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Mice Lacking the Multidrug Resistance Protein 1 Are Resistant to Streptococcus pneumoniae-Induced Pneumonia

Marc J. Schultz,† Jan Wijnholds,‡ Maikel P. Peppelenbosch,† Margriet J. B. M. Vervoordeldonk,‡ Peter Speelman,† Sander J. H. van Deventer,‡ Piet Borst,‡ and Tom van der Poll*†

Leukotrienes (LTs) are considered important for antibacterial defense in the lung. Multidrug resistance protein 1 (mrp1) is a transmembrane protein responsible for the cellular extrusion of LTC\textsubscript{4}. To determine the role of mrp1 in host defense against pneumonia, mrp1\textsuperscript{−/−} and wild-type mice were intranasally inoculated with Streptococcus pneumoniae. mrp1\textsuperscript{−/−} mice displayed a diminished outgrowth of pneumococci in lungs and a strongly reduced mortality. These findings were related to an effect of mrp1 on LT metabolism, because survival was similar in mrp1\textsuperscript{−/−} and wild-type mice treated with the 5-lipoxygenase-activating protein inhibitor MK-886. Although LTC\textsubscript{4} levels remained low in the bronchoalveolar lavage fluid of mrp1\textsuperscript{−/−} mice, LTB\textsubscript{4} concentrations were higher than in wild-type mice. These elevated LTB\textsubscript{4} concentrations were important for the relative protection of mice, because the LTB\textsubscript{4} antagonist LTB\textsubscript{4}-dimethyl amide abolished their survival advantage. In vitro experiments suggested that the intracellular accumulation of LTC\textsubscript{4} in mrp1\textsuperscript{−/−} mice results in product inhibition of LTC\textsubscript{4}-synthase, diminishing substrate competition between LT\textsubscript{A\textsubscript{4}}-hydrolase (which yields LTB\textsubscript{4}) and LTC\textsubscript{4}-synthase for the available LT\textsubscript{A\textsubscript{4}}. We conclude that mrp1\textsuperscript{−/−} mice are resistant against pneumococcal pneumonia by a mechanism that involves increased release of LTB\textsubscript{4}. These results identify mrp1 as a novel target for adjunctive therapy in pneumonia.

The Journal of Immunology, 2001, 166: 4059–4064.

Leukotrienes (LTs), comprising the group of highly potent lipid mediators synthesized by 5-lipoxygenase (5-LO), are important for host defense against bacterial infection, especially in the lung. LT synthesis in human leukocytes is induced by various microorganisms under different conditions (7, 8). High levels of LTB\textsubscript{4} are measured in lung tissue and bronchoalveolar lavage fluids (BALF) derived from animals infected with Pseudomonas aeruginosa (9) or Klebsiella pneumoniae (10), and in BALF from patients with pneumonia (11). Moreover, alveolar macrophages from patients infected with the HIV demonstrate a marked reduction in LT synthesis, which has been considered to contribute to the higher occurrence of pneumonia in these subjects (12). The most dramatic proof for the action of LTs in pneumonia comes from studies in mice bearing two inactivated copies of the 5-LO gene, manifest enhanced bacterial outgrowth and lethality from K. pneumoniae pneumonia (10). However, 5-LO\textsuperscript{−/−} mice can produce neither LTB\textsubscript{4} nor cysteinyll LTs, and thus the distinct roles of these LT pathways in host defense against pneumonia cannot be deduced from these mice.

The three cysteinyll LTs have been implicated as mediators of host defense against bacterial infection, especially in the lung. LT synthesis in human leukocytes is induced by various microorganisms under different conditions (7, 8). LT\textsubscript{A\textsubscript{4}} is a fellows of the Royal Dutch Academy of Arts and Sciences. [1] Address correspondence and reprint requests to Dr. Marc J. Schultz, Academic Medical Center, University of Amsterdam, C33-324, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail address: m.j.schultz@amc.uva.nl

[2] Abbreviations used in this paper: LT, leukotrienes; 5-LO, 5-lipoxygenase; mrp, multidrug resistance protein; BALF, bronchoalveolar lavage fluid.

Materials and Methods

Animals

The animals used were female mrp1\textsuperscript{−/−} mice, generated by gene targeting in embryonic stem cells as described previously (6). Control and mutant mice were on the genetic background (129/Ola)/FVB (50%/50%). The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

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This work was supported in parts by grants from the Dutch Cancer Society. T.v.d.P. is a fellow of the Royal Dutch Academy of Arts and Sciences.

Abbreviations used in this paper: LT, leukotrienes; 5-LO, 5-lipoxygenase; mrp, multidrug resistance protein; BALF, bronchoalveolar lavage fluid.
ROLE OF MULTIDRUG RESISTANCE PROTEIN 1 IN PNEUMONIA

Reagents

LTB₄ dimethyl amide (a specific LTB₄ antagonist) was obtained from ICN Pharmaceuticals (Aurora, OH). MK-886 (a 5-LO-activating protein inhibitor) was purchased from Alexis (San Diego, CA). The doses of LTB₄ dimethyl amide (0.4 nmol/mouse) and MK-886 (1 nmol/mouse) used in the in vivo experiments were extrapolated from in vitro studies in which the effectiveness of the inhibitors was assessed (18). The concentrations used represent a 10-fold excess of those required for an optimal in vitro effect. Arachidonic acid, LTB₄, and LTC₄ were acquired from Sigma (St. Louis, MO).

Induction of pneumonia

Pneumonia was induced as described previously (16, 17). Briefly, S. pneumoniae serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Manassas, VA). Pneumococci were grown for 6 h to mid-logarithmic phase at 37°C in 5% CO₂ using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 × g for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of ∼2 × 10⁶ CFU/ml, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands, U.K.), according to the instructions of the manufacturer. (10⁷ CFU/ml), after which 200 μl whole blood was added. Tubes were gently mixed and placed in the incubator at 37°C for 20 min. After incubation, supernatant was stored at −20°C until measurements were performed.

Preparation of lung homogenates

At 24 and 48 h after inoculation mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the vena cava inferior. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile 0.9% NaCl in a tissue homogenizer that was carefully cleaned and disinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates (and blood), and 50 μl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO₂. CFU were counted after 24 h. For cytokine measurements, lung homogenates were spun at 1500 × g for 15 min at 4°C, and supernatants were filtered through a 35 μm filter (Becton Dickinson, Lincoln Park, NJ) and frozen at −20°C until cytokine measurement.

Bronchoalveolar lavage

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbott-Catheter (Abbott Laboratories, Sligo, Ireland). Bronchoalveolar lavage was performed by instilling two 0.5-ml aliquots of 0.9% NaCl in a tissue homogenizer that was carefully cleaned and disinfected with 70% alcohol after each homogenization. At the end of each incubation, LTs were extracted as described below. In a separate experiment, peritoneal macrophages were stimulated with heat-killed S. pneumoniae. For these experiments, polypropylene tubes were prefilled with 100 μl RPMI 1640 containing heat-kill S. pneumoniae (10⁷ CFU/ml), after which 250 μl of the peritoneal washing was added. Tubes were gently mixed and placed in the incubator at 37°C for 20 min. After incubation, supernatant was stored at −20°C until assays were performed.

Determination of LTB₄ dimethylamide and MK886 activity in vivo

Normal wild-type mice received an i.p. injection with 0.4 nmol LTB₄ dimethyl amide, and were inoculated intranasally with 2 × 10⁵ CFU LTB₄ 24 h after. BALF was obtained 4 h after the administration of LTB₄, and granulocytes were counted according to the methods described above. In another experiment, mice received an i.p. injection with 1 nmol MK886. After 24 h, heparinized whole blood was obtained and stimulated with heat-killed S. pneumoniae. For these experiments, polypropylene tubes were prefilled with 100 μl RPMI 1640 containing heat-kill S. pneumoniae (10⁷ CFU/ml), after which 200 μl whole blood was added. Tubes were gently mixed and placed in the incubator at 37°C for 20 min. After incubation, plasma was prepared by centrifugation and stored at −20°C until assays were performed.

Assays

Levels of the following cytokines and chemokines were measured by ELISA according to the manufacturer’s recommendations: TNF-α (Genzyme, Cambridge, MA), IL-6 (PharMingen, San Diego, CA), IFN-γ (R&D Systems, Minneapolis, MN), IL-12 p40 (PharMingen), macrophage-inflamma
tory protein-2 (R&D Systems), and KC (R&D Systems). LTB₄ and LTC₄ levels were measured by enzyme immunoassays (Amersham, Buckinghamshire, U.K.), according to the instructions of the manufacturer.

LTB₄ production by peritoneal macrophages

Peritoneal macrophages from mrp1−/− and wild-type mice were isolated by washing the peritoneal cavity with RPMI 1640 according to routine procedures. After isolation, macrophages were allowed to adhere to 6-well plates (10⁶ cells/cm²) for 1 h at 37°C, washed, and maintained in RPMI 1640/10% FCS until experimentation. For experiments in which LT production was stimulated, the medium was replaced by 2 ml RPMI 1640/10% FCS containing arachidonic acid. This medium was immediately aspirated again, resulting in a film of medium over the cells containing the stimuli. This method was used to minimize the amount of liquid in the culture system during stimulation, facilitating subsequent LT extraction by ethanol and allowing a more accurate measurement of LTs produced within cells and excreted from cells. Hence, this low volume method was used to en
duce simultaneous measurement of intracellular and extracellular LTs. Stimulation was performed for 20 min at 37°C with arachidonic acid (20 μM, final concentration). Arachidonic acid was chosen to stimulate lipoxy
genase metabolism rather than a calcium-raising agent (e.g., a ionophore), because calcium preferentially stimulates LTC₄ synthesis over LTB₄ generation (19). At the end of the 20-min incubation period, LTs were extr
tacted as described below. In a separate experiment, peritoneal macrophages were stimulated with heat-killed S. pneumoniae. For these experiments, polypropylene tubes were prefilled with 100 μl RPMI 1640 containing heat-kill S. pneumoniae (10⁷ CFU/ml), after which 250 μl of the peritoneal washing was added. Tubes were gently mixed and placed in the incubator at 37°C for 20 min. After incubation, supernatant was stored at −20°C until assays were performed.

Statistical analysis

Comparisons of survival data were made using the log-rank test. All other data are expressed as means ± SEM. Comparisons between means were conducted using the Wilcoxon test. Significance was set at p ≤ 0.05.

Results

mrp1−/− mice are resistant to S. pneumoniae pneumonia

To determine the role of mrp1 in host defense against pneumonia, we first assessed the outgrowth of pneumococci in lungs of mrp1−/− and wild-type mice after intranasal inoculation with 10⁴ CFU of S. pneumoniae. Although at 24 h postinoculation the number of CFUs in lungs was similar in both mouse strains, after 48 h wild-type mice had two orders of magnitude more CFUs in lungs.
Mice surviving for 14 days postinoculation appeared permanent

than \(mrp1 \sim /\) mice (1.9 ± 0.9 × 10⁸ and 1.0 ± 0.7 × 10⁶ CFU/ml, respectively; \(p < 0.05\); Fig. 1A). In addition, blood from \(mrp1 \sim /\) mice contained fewer pneumococci than wild-type mice at this time point (1.6 ± 1.6 × 10⁸ vs 6.1 ± 2.6 × 10⁶ CFU/ml; \(p < 0.05\)) with 37% of \(mrp1 \sim /\) mice and 75% of wild-type mice having positive blood cultures. The reduced growth of \(S. pneumoniae\) in \(mrp1 \sim /\) mice corresponded with an enhanced survival, the difference with wild-type mice becoming evident at 72 h postinoculation (\(p < 0.05\), Fig. 1B). 14-day survival rates were 7/16 (43.8%) for \(mrp1 \sim /\) mice and 2/16 (12.5%) for wild-type mice. Mice surviving for 14 days postinoculation appeared permanent survivors.

\(mrp1 \sim /\) mice display a reduced inflammatory response to \(S. pneumoniae\) within the pulmonary compartment, but increased LT\(B_4\) concentrations

Pneumonia elicits a number of inflammatory responses within the lungs that have been found to contribute to local antibacterial host defense. These “protective” responses include the influx of neutrophilic granulocytes, and the production of certain cytokines (TNF, IL-6, IL-12, IFN-\(\gamma\)) and chemokines (macrophage-inflammatory protein-2, KC) (17, 21–25). In a first attempt to obtain insight in the mechanism by which \(mrp1 \sim /\) mice are resistant against pneumococcal pneumonia, we compared these responses in \(mrp1 \sim /\) and wild-type mice. \(mrp1 \sim /\) mice demonstrated a reduced influx of granulocytes in BALF (Table 1), and a reduced production of protective cytokines and chemokines in lung homog- enates (Table II), when compared with wild-type mice. Hence, these data suggested that other mechanisms likely are responsible for the relative protection of \(mrp1 \sim /\) mice. As expected, LTC\(_4\) was barely detectable in BALF of \(mrp1 \sim /\) mice, while high levels of LTC\(_4\) were observed in BALF of wild-type mice (Table I). LT\(B_4\) release followed different kinetics than the release of LTC\(_4\), elevated LT\(B_4\) levels especially being detected 24 h postinoculation. Remarkably, at this time point, but not after 48 h, significantly higher LT\(B_4\) concentrations were found in BALF from \(mrp1 \sim /\) mice than in BALF from wild-type mice.

**Increased LT\(B_4\) production in \(mrp1 \sim /\) mice mediates increased resistance during pneumonia**

\(5-LO \sim /\) mice displayed reduced survival during \(Klebsiella pneumoniae\), and alveolar macrophages from these mice exhibited impaired bacterial phagocytosis and killing in vitro, which could be overcome by the addition of exogenous LT\(B_4\) (10). Therefore, to determine whether the increased LT\(B_4\) levels in \(mrp1 \sim /\) mice played a role in the relative protection of these mice during pneumonia, we treated \(mrp1 \sim /\) mice with the LT\(B_4\) receptor antagonist LT\(B_4\)-dimethyl amide. As evident from Fig. 2A, this treatment resulted in the complete abolition of the survival advantage of \(mrp1 \sim /\) mice over wild-type mice. In addition, we treated \(mrp1 \sim /\) and wild-type mice with MK-886 to determine whether the difference in survival between wild-type and \(mrp1 \sim /\) mice indeed was due to altered LT synthesis, or to an unknown function of mrp1 unrelated to LT release. In case the latter possibility is true, differences between wild-type and \(mrp1 \sim /\) mice should still be detectable after MK-886 treatment. However, mortality of wild-type and \(mrp1 \sim /\) mice was similar after treatment with MK-886 (Fig. 2B), suggesting that disruption of the mrp1 function in LT release mediates increased resistance of \(mrp1 \sim /\) mice to \(S. pneumoniae\)-induced pneumonia. Administration of MK-886 to mice without pneumonia for 2 wk did not produce any detectable toxicity. To ascertain that LT\(B_4\)-dimethyl amide blocks LT\(B_4\) effects in vivo, and that MK-886 inhibits LT production in vivo, we performed the following experiments. First, we established that i.p. injection of LT\(B_4\)-dimethylamide strongly reduced granulocyte influx into BALF after intranasal inoculation with LT\(B_4\) (Fig. 3A). Second, we found that i.p. injection of MK886 strongly attenuated LT\(B_4\) production by whole blood harvested 24 h later and stimulated ex vivo with heat-killed \(S. pneumoniae\) (Fig. 3B).

### Table I. LT levels and number of neutrophil cells in BALF

<table>
<thead>
<tr>
<th></th>
<th>LTC(_4) (pg/ml)</th>
<th>LT(B_4) (pg/ml)</th>
<th>Neutrophils, (\times 10^6/ml) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>(mrp1 \sim /)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>3 ± 2</td>
<td>16 ± 5</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>Control (mrp1 \sim /)</td>
<td>1 ± 1</td>
<td>228 ± 39*</td>
<td>24 ± 4*</td>
</tr>
<tr>
<td>Control wild type</td>
<td>12 ± 7</td>
<td>0 ± 0</td>
<td>14 ± 1</td>
</tr>
</tbody>
</table>

* Mice were intranasally inoculated with \(S. pneumoniae\) (10⁶ CFU) or sterile saline (control mice) at 0 h. The data represent means ± SEM of five or more mice per group.

* \(p < 0.05\), compared to \(mrp1 \sim /\) mice.
incubated semipermeabilized cells from the 4-4 clone of VN11

2

mrp1

injection for 14 consecutive days with either 0.4 nmol LTB 4 -dimethyl

mrp1

survival advantage of

Table II. Cytokine and chemokine concentrations in lung homogenates at 24 and 48 h after induction of pneumonia a

Table

<table>
<thead>
<tr>
<th>Cytokine and chemokine</th>
<th>mrp1−/− (24 h)</th>
<th>Wild Type (24 h)</th>
<th>mrp1−/− (48 h)</th>
<th>Wild Type (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF (pg/ml)</td>
<td>159 ± 30</td>
<td>121 ± 29</td>
<td>132 ± 12</td>
<td>256 ± 64</td>
</tr>
<tr>
<td>IL-6 (ng/ml)</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.05</td>
<td>0.43 ± 0.48</td>
<td>5.7 ± 2.0*</td>
</tr>
<tr>
<td>IL-12 (ng/ml)</td>
<td>0.68 ± 0.33</td>
<td>0.58 ± 0.19</td>
<td>0.79 ± 0.16</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>214 ± 41</td>
<td>148 ± 58</td>
<td>190 ± 37</td>
<td>412 ± 149</td>
</tr>
<tr>
<td>MIP-2 (pg/ml)</td>
<td>591 ± 183</td>
<td>271 ± 166</td>
<td>60 ± 43</td>
<td>4046 ± 2154*</td>
</tr>
<tr>
<td>KC (pg/ml)</td>
<td>279 ± 60</td>
<td>504 ± 146</td>
<td>375 ± 98</td>
<td>1610 ± 484*</td>
</tr>
</tbody>
</table>

a Mice were intranasally inoculated with S. pneumoniae (106 CFU) at 0 h. The data represent means ± SEM of five or more mice per group.

*, p < 0.05, compared to mrp1−/− mice.

an explanation for this phenomenon, we investigated the possibility that the increased intracellular accumulation of LTC4 in

mrp1−/− cells may reduce substrate competition between LTA4 hydroxase (the LTB4 producing enzyme) and LTC4 synthase for

the available LTA4 (the precursor of both LTB4 and LTC4). We tested the effect of increasing intracellular LTC4 concentrations on

LTB4 generation (see Materials and Methods). To this end we incubated semipermeabilized cells from the 4-4 clone of VN11

retrovirus-immortalized macrophages (20) with 100 ng/ml LTC4. Cells were stimulated for various time periods with arachidonic

acid and, subsequently, LTB4 production was measured. As shown in Fig. 4C, increasing intracellular LTC4 levels resulted in enhanced LTB4 release.

Discussion

mrp1 is involved in the cellular excretion of many different (anti-
cancer) drugs, glutathione-S-conjugates of lipophilic xenobiotics and cysteinyl LTs (4, 5, 14, 15). The recent generation of mrp1−/− mice has enabled detailed analysis of the function of this cellular pump in vivo. mrp1−/− mice appeared hypersensitive to the anti-
cancer drug etoposide (6, 26, 27), and had an impaired response to

5-LO

abolishes the survival advantage of

mrp1

; in this study we evaluated the pathophysiologic relevance of this defect in a well established model of pneumonia induced by S.

pneumoniae. mrp1−/− mice were found to have an increased resis-
tance against pneumococcal pneumonia, as reflected by an en-
hanced clearance of bacteria from the lungs and an increased sur-
vival, by a mechanism that involved increased LTB4 synthesis.

The local production of LTs, and in particular of LTB4, has been

considered important for an effective host defense against invading pathogens in the pulmonary compartment. The alveolar macro-

phage is a major source of LTB4 in the lung (28, 29), where this

LT can stimulate microbicidal activities of phagocytic cells (10,

30). 5-LO−/− mice, which have a general deficiency of all LTs,
demonstrated an enhanced lethality from Klebsiella pneumonia in association with an increased outgrowth of bacteria in lungs (10).

We report here that mrp1−/− mice behave completely opposite to

5-LO−/− mice during pneumonia. By treating mrp1−/− mice with

MK-886, we confirmed that their increased resistance was related to a selective disruption of the cysteinyl LT pathway. Indeed, MK-

886 administration abolished the survival advantage of mrp1−/− mice, indicating that the relative protection of these mice was not

mediated by a function of mrp1 unrelated to LT metabolism. In addition, we confirmed that mrp1−/− mice are unable to release

significant quantities of LTC4 in BALF during pneumonia. Quite

FIGURE 2. A. The LTB4-antagonist LTB4-dimethyl amide reverses the survival advantage of mrp1−/− mice. mrp1−/− mice received a daily i.p.
injection for 14 consecutive days with either 0.4 nmol LTB4-dimethyl amide (●; n = 18) or isotonic saline (□; n = 17). Wild-type mice were
injected daily with isotonic saline (●; n = 14). B. Inhibition of 5-LO abolishes the survival advantage of mrp1−/− mice. mrp1−/− mice (□; n = 17) and wild-type mice (●; n = 14) received a daily i.p. injection with MK-886 (1 nmol) for 14 consecutive days. In all experiments shown, mice were intranasally inoculated with 104 CFU S. pneumoniae at 0 h.

injection of LTB4-dimethyl amide (0.4 nmol) strongly reduced granulocyte influx into BALF after intranasal inoculation with LTB4 (2 × 10−4 μmol) (●). Data are mean ± SE of four determinations for each point. *, p < 0.05. B. Compared with i.p. injection of sterile saline (□), i.p. injection of MK886 (1 nmol) strongly attenuated LTB4 production by whole blood harvested 24 h later and stimulated ex vivo with heat-killed S. pneumoniae (105 CFU) (●). Data are mean ± SE of seven to nine determinations for each point. *, p < 0.05.
unexpectedly, LTB$_4$ concentrations in BALF were higher in mrp$^{1/−}$ mice than in wild-type mice early after the induction of pneumonia. Additional experiments revealed that these increased LTB$_4$ levels were essential for the protection of mrp$^{1/−}$ mice, as indicated by the finding that treatment with the LTB$_4$ receptor antagonist LTB$_4$-dimethyl amide reversed the survival advantage of mrp$^{1/−}$ mice. Alternatively, the enhanced bacterial clearance of mrp$^{1/−}$ mice may be a direct consequence of intracellular accumulation of LTC$_4$ in these mice, and may be independent of the observed enhanced LTB$_4$ release. Increased intracellular levels of LTs have been associated with altered cellular Ca$^{2+}$ influx (31) and actin reorganization (31), and hence it is well envisionable that such accumulation would result in enhanced bactericidal activity of phagocytes. Contradicting with this hypothesis is the remarkably negative effect of LTB$_4$ antagonists in mrp$^{1/−}$ mice. Therefore, we favor the hypothesis that the increased LTB$_4$ release mediates enhanced bacterial clearance in these mice, but obviously definitive proof of this notion awaits experiments in LTC$_4^{−/−}$ synthase mice.

MK886 treatment did not influence survival in normal wild-type mice. This finding contrasts with the finding of increased mortality of 5-LO$^{−/−}$ mice during pneumonia caused by K. pneumoniae (10). Possible explanations for this discrepancy include incomplete inhibition of 5-LO by MK886 and/or the fact that the model may be too severe to detect a harmful effect of MK886 in normal mice (i.e., <20% of wild-type mice recovered from pneumococcal pneumonia).

It should be noted that in wild-type mice, LTB$_4$ release in BALF occurred earlier than LTC$_4$ release. This finding is surprising in light of the fact that both lipids are probably predominantly derived from alveolar macrophages. We do not have a clear explanation for this observation; further studies are warranted to determine possible underlying mechanisms. In addition, the finding at 48 h of similar LTB$_4$ concentrations in BALF of wild-type and mrp$^{1/−}$ mice is remarkable in light of the presence of up to 4-fold more neutrophils in BALF of the former strain, considering that neutrophils can be a source of LTB$_4$. Taken together with the fact that LTB$_4$ levels were already significantly elevated at 24 h (i.e., when neutrophil influx was still marginal), these data suggest that during pneumonia alveolar macrophages are a more important source of LTB$_4$ than neutrophils.

Two of us (J.W. and P.B.) have reported that the capacity of unstimulated mrp$^{1/−}$ bone-marrow derived mast cells to secrete LTB$_4$ is unaltered in comparison to wild-type cells (6). In the present study, we documented enhanced LTB$_4$ secretion in BALF of intact mrp$^{1/−}$ mice during pneumonia. In accordance, one of us (S.J.H.v.D.) recently found higher LTB$_4$ concentrations in colon homogenates of mrp$^{1/−}$ mice than in colon homogenates of wild-type mice in a model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis (T. Len Hove, P. Drillenburg, F. Wynholds, A. te Velde, and S. van Devenstir, manuscript in preparation). Importantly, also macrophages isolated from mrp$^{1/−}$ mice displayed increased LTB$_4$ synthesis, demonstrating that enhanced LTB$_4$ production is an inherent property of these cells and is not secondary to other effects induced by the absence of mrp1. The absence of mrp1 results in enhanced bacterial clearance in intracellular LTC$_4$ (6). Here we provide evidence that this increased intracellular LTC$_4$ concentrations can stimulate LTB$_4$ production. Indeed, semipermeabilized macrophages exposed to high concentrations of LTC$_4$ released more LTB$_4$ upon stimulation. It should be noted that immortalized macrophages and peritoneal, rather than alveolar, macrophages from wild-type and mrp$^{1/−}$ mice were used for metabolic LT studies in vitro. Although alveolar macrophages may have been more appropriate for these studies, we consider our in vitro data reliable especially because they are in accordance with the in vivo observation of elevated LTB$_4$ concentrations in BALF of mrp$^{1/−}$ mice at 24 h after the induction of pneumonia. Hence, together, these data suggest that the increased intracellular LTC$_4$ levels, due to the absence of mrp1, enhance LTB$_4$ generation, which then results in enhanced resistance to pneumococcal pneumonia. Enhanced LTB$_4$ synthesis could occur in two ways. Intracellular LTC$_4$ accumulation may give rise to product inhibition of LTC$_4$ synthase thus removing substrate competition between LTC$_4$ synthase and LTA$_4$ hydrolase (the LTB$_4$ producing enzyme) for LTA$_4$ (the precursor of both LTB$_4$ and LTC$_4$), yielding enhanced LTB$_4$ synthesis. Alternatively, LTC$_4$ may be converted into a LTB$_4$ precursor. Because an enzymatic activity supporting such a reaction has hitherto not been described, we favor the former explanation.
Normal wild-type mice, rather than heterozygous mrp1+/− mice, were used as controls in our investigations. Although Western blot results indicate that heterozygous mrp1(−/−) mice show less expression of mrp1 protein than wild-type mice, heterozygous mrp1(−/−) mice are identical with normal wild-type mice with respect to relevant physiological functions, in particular LTC4 transport (6). Therefore, we consider it less likely that heterozygous mrp1(−/−) mice have a phenotype that is different from wild-type mice in models in which differences in LT metabolism determine the difference in outcome between mrp1(−/−) mice and wild-type mice.

Although mrp1(−/−) mice had elevated LTβ2 levels in BALF at 24 h after infection, granulocyte numbers in BALF were similar in both mouse strains at that time point, while at 48 h wild-type mice even had significantly more granulocytes in their BALF than mrp1(−/−) mice. These findings are unexpected in light of the fact that LTβ2 is considered a potent chemoattractant for neutrophils. However, it should be noted that LTβ2 also can enhance bacterial killing by phagocytic cells, which at least in part may have increased antibacterial defense of mrp1(−/−) mice at early time points after infection. Indeed, the addition of exogenous LTβ2 to peritoneal or alveolar macrophages increased bacterial phagocytosis and killing of Salmonella typhimurium, P. aeruginosa and K. pneumoniae (10, 30). At later time points, the extent of inflammation (i.e., number of neutrophils in BALF, cytokine levels in lungs) (10, 30). At later time points, the extent of inflammation (i.e., number of neutrophils in BALF, cytokine levels in lungs) likely merely is a reflection of the bacterial load present in the pulmonary compartment. Hence, at 48 h, the presence of many more S. pneumoniae CFUs in lungs of wild-type mice, caused by a relatively reduced defense against pneumococci early after the infection, likely are responsible for the higher granulocyte counts and cytokine levels in these mice. This is also why we consider measurements of inflammatory parameters later than 48 h after the induction of pneumonia of little value, i.e., mice dying from pneumococcal pneumonia invariably show extensive inflammation in lungs (our unpublished observation), and comparisons between mrp1(−/−) and wild-type mice would have been biased by an unbalanced mortality in these strains.

mrp1 has attracted widespread interest due to its action as a multidrug-resistance protein. In the present study, we have demonstrated that mice lacking functional mrp1 are less sensitive to pneumonia and wild-type mice. Indeed, the addition of exogenous LTB4 to peritoneal macrophages induced bacterial phagocytosis and killing of Salmonella typhimurium, P. aeruginosa and K. pneumoniae (10, 30). At later time points, the extent of inflammation (i.e., number of neutrophils in BALF, cytokine levels in lungs) likely merely is a reflection of the bacterial load present in the pulmonary compartment. Hence, at 48 h, the presence of many more S. pneumoniae CFUs in lungs of wild-type mice, caused by a relatively reduced defense against pneumococci early after the infection, likely are responsible for the higher granulocyte counts and cytokine levels in these mice. This is also why we consider measurements of inflammatory parameters later than 48 h after the induction of pneumonia of little value, i.e., mice dying from pneumococcal pneumonia invariably show extensive inflammation in lungs (our unpublished observation), and comparisons between mrp1(−/−) and wild-type mice would have been biased by an unbalanced mortality in these strains.

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