampled derives from the spleen only, but the visual verification in every experiment of the lymph vessel origin and the dye injection experiments make us confident that we sampled lymph originating from the spleen. To exclude an effect of the lymph node on the composition of sampled lymph, we should ideally have cannulated efferent spleen tributaries (afferents to the splenic node), but such cannulation was deemed impracticable. Because our samples were from a lymphatic draining the splenic node, the lymph was postnodal, a fact that has to be taken into consideration when interpreting the results. Previous data, however, suggest minimal node influence. In the kidney, we found that albumin injected prenodally in a hilar lymph vessel was completely recovered in the thoracic duct (16). Since lymph node protein and fluid exchange decrease with increased flow (17), and the spleen lymph flow rates were on average ~four times higher than that of the kidney, our kidney data suggest minimal exchange of fluid and proteins also in the spleen nodes. One way to show that spleen lymph was the dominant component in the collected lymph might have been to compare with thoracic duct lymph, which derives from the entire abdominal area (18). We have sampled thoracic duct lymph in a previous study (16), and preliminary experiments showed that its protein concentration was ~65% of that in plasma, that is, lower than in sampled spleen lymph, indicating that the origin of the sampled lymph cannot be verified by such a comparison.

Another issue is the cellular composition of efferent lymph, which has been studied quantitatively in a sheep model (reviewed in Ref. 19). These studies show that the majority of the lymphocytes found in efferent lymph draining the popliteal node originate from blood and enters the lymph node through specialized endothelium...
in the so called high endothelial venules. It is thus likely that some of the lymphocytes isolated derived from venules in the lymph node and not from the spleen. Interestingly, whereas efferent lymph consists of lymphocytes only (19), our finding of other leukocyte populations (Fig. 5) similar to that of afferent lymph (19) may suggest that a significant proportion of the cells isolated in our lymph samples derive from the spleen. The exact origin of the cells will, however, have to be sought in additional experiments.

Extensive studies of splenic circulation and the role of the spleen in control of the intravascular fluid volume have been performed by Kaufman and coworkers. They have found that the spleen has a very high blood flow (∼8 ml/min/g) (20), and also that there is a significant arteriovenous volume difference that is filtered out of the blood into the lymphatic system (2, 20). Such filtration is exaggerated during volume loading (2) and LPS infusion (21), favoring lymph formation even further in these conditions. In line with these observations, we found that spleen lymph accumulated avidly at a rate of ∼2 µl/min in control situation and increasing 8-fold this value 3 h after LPS exposure. Because of anatomical variation in lymph vessels draining the spleen and the need to ligate vessels during the preparation process, it is not possible to measure total lymph flow in the rat using our approach. With the reservations discussed previously regarding the origin of the lymph, the present lymph vessel cannulation enabled us to access the microenvironment of the spleen, namely, the fluid bathing the spleen cells and other cells residing in the splenic lymphatic bed and to monitor the quantitative role of mediators released from the spleen during inflammation. Comparing the cytokine ratio in lymph and plasma revealed a discrepancy between the two compartments, and demonstrated a variable response for each cytokine. The concentrations of the proinflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokine IL-10 were significantly elevated in lymph compared with plasma after administration of LPS. Accordingly, these cytokines must be produced and released from cells within the spleen (with a possible contribution from splenic lymph nodes), because any solute being transported across the microvasculature from plasma to interstitial fluid and lymph will be present in lower concentrations in lymph than in plasma (22). That the spleen production is quantitatively important on an organism level was shown by a drastically reduced IL-6 response in plasma after LPS-exposure in splenectomized animals shows the quantitative role of the spleen in mounting an inflammatory response that may be of vital importance and predict survival.
Considering the substantial increase in IL-10 in spleen lymph after LPS exposure, it was somewhat surprising that there was no difference between the serum concentrations of IL-10 in splenectomized and intact animals after treatment. The explanation is probably a difference in sympathetic nervous system activation in the two experimental situations. Spleen vessel cannulation requires more extensive surgery than a simple removal of the spleen. It is thus likely that there is a stronger activation of the sympathetic nervous system and thus a drive for IL-10 secretion similar to what has been observed in acute brain injury (24) in the former situation.

Whereas we are not aware of any previous studies on cytokine secretion to spleen lymph in vivo, Straub and coworkers have studied the regulation of spleen cytokine secretion in vitro using a micro-supersfusion chamber (25). With this model they found that stimulation by bacteria (similar to LPS in our experiments) increased secretion of TNF-α, that after a delay period further stimulated the secretion of IL-6 (26). Although the timing of their response did not match ours exactly, the overall response pattern was similar.

Function of cells secreted to lymph

In addition to what has been discussed previously, the LPS stimulus will also elicit a cellular response. The spleen has a role in innate as well as adaptive immunity (4, 27), linked by DCs that mature and differentiate in the spleen microenvironment (28, 29). The precise mechanisms by which lymphocytes leave the white pulp and the exact anatomical route they use are, however, unknown (4). We clearly showed that a significant amount of CD4+, CD8+ T cells, and other immune cells leave the spleen via efferent lymphatics and eventually enter the systemic circulation. Although the volume of lymph and thus clearance of cells from the spleen may not reflect the total drainage due to variable lymphatic anatomy and sampling efficiency, the relative distribution between the various fractions of cells should be representative for cells drained. We notice that compared with blood, CD4+ and CD8+ T cell fractions were higher in lymph whereas B cells were similar when compared with blood. Of interest, LPS resulted in rapid reduction in B cell fraction in lymph with a corresponding increase in blood.

Previous data may indicate that CD4+ and CD8+ T cells have a blood to lymph transit time of ~3 h (30), suggesting that cells exposed to a splenic microenvironment entered the systemic circulation in our experiments, a microenvironment which may induce specific cell differentiation (29). That exposure to the microenvironment drained by spleen lymphatics actually induces changes in cell signaling was shown by our phosphosignaling experiments. By presenting cells isolated from lymph to potentiating inputs, we could discern unique leukocyte network profiles. Thus, single-cell measurements of phosphoprotein responses reveal shifts in signaling potential of a phosphoprotein network, allowing for categorizing of cell network phenotypes by multidimensional molecular profiles of signaling.

LPS signaling acts through TLRs and initiates transcription and release of cytokines like TNF-α, IL-1β, IL-6, and IL-10 (31). IL-10 and IL-1β mainly signal via activation JAK2 and subsequently phosphorylation of transcriptional factor STAT3 (32). The anti-inflammatory activity of IL-10 thought to go through TNF-α suppression may be stronger compared with IL-6 (33, 34). The decrease in TNF-α after 75 min of LPS exposure may reflect the increase in IL-6 and IL-10 (Fig. 3). An inhibition of TNFα production and secretion is postulated to be due to the limited amount of CBP and the opposite regulation of glycogen synthase kinase 3 (33). A downregulation of glycogen synthase kinase 3 leads to AP-1 and CREB phosphorylation and binding to CBP coactivator with a higher affinity than NFκB p65 (35). This IL-6 and IL-10 production/release and signaling response is reflected by activation of STAT3 and CREB (Fig. 8a–c). The implication of STAT3 activation of spleen-released lymphocytes may be to increase survival (32). We suggest that in vivo, lymphocytes in spleen lymph are activated by the locally produced spleen cytokines IL-6 and IL-10. In vitro, the signaling responses to LPS stimulation in CREB, ERK1/2, and p38 were practically absent (Fig. 8e).

To summarize, we have gained access to the microenvironment in the spleen by cannulating an efferent lymphatic, and thus been able to monitor circulatory fluid balance as well as cellular function in the splenic lymphatic bed. The spleen has a relatively high lymph flow, a permeable microvasculature exhibiting a low sieving of plasma proteins. During an LPS-induced systemic inflammation, the spleen produces inflammatory mediators that may actually determine the systemic level and thus the ensuing inflammatory response. A significant amount of lymphocytes exit the spleen via draining lymphatics, and exposure to the spleen microenvironment affects their signaling status. The lymphatic path is accordingly important in lymphocyte recirculation and thus immune function.

Disclosures

The authors have no financial conflicts of interest.

References

Testing p-Ab on rat full blood

- Lymphocytes
  - p-STAT3 (pY705)
  - p-STAT5 (pY694)
  - p-CREB (pS133)
  - p-p38 (pT180/pY182)
  - p-Erk1/2 (pT202/pY204)
  - p-Zap70/Syk (pY319/pY352)
  - p-Akt (pS473)

- Monocytes
  - p-STAT3 (pY705)
  - p-STAT5 (pY694)
  - p-CREB (pS133)
  - p-p38 (pT180/pY182)
  - p-Erk1/2 (pT202/pY204)
  - p-Zap70/Syk (pY319/pY352)
  - p-Akt (pS473)

- Granulocytes
  - p-STAT3 (pY705)
  - p-STAT5 (pY694)
  - p-CREB (pS133)
  - p-p38 (pT180/pY182)
  - p-Erk1/2 (pT202/pY204)
  - p-Zap70/Syk (pY319/pY352)
  - p-Akt (pS473)
Supplementary Figure.

**Anti human phospho antibodies verified for use in rat.** 7 phospho Ab were tested on rat full blood. Full blood from rat was stimulated with respective stimulants for given time (see table below), and BD Phosflow Lyse/Fix buffer was added (20x volume) to stop the reaction (37°C for 10min). After washing the samples twice in PBS (500g – 8min, RT), 1 ml of 100% MetOH was added, the pre-vertexed cell pellet and incubated on ice for 10 minutes. Rehydrated and washed cells were stained with phospho specific Ab in 1% BSA solution for 30 min at RT and collected on a FACSArria flow cytometer (BD Biosciences). Analysis was done in FlowJo 7.5.5 (Tree Star Inc.).

<table>
<thead>
<tr>
<th>Phospho Ab</th>
<th>Stimulant</th>
<th>Concentration [µg/ml]</th>
<th>Time [minutes]</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3(pY701)</td>
<td>IL-6</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>STAT5(pY694)</td>
<td>GM-CSF</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>CREB(pS133)</td>
<td>PMA</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>p38(pT180/pY182)</td>
<td>LPS</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Erk1/2(pT202/pY204)</td>
<td>LPS</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>pZap70/Syk(pY319/pY352)</td>
<td>LPS and PMA or H₂O₂</td>
<td>100 and 5 or 0.1mM</td>
<td>2 / 2</td>
</tr>
<tr>
<td>pAkt(pS473)</td>
<td>GM-CSF or H₂O₂</td>
<td>0.05 or 0.1mM</td>
<td>10 / 10</td>
</tr>
</tbody>
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