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CHAPTER 9

Differential Role of Interleukin-6 in Lung Inflammation induced by Lipoteichoic Acid and Peptidoglycan from *Staphylococcus aureus*

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Lipoteichoic acids and peptidoglycans are major components of the cell walls of gram-positive bacteria that trigger inflammatory responses in vitro. To study the in vivo effects of lipoteichoic acid and peptidoglycan from *S. aureus* in lungs, and to determine the role of IL-6 herein, these compounds were intranasally administered to IL-6 gene deficient (IL-6-/-) and wild-type (IL-6+/+) mice. In IL-6+/+ mice lipoteichoic acid and peptidoglycan induced acute pulmonary inflammation in a dose-dependent way, characterized by neutrophilic influx and IL-6 production in the bronchoalveolar lavage fluid. Endogenously produced IL-6 attenuated inflammation induced by 10 μg lipoteichoic acid, as reflected by enhanced neutrophil influx, and increased TNFα, MIP-1α, and KC release into bronchoalveolar lavage fluid of IL-6-/- mice compared to IL-6+/+ mice. By contrast, pulmonary inflammation induced by 100 μg lipoteichoic acid was similar (neutrophil influx) or even tended to be attenuated (cytokine and chemokine release) in IL-6-/- mice. Endogenous IL-6 increased inflammation induced by peptidoglycan, as reflected by decreased neutrophil influx into lungs of IL6-/- mice compared to IL-6+/+ mice. These data suggest that IL-6 plays an anti-inflammatory role during lipoteichoic acid-induced pulmonary inflammation, which is dependent on the severity of the inflammatory challenge, and a pro-inflammatory role in peptidoglycan-induced acute lung inflammation. Thus, the contribution of IL-6 to lung inflammation may vary with the stimulus used.
INTRODUCTION

The incidence of gram-positive infections has increased considerably over the past few years, and gram-positive organisms are now as common as gram-negative bacteria in causing sepsis (1-3). Staphylococcus aureus is the most frequently isolated gram-positive pathogen in nosocomial infections associated with severe complications (4). In addition, S. aureus accounts for 6-33% of bacterial isolates from patients with hospital-acquired pneumonia (5,6). Gram-positive inflammation is presumed to be due to bacterial cell wall components, such as lipoteichoic acid (LTA) and peptidoglycan (PepG). LTAs are phosphate-containing polymers, which are considered as surface antigens, as well as membrane components, that mediate the attachment of certain bacteria to host cells (7,8). PepG is a glycosyl macromolecule interlinked by peptide bridges, which is especially abundant in gram-positive bacteria where it provides stress-resistance and determines the form of the bacterial cell wall. Both LTA (9,10) and PepG (10-12) can induce inflammatory responses in vitro. Furthermore, intravenous administration of S. aureus LTA and PepG resulted in a systemic inflammatory response syndrome in rats (13). Knowledge of the in vivo effect of LTA or PepG from S. aureus within the lungs is highly limited. Such knowledge is important, considering the role of S. aureus in nosocomial pneumonia.

Several lines of evidence suggest that the pleiotropic cytokine interleukin (IL)-6 is involved in regulation of inflammatory responses during gram-positive bacterial infection. Elevated IL-6 levels were detected in plasma of patients with gram-positive sepsis (14), and in bronchoalveolar lavage fluid (BALF) of patients with pneumonia (15). In a murine model of pneumococcal pneumonia, this endogenously released IL-6 played an important role in antibacterial host defense, as reflected by enhanced bacterial outgrowth and increased mortality in IL-6 gene deficient mice (16). The role of IL-6 in the pathophysiology of inflammation in general, and of inflammation in the pulmonary compartment in particular, has not been elucidated completely. Indeed IL-6 has been reported to have both pro-inflammatory and anti-inflammatory effects (17,18).

In the present study we sought to determine the acute pulmonary inflammatory response caused by local exposure to LTA or PepG from S. aureus and the regulatory role of IL-6 herein. For this purpose, IL-6 gene deficient (IL-6-/-) and wild type (IL-6+/+) mice received LTA or PepG via the intranasal route.
METODS

Mice
For experiments with LTA 8 to 10-week old female IL-6/- mice on a BALB/c background were kindly donated by Dr. Manfred Kopf (Basel Institute of Immunology, Basel, Switzerland). IL-6+/+ BALB/c mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). At the time when the studies with PepG were done, IL-6/- BALB/c mice were not available anymore. For these experiments, 8-10 week old female IL-6/- mice on a C57Bl/6 background and their normal C57Bl/6 wild type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The Animal Care and Use Committee of the University of Amsterdam, the Netherlands, approved all experiments.

Materials
LTA from S. aureus was purchased from Sigma (St. Louis, Mo). PepG was prepared from S. aureus (a clinical isolate derived from a patient with catheter-related sepsis) according to the method of Peterson et al. (19) as described earlier (12). The amount of lipopolysaccharide (LPS) present in LTA and PepG was determined with the chromogenic Limulus Amoebocyte Lysate assay (Chromogenix, Mölndal, Sweden) and was respectively 4.15 pg LPS/mg LTA and below the detection limit (2.5 pg/ml). Hence, 100 μg LTA (the highest dose used in our experiments) contained <1 pg LPS. This LPS dose is not capable of eliciting an inflammatory reaction in the lung (data not shown).

Experimental design
Briefly, mice were anaesthetized by inhalation with isoflurance (Abbott Laboratories Ltd., Kent, U.K.), after which sterile saline, LTA or PepG dissolved in saline was administered intranasally (i.n.). After 4h., mice were anaesthetized by intraperitoneal injection of Hypnorm (Janssen Pharmaceutica, Beere, Belgium) and midazolam (Roche, Mijdecht, the Netherlands), sacrificed by bleeding from the vena cava inferior and lungs were lavaged.

Analysis of BALF
Broncho-alveolar lavage (BAL) and leukocyte differentiation was done as described
previously (20,21). BALF cytokines were measured by specific ELISAs according to the manufacturers' instructions (R&D Systems, Minneapolis, MN). The detection limits were 37 pg/mL (IL-6), 31 pg/mL (tumor necrosis factor-alpha: TNFα), 8 pg/ml (IL-10), 8 pg/ml (IL-1α), 8 pg/ml (IL-1β), 31 pg/ml (macrophage inflammatory protein-1-alpha: MIP-1α), 12 pg/ml (KC), and 47 pg/ml (MIP-2).

Histological analyses
Lungs for histologic examination were harvested at 4h after infection, fixed in 4% formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E). Granulocyte staining was done with FITC-labeled anti-mouse Ly-6 G mAb (Pharmingen, San Diego, CA) exactly as described previously (22). The inflammatory infiltrate was scored semi-quantitatively and the amount of abscesses (well-defined inflammatory collections) was counted in 12 mm² (5 fields of x10).

Stimulation of peritoneal macrophages
Peritoneal macrophages from IL-6+/+ (C57Bl/6) and IL-6-/- mice (C57Bl/6 background) were isolated by washing the peritoneal cavity with RPMI 1640 (Bio Whittaker, Verviers, Belgium). Collected cells maintained in medium (RPMI 1640, 10% fetal calf serum, 1% antibiotic-antimycotic (GibcoBRL, Life Technologies, Rockville, MD)) were allowed to adhere to 96-wells tissue culture plates (1x10⁴ cells) for 1 hour at 37°C, after which nonadherent cells were removed by rinsing the monolayer. Macrophages were incubated with medium containing either LTA or PepG.

Statistical analysis
All values are expressed as mean ± SEM. Differences between groups were analysed by Mann-Whitney U test. P ≤ 0.05 was considered statistically significant.

RESULTS

LTA induces a dose-dependent influx of PMNs and release of IL-6 in BALF
To determine pulmonary inflammatory responses to LTA in vivo, IL-6+/+ mice were intranasally inoculated with either sterile saline (control mice), or 10, 100 or 150 μg LTA, and BAL was performed after 4h. LTA induced a dose-dependent increase in leukocytes in BALF. When compared to controls, BALF leukocyte counts were 5-fold higher after
administration of 10 µg LTA, 20-fold higher after instillation of 100 µg and 38-fold higher after inoculation with 150 µg LTA (Fig. 1A). The LTA-induced increase in the number of cells in BALF mainly resulted from influx of PMNs, although numbers of alveolar macrophages (AMs) were also increased. Lymphocytes were present in low numbers and were similar in all groups. Inhalation of LTA was also associated with a dose-dependent release of IL-6 in BALF (Fig 1B), whereas IL-6 remained undetectable in BALF of saline-treated mice. In subsequent experiments, the role of IL-6 in LTA-induced lung inflammation was studied after administration of either 10 or 100 µg LTA.

Inflammatory responses in IL-6-/− and IL-6+/+ mice after low dose of LTA

To investigate the role of IL-6 in the pulmonary effects of LTA at a dose that caused mild lung inflammation, IL-6+/+ and IL-6-/− mice received 10 µg LTA i.n. and BALF was obtained 4h. later. The absence of IL-6 resulted in higher leukocyte counts in BALF, which was mainly caused by a greater influx of PMNs (Fig. 2).
In accordance with cell counts in the BALF, the lungs of IL-6-/- mice (Fig. 3A) treated with low dose LTA showed more diffuse and dense inflammatory infiltrates than IL-6+/+ mice (Fig. 3B) 4 h after inoculation. The influx of PMNs was also increased in IL-6-/- (inset Fig. 3A) mice compared to IL-6+/+ mice (inset Fig. 3B).

To understand the impact of IL-6 deficiency on cytokine and chemokine responses, TNF-α, IL-1α, IL-1β, IL-10, MIP-1α, KC and MIP-2 were measured in BALF. While LTA induced a TNFα response in both groups of mice, BALF TNFα concentrations...
were more than 5-fold higher in IL-6-/- mice than in IL-6+/+ mice \((P=0.002)\) (Fig. 2). Further, in IL-6-/- animals, MIP-1-\(\alpha\) and KC levels were respectively 2.6-fold \((P=0.01)\) and 1.5-fold higher \((P=0.07)\), than in IL-6+/+ mice, whereas MIP-2 levels were not different between both groups (Fig. 2). LTA did not induce detectable release of IL-1\(\alpha\), IL-1\(\beta\) or IL-10 in BALF of either mouse strain.

**Inflammatory responses in IL-6-/- and IL-6+/+ mice after high dose LTA**

In contrast to pulmonary inflammation induced by 10 \(\mu\)g LTA, administration of 100 \(\mu\)g LTA was not associated with differences in leukocyte influx in IL-6-/- and IL-6+/+ mice (Fig. 2). Furthermore, TNF-\(\alpha\), MIP-1-\(\alpha\) and MIP-2 \((P=0.012)\) tended to be lower in IL-6-/- mice, while KC concentrations were similar in BALF of both mice strains.

**PePG induces a dose-dependent influx of PMNs and release of IL-6 in BALF**

Next, we wished to determine the role of IL-6 in pulmonary inflammatory responses to PePG from *S. aureus*. To determine the dose of PePG that induced a comparable immune response as 10 \(\mu\)g LTA, IL6+/+ mice were intranasally inoculated with either 50 or 180 \(\mu\)g PePG, after which BAL was performed after 4 hours. Inoculation with PePG resulted in a dose dependent increase in numbers of leukocytes and IL-6 levels in the BALF (Fig. 4). Leukocyte differentiation revealed that the increase in BALF cells resulted from an influx of PMNs. Additional experiments using IL-6+/+ and IL-6-/- mice were performed with 50 \(\mu\)g PePG.
Inflammatory responses in IL-6-/- and IL-6+/+ mice after PepG

Having established that PepG induced an influx of PMNs into lungs, we were interested in the role of IL-6 herein. Surprisingly, the number of recruited PMNs in the lungs was markedly decreased in IL-6-/- mice compared to IL-6+/+ mice at 4 h. after inoculation (P=0.004) (Fig. 5).

Histopathological analysis of the lungs of mice inoculated with PepG revealed numerous well-defined collections of leukocytes (abscesses) generally centred around small bronchi together with a slight interstitial inflammatory infiltrate. In line with the results of cell counts, IL-6-/ - mice (Fig 6A) displayed less abscesses than IL-6+/+ mice (Fig 6B) (13.1 ± 2.4 vs 17.4 ± 3.3 per 12 mm², not statistically significant) partly consisting of PMNs (see insets Fig 6).

To determine the role of IL-6 in PepG-induced cytokine and chemokine release in the lungs, the concentrations of TNFα, MIP-1α, MIP-2 and KC were measured in BALF of IL-6-/ - and IL-6+/+ mice, 4h. after PepG administration. No significant differences were found between IL-6+/+ and IL-6-/ - mice (Fig. 5).
Role of IL-6 in TNFα release by isolated macrophages

Having established that endogenous IL-6 inhibited TNFα release in BALF in response to LTA but not to PepG, we were interested in the role of IL-6 by TNFα production by isolated macrophages. To determine whether LTA and PepG of *S. aureus* can induce IL-6 release *in vitro*, mouse peritoneal IL-6+/+ macrophages were exposed to increasing concentrations LTA and PepG during 4h and the levels of IL-6 were evaluated in culture supernatants. As shown in Fig. 7A and B, both LTA and PepG induced IL-6 in a dose-dependent manner. In order to investigate the role of IL-6 in macrophage responses to LTA and PepG, we examined the responsiveness of IL-6+/+ and IL6-/- macrophages to increasing concentrations of these bacterial components. The production of TNFα in response to *S. aureus* LTA was significantly higher in IL-6-/- mice at the two highest doses (Fig. 7C). In contrast, IL-6+/+ and IL-6-/- macrophages produced comparable amounts of TNFα in response to *S. aureus* PepG (Fig. 7D).

**DISCUSSION**

*Staphylococcus aureus* is a major cause of hospital-acquired pneumonia (6). LTA and PepG are cell wall components of *S. aureus*, which have pro-inflammatory activities *in vitro*. The present study reports on the *in vivo* effects of LTA and PepG from *S. aureus* in the lung. Intranasal administration of LTA or PepG led to enhanced leukocyte influx predominated by PMNs. Increasing the dose clearly led to an increase in pulmonary inflammation. In addition, LTA and PepG induced elevated lung levels of IL-6 in a dose-dependent manner. Endogenous IL-6 was found to exert a negative feedback effect on PMN recruitment and the local release of TNFα, MIP-1α and KC at the lower
but not the higher, LTA dose tested. In contrast to its role in the pulmonary response to low dose LTA, endogenous IL-6 positively influenced PMN influx into the lungs after PepG instillation.

LTA partially can signal via the same mechanisms as LPS from gram-negative bacteria. CD14 functions as a ligand binding receptor of LPS (23), but was also shown to recognize LTA (24). In addition, the LPS signalling receptor toll-like receptor 4 (TLR4) is likely also involved in the recognition of LTA (25). Although data exist indicating that TLR2 can be a signal transducer for LTA from *Bacillus subtilis, Streptococcus pyogenes, and Streptococcus sanguis* (26), others have shown that TLR4 and not TLR2 is required for signal transduction of *S. aureus* LTA (25,27,28). TLR2 has been reported to be the signalling receptor for PepG from *S. aureus* (25,29). The data of the current study suggest that intrapulmonary administration of LTA provokes an inflammatory response that is very similar to that induced by LPS. LPS administered to the pulmonary compartment caused a PMN influx and induction of TNF-α and IL-6 in BALF of mice (30,31). Likewise, in this study, intranasal administration of LTA resulted in an increase in PMNs and elevated BALF levels of TNF-α and IL-6. Like LTA, the intranasal inoculation of
PepG also resulted in increased PMN influx and cytokine levels, but pathological analysis of the lungs of these mice revealed extensive differences when compared to lungs of LTA-inoculated mice. LTA-inoculation induced an interstitial inflammatory infiltrate in the lungs whereas PepG exposure led to the formation of numerous abscesses generally centred around small bronchi.

Several studies have reported on the importance of IL-6 for inflammatory responses during bacterial pulmonary inflammation. High IL-6 levels were found in BALF of pneumonia patients (15) and in lung and plasma of mice infected with *Streptococcus pneumoniae* (16). Moreover, elevated IL-6 levels were found in mice during acute lung and systemic inflammation caused by LPS (32). IL-6 has pro-inflammatory as well as anti-inflammatory properties. In a carrageenan-induced pleurisy model, endogenous IL-6 played a pro-inflammatory role, as reflected by reduced PMN infiltration and diminished lung injury in IL-6/-/- mice (33). In contrast, IL-6 played an anti-inflammatory role in an LPS-induced acute lung inflammation model (32). The data of the current study suggest that IL-6 plays an anti-inflammatory role in lung inflammation caused by low dose LTA, but that this anti-inflammatory role is lost (PMN recruitment) or even is converted into a modest pro-inflammatory role (cytokine and chemokine release) during lung inflammation induce by high dose LTA. Interestingly, our findings demonstrate that IL-6 plays a pro-inflammatory role (PMN recruitment, abscess formation) in pulmonary inflammation induced by PepG of *S. aureus*. Together, these data suggest that the role of IL-6 in inflammation depends on the stimulus and/or the model of inflammation used. As mentioned earlier, LTA and PepG can signal via different receptors, which could be the basis for the pleiotropic characteristics of IL-6. During inflammation induced by LPS (32) and LTA (this study), bacterial components that signal at least in part via TLR4, IL-6 plays an anti-inflammatory role. However, a pro-inflammatory role of IL-6 has been demonstrated in mice with a PepG-induced pulmonary inflammation (this study) and in mice with a non-septic shock induced by zymosan (34). Interestingly, zymosan and PepG are both ligands for TLR2 (27).

TNFα production by IL-6/-/- macrophages was higher than IL-6+/+ macrophages after incubation with LTA. In addition, BALF TNFα levels were higher in IL-6/-/- than in IL-6+/+ mice after low dose LTA, suggesting that endogenous IL-6 inhibits TNFα production after LTA stimulation. This is in line with observations that IL-6 inhibits transcription of the TNFα gene (35). Moreover, recombinant IL-6 reduced TNFα release in mice intratracheally administered with LPS (36). It is furthermore
known that TNFα is an inducer of IL-6, therefore the inhibitory effect of IL-6 on TNFα production elicited by low dose LTA may be the negative arm of a regulatory circuit. In contrast, TNFα levels in supernatants of PepG-stimulated IL-6-/− macrophages and BALFs of PepG-inoculated IL-6-/− mice were not significantly different from IL-6+/+ macrophages and mice. In a model of carrageenan-induced pleurisy (33) and collagen-induced arthritis (37) IL-6-/− mice had even lower TNFα levels than IL-6+/+ mice. Together, this indicates that TNFα production is regulated in a different way during LPS- (32) and low-dose LTA-induced acute lung inflammation versus high-dose LTA-and PepG-induced pulmonary inflammation.

Elevated levels of MIP-1α in the lung after low dose LTA could be a consequence of increased TNFα levels. Exogenous TNFα has been shown to be a potent stimulator for MIP-1α secretion by human PMNs and neutralizing anti-TNFα antiserum partially blocked this expression (38). Interestingly, IL-6 deficiency resulted in increased KC release in BALF without influencing MIP-2 levels after low dose LTA, suggesting that IL-6 influences the production of these chemokines differentially. Although a clear explanation for this finding is not available, a recent investigation also reported on differential expression of MIP-2 and KC during lung inflammation. Indeed, mice deficient for both type I TNFα receptor and type I IL-1 receptor demonstrated reduced release of KC, but unaltered MIP-2 secretion into BALF upon pulmonary exposure to E. coli (39).

These data indicate that IL-6 plays a role in increasing PMN accumulation at sites of low dose LTA-induced lung inflammation, probably at least in part by increasing the local induction of cytokines/chemokines. Although MIP-2 levels were moderately decreased in IL-6-/− mice, alterations in cytokine/chemokine concentrations could not explain the reduced PMN recruitment after PepG administration.

We chose to investigate inflammatory responses in BALF at 4 h after intranasal administration of LTA or PepG for several reasons. First, this time point seems most suitable to concurrently evaluate neutrophil influx and cytokine release (32,40). Second, Xing et al. demonstrated that this time point is suitable to reliably study the role of endogenous IL-6 in LPS-induced lung inflammation (32). Third, many previous studies investigated the regulation of LPS-induced pulmonary inflammation 3-6 h after the challenge, allowing easy comparison with the present data (41,42,43).
In conclusion, we demonstrate that in vivo administration of LTA and PepG from *S. aureus* to the pulmonary compartment triggered acute lung inflammation, characterized by PMN influx and a strong induction of IL-6 and other cytokines and chemokines in BALF. The absence of IL-6 resulted in a more profound pro-inflammatory response at a LTA dose that caused relatively mild inflammation. At a higher LTA dose, however, IL-6 deficiency was not associated with anti-inflammatory effects. The absence of IL-6 during PepG-induced pulmonary inflammation resulted in a reduced PMN infiltration. These data suggest that the role of IL-6 in lung inflammation induced by *S. aureus* LTA and PepG depends on the severity of the challenge and on the stimulus used.

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