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Pulmonary Immunostimulation with MALP-2 in Influenza Virus-Infected Mice Increases Survival after Pneumococcal Superinfection

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Pulmonary infection with influenza virus is frequently complicated by bacterial superinfection, with *Streptococcus pneumoniae* being the most prevalent causal pathogen and hence often associated with high morbidity and mortality rates. Local immunosuppression due to pulmonary influenza virus infection has been identified as a major cause of the pathogenesis of secondary bacterial lung infection. Thus, specific local stimulation of the pulmonary innate immune system in subjects with influenza virus infection might improve the host defense against secondary bacterial pathogens. In the present study, we examined the effect of pulmonary immunostimulation with Toll-like receptor 2 (TLR-2)-stimulating macrophage-activating lipopeptide 2 (MALP-2) in influenza A virus (IAV)-infected mice on the course of subsequent pneumococcal superinfection. Female C57BL/6N mice infected with IAV were treated with MALP-2 on day 5 and challenged with *S. pneumoniae* on day 6. Intratracheal MALP-2 application increased proinflammatory cytokine and chemokine release and enhanced the recruitment of leukocytes, mainly neutrophils, into the alveolar space of IAV-infected mice, without detectable systemic side effects. Local pulmonary instillation of MALP-2 in IAV-infected mice 24 h before transnasal pneumococcal infection considerably reduced the bacterial number in the lung tissue without inducing exaggerated inflammation. The pulmonary viral load was not altered by MALP-2. Clinically, MALP-2 treatment of IAV-infected mice increased survival rates and reduced hypothermia and body weight loss after pneumococcal superinfection compared to those of untreated coinfected mice. In conclusion, local immunostimulation with MALP-2 in influenza virus-infected mice improved pulmonary bacterial elimination and increased survival after subsequent pneumococcal superinfection.

Pneumonia is a significant cause of morbidity and the fourth leading cause of death worldwide (http://www.who.int/mediacentre/factsheets/fs310/en/), with *Streptococcus pneumoniae* being the most prevalent causative agent identified in lower respiratory tract infections (1, 2). The risk of pneumonia is greatly enhanced in specific pathological situations with an impaired pulmonary host defense, including long-term ventilation (3), stroke-induced immune depression (4, 5), sepsis-associated immune paralysis (6), and viral lung infections (7). In particular, pulmonary infections with seasonal circulating (8) or pandemic (9) influenza viruses are frequently complicated by bacterial superinfection, resulting in a severe pneumonia often associated with high mortality rates. *S. pneumoniae* is one of the most common bacterial pathogens of severe postinfluenza bacterial pneumonia (8).

Influenza viruses have been reported to impair the pulmonary host defense against bacteria via different mechanisms, thus promoting secondary bacterial infections. Bacterial adherence was shown to be facilitated by influenza virus-induced cytolysis and apoptosis (10, 11) and by upregulation of platelet-activating factor receptor expression in pulmonary epithelial cells (12). Influenza virus-induced type I interferons (IFNs) attenuated neutrophil recruitment and activation in murine lungs by impairing the production of neutrophil chemoattractants, thereby sensitizing the host to secondary bacterial pneumonia (13, 14). Moreover, infection with influenza virus decreased phagocytosis and intracellular reactive oxygen species generation in neutrophils (15), and IFN-α/β (type I) (16) and IFN-γ (type II) (17) mediated reduced bacterial clearance by macrophages. In addition, the anti-inflammatory cytokine interleukin-10 (IL-10) has been reported to be an important mediator of the immunosuppressive state during pulmonary influenza virus infection (18). Importantly, recent experimental studies suggested that local treatment with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (19) or its intrinsic overexpression (20) improves the antibacterial defense in influenza virus-infected mice by increasing the antimicrobial capacity of phagocytic cells. Thus, therapeutic strategies aimed at compensating the locally compromised immune responses and reestablishing the early antibacterial innate host defense during pulmonary influenza virus infection may be promising.

The recognition of highly conserved pathogen-associated molecular patterns (PAMPs) of bacteria invading the respiratory tract

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by pattern recognition receptors of local host cells, including Toll-like receptors (TLRs), is a crucial step in host defense during infection and leads to the induction of local and systemic inflammatory responses (21). Preactivation of TLRs by bacterial lysates (22, 23) or specific TLR agonists (24, 25) in naive lungs has been reported to stimulate a protective pulmonary innate immune response in various experimental models.

In pneumococcal infection, TLR-2 activation induces an early innate immune response (26) and enhances pneumococcal clearance (27). In a previous study using a murine model of pneumococcal pneumonia (28), we demonstrated that local delivery of the chemically synthesized TLR-2/6 agonist macrophage-activating lipopeptide of 2 kDa (MALP-2) (29, 30), which was originally derived from *Mycoplasma fermentans* (31), evoked an upregulation of TLR-2 expression in pulmonary cells and induced the release of proinflammatory mediators and leukocyte recruitment into the naive lung. Moreover, pulmonary MALP-2 pretreatment increased the local host defense and survival in mice with pneumococcal pneumonia (28).

In the present study, it was hypothesized that pulmonary treatment with MALP-2 may also increase the pulmonary innate immune response in influenza virus-infected mice and improve the pulmonary host defense against *S. pneumoniae* superinfection. Therefore, influenza A virus (IAV)-infected mice were treated by intratracheal MALP-2 instillation 24 h prior to pneumococcal infection. The pulmonary inflammatory response to MALP-2 in influenza virus-infected lungs, as well as the course of combined influenza and pneumococcal pneumonia, was examined with respect to survival, inflammatory response, pathological lung alterations, and pathogen clearance.

MATERIALS AND METHODS

Animals. Female C57BL/6N mice (8 to 9 weeks, 18 to 20 g; Charles River, Sulzfeld, Germany), housed under specific-pathogen-free conditions, were used in all experiments. All animal procedures were approved by local institutional (Charité-Universitätsmedizin Berlin) and governmental (Landesamt für Gesundheit und Soziales Berlin; approval ID G 0044/11) authorities. Animal housing and experimental procedures complied with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals.

Infection with influenza A virus. Influenza A/PR/8/34 virus (H1N1; PR8) was grown in the allantoic cavity of 11-day-old embryonated chicken eggs. Mice were transnasally infected with 10^2 PFU PR8 in 50 μl sterile phosphate-buffered saline (PBS) under inhaled isoflurane anesthesia (Forene; Abbott, Wiesbaden, Germany). Control mice received sterile PBS. All mice were monitored at 12-h intervals throughout the experiment to assess appearance, behavior, grooming, respiration, body weight, and rectal temperature (BAT-12 microprobe thermometer; Physitemp Instruments, Clifton, NJ).

Treatment with MALP-2. MALP-2 was synthesized and purified as described previously (29), dissolved in 30% 2-propanol–water (1 mg/ml), and diluted in 0.9% saline solution for application. Five days after IAV infection, the mice were anesthetized intraperitoneally (i.p.) with ketamine (80 mg/kg of body weight) (Ketavet; Pfizer, Berlin, Germany) and xylazine (25 mg/kg) (Rompun; Bayer, Leverkusen, Germany). After transoral tracheal intubation (32), MALP-2 (0.5 μg in 30 μl) was instilled intratracheally (i.t.). Solvent-treated mice received 30 μl of saline-diluted 2-propanol (0.15 μl 2-propanol in 30 μl saline) .

Transnasal infection with *S. pneumoniae*. *Streptococcus pneumoniae* (serotype 3, strain NCTC7978) was cultured as described previously (33) and resuspended in sterile PBS. Mice were anesthetized i.p. with ketamine and xylazine and transnasally inoculated with 10^3 CFU *S. pneumoniae* in 20 μl PBS 6 days after viral infection. Control mice received 20 μl of sterile PBS transnasally.

TABLE 1 Lung scoring parameters

Parameter	Scale/score range
Total lung area affected	1–100%
Bronchitis	0–4
Peribronchial inflammation	0–4
Interstitial inflammation	0–4
Intra-alveolar inflammation	0–4
Alveolar necrosis	0–4
Bronchial necrosis	0–4
Alveolar edema	0–4
Perivascular edema	0–4
Infiltration by neutrophils	0–4
Infiltration by macrophages	0–4
Infiltration by lymphocytes	0–4

Dissection and sampling. At the indicated time points, mice were anesthetized with ketamine (160 mg/kg) and xylazine (75 mg/kg), tracheotomized, and ventilated as previously described (34). After heparinization, blood was drawn from the vena cava caudalis. Lungs were flushed with sterile 0.9% saline via the pulmonary artery and dissected. To analyze leukocytes and cytokines, bronchoalveolar lavage (BAL) was performed twice, using 800 μl PBS containing a protease inhibitor each time (Roche, Mannheim, Germany).

Leukocyte quantification and differentiation in BALF and blood. Total BAL fluid (BALF) leukocytes were quantified by use of a Neubauer hemocytometer cell counting chamber and differentiated by flow cytometric analysis (FACSCalibur; BD Biosciences, Heidelberg, Germany), using forward versus side scatter characteristics and staining with CD45-peridinin chlorophyll protein (PerCP) (clone 30-F11; BD Biosciences), GR-1–phycoerythrin (PE) (clone RB6-8C5; BD Biosciences), and F4-80–allophycocyanin (APC) (clone BM8; Invitrogen, Karlsruhe, Germany). Total blood leukocytes were counted by flow cytometric analysis using BD TruCOUNT tubes and differentiated using forward versus side scatter characteristics and staining with CD45-PerCP and GR-1–PE.

Quantification of cytokine levels in BALF. Cytokines (IL-1β, tumor necrosis factor alpha [TNF-α], IFN-γ, IL-10, keratinocyte chemoattractant [KC], monocyte chemoattractant protein 1 [MCP-1], RANTES [regulated upon activation, normal T cell expressed and secreted], and macrophage inflammatory protein 2 [MIP-2]) were measured from BALF supernatant according to the manufacturer's instructions, using a multiplex cytokine assay (Bioplex; Bio-Rad, Hercules, CA).

Survival studies. Influenza virus-infected mice were treated intratracheally with MALP-2 or solvent 5 days after infection, as described above. For evaluation of survival after pneumococcal superinfection, IAV-infected mice were transnasally inoculated with *S. pneumoniae* 24 h after treatment. Disease severity was monitored at 12-h intervals, as described above, for 10 days after viral infection. Mice were euthanized when they reached at least one of the predefined criteria (body temperature of <30°C, body weight loss of 20%, cumbersome breathing, and accelerated breathing in combination with staggering, pain, or paleness) by exsanguination via the caudal vena cava after i.p. injection of ketamine (160 mg/kg body weight) and xylazine (75 mg/kg).

Histopathology and immunohistochemistry. For histopathological analysis, anesthetized mice were sacrificed on day 7, and lungs were removed after the trachea was ligated to prevent alveolar collapse, as described previously (34). The lungs were immersion fixed in formalin, embedded in paraffin, cut into 2-μm-thick sections, and stained with hematoxylin and eosin. Three evenly distributed sections per lung were scored microscopically for specified lung inflammation parameters (Table 1) to assess the dissemination and quality of pathological altera-

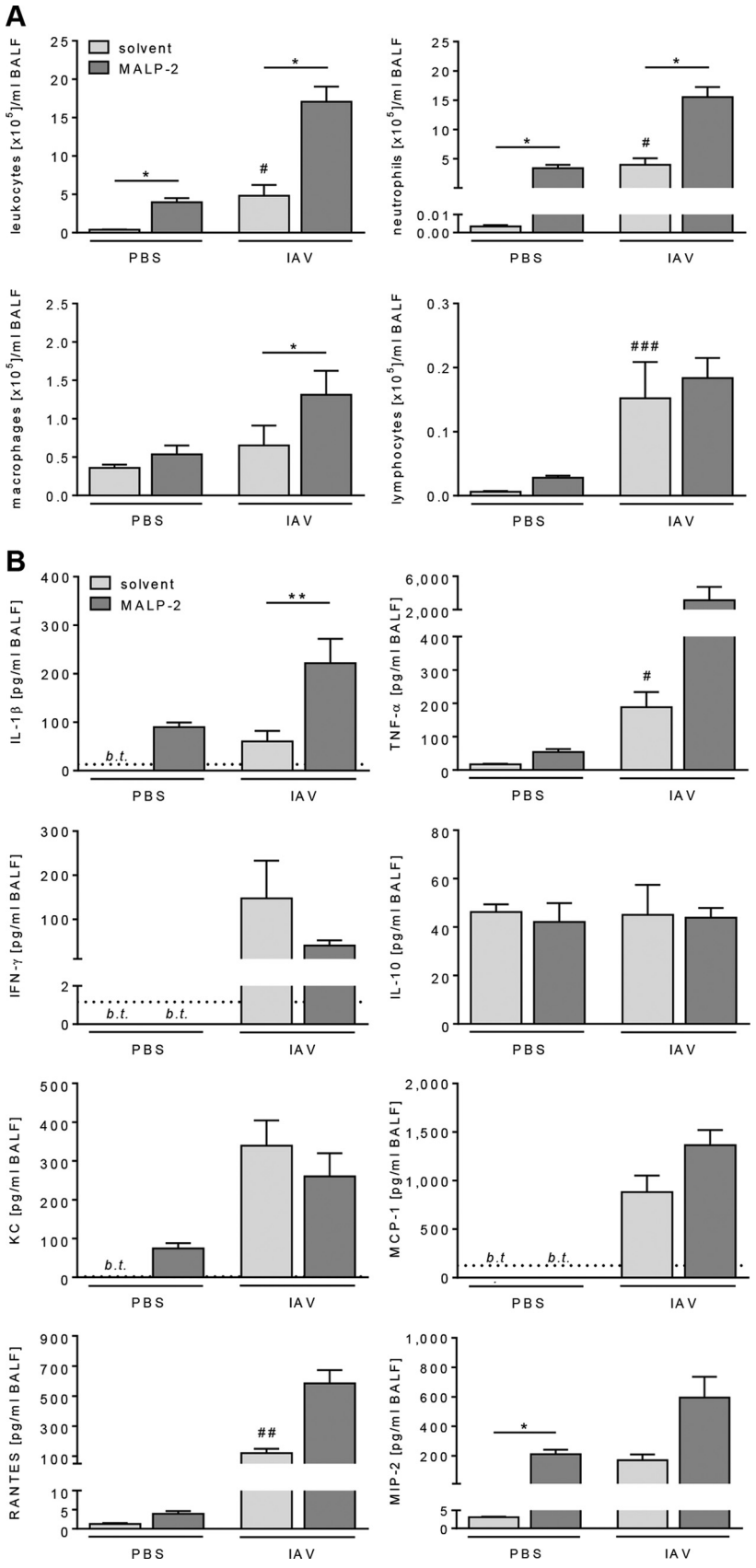

FIG 1 MALP-2 increased leukocyte recruitment and cytokine release in IAV-infected lungs. Mice were treated intratracheally with 0.5 μg MALP-2 or solvent 5 days after infection with 10^2 PFU IAV PR8 or sham infection with PBS. At 6 days postinfection, BAL was performed, and BALF leukocytes (A) and cytokines (B) were quantified. Dotted lines indicate the lower limit of the cytokine assay working range and are missing if all values were within the working range. *b.t.*, values below the threshold. Values are given as means plus SEM ($n = 6$ to 8 [A] or 5 to 8 [B] for each group). *, $P < 0.05$; **, $P < 0.01$ (for the indicated comparisons). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (versus the corresponding sham-infected group).

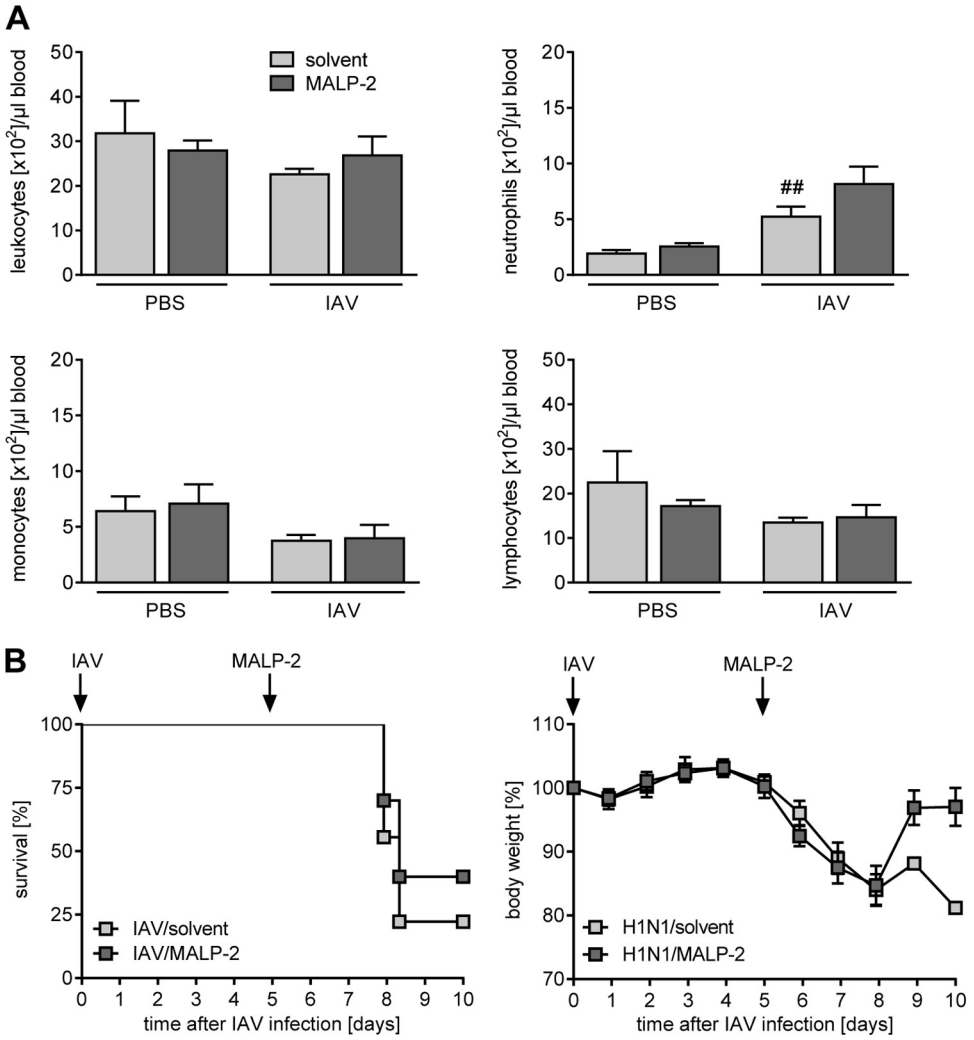

FIG 2 Pulmonary MALP-2 stimulation did not affect the systemic leukocyte response and clinical parameters in IAV-infected mice. Mice were intratracheally treated with 0.5 μg MALP-2 or solvent 5 days after infection with 10^2 PFU IAV or sham infection with PBS. (A) At 6 days postinfection, blood leukocytes were analyzed by flow cytometry. Values are given as means plus SEM ($n = 5$ to 7 for each group). ##, $P < 0.01$ versus the corresponding sham-infected group. (B) Survival and body weight loss were monitored for 10 days after IAV infection. Body weight values are given as means plus SEM ($n = 10$ for MALP-2 group and 9 for solvent group).

tions (35–38). For immunohistochemical detection of IAV and *S. pneumoniae*, lung sections were processed as described previously (39) and incubated with purified goat anti-IAV (1:4,000) (OBT155; Serotec) or rabbit anti-*S. pneumoniae* (1:2,000) (40) antibody at 4°C overnight. Incubation with an immunopurified, irrelevant rabbit or goat antibody at a similar dilution served as a negative control. Subsequently, slides were incubated with an alkaline phosphatase-conjugated horse anti-goat (1:1,000) (AP-9500; Vector, Burlingame, CA) or goat anti-rabbit (1:500) (AP-1000; Vector, Burlingame, CA) secondary antibody for 30 min at room temperature. The alkaline chromogen triamino-tritolyl-methanechloride (Neufuchsin) was used as a phosphatase substrate for color development. The slides were counterstained with hematoxylin, dehydrated through increasing concentrations of ethanol, cleared in xylene, and covered with coverslips.

Viral load in lungs. For standard plaque assay, whole lungs were each homogenized in 2 ml ice-cold PBS^{++} (with $CaCl_2$ and $MgCl_2$; Biochrom, Berlin, Germany), and supernatants were deep-frozen. Madin-Darby canine kidney (MDCK) cells were grown in 12-well plates with minimum essential medium (MEM) (with Earle's salts and L-glutamine; PAA, Pasching, Austria) supplemented with 10% fetal calf serum (FCS) (PAA), 2 mM L-glutamine (Invitrogen), and antibiotics at 37°C and 5% CO_2 to about 90% confluence. Lung supernatants were gently thawed on ice and serially diluted in PBS^{++} with 0.2% bovine serum albumin (BSA) (PAA). MDCK cells were washed with PBS (Dulbecco's; PAA) and infected in duplicate with 150-μl samples in 10-fold dilutions for 45 min. Cell culture supernatants were replaced by MEM (Gibco, Life Technologies, Paisley, United Kingdom) supplemented with 0.2% BSA, 0.05% $NaHCO_3$, 0.01% dextran, 1 μg/ml trypsin (tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK]-treated trypsin; Sigma, Taufkirchen, Germany), and 1.25% Avicel RC/CL microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA). Plates were incubated for 72 h (37°C, 5% CO_2). Afterwards, cells were washed, fixed in formalin, and stained with 0.1% crystal violet (Roth, Karlsruhe, Germany). The number of PFU per milliliter was calculated from the counted plaques.

Bacterial burdens in lungs and blood. Lungs were homogenized by passage through a cell strainer (100 μm; BD Biosciences). Serial dilutions of lung homogenates and blood were plated on Columbia agar with 5% sheep blood and incubated at 37°C under 5% CO_2 for 24 h to count the CFU.

Data analysis. Data are expressed as means and standard errors of the means (SEM) or as individual data and means (in scatterplots). One-way

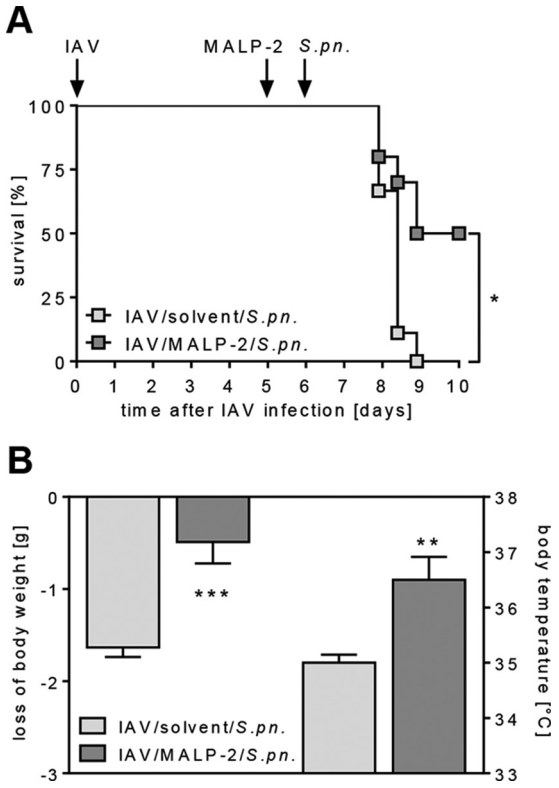

FIG 3 Pulmonary MALP-2 treatment of IAV-infected mice improved survival after pneumococcal superinfection. Mice infected with 10^2 PFU IAV were treated with 0.5 μg MALP-2 or solvent on day 5. Secondary infection with 10^3 CFU *S. pneumoniae* (S.pn.) was performed on day 6. (A) Survival was monitored every 12 h for 10 days after IAV infection. (B) Body weight loss within the first 24 h of secondary pneumococcal infection and body temperature were measured on day 7. Values are given as means plus SEM ($n = 10$ for MALP-2 group and 9 for solvent group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus the corresponding solvent-treated group).

analysis of variance (ANOVA) followed by Dunn's *post hoc* test for multiple comparisons, two-way ANOVA for comparison of continuously measured data between groups, and the two-tailed Mann-Whitney U test for comparison of two experimental groups were performed by using GraphPad Prism 4.02 software. Statistical analysis of cytokine levels was applied to experimental groups whose values were all detected within the working range. Kaplan-Meier survival curves were analyzed by the log rank test. *P* values of <0.05 were considered to be significant.

RESULTS

The TLR-2 agonist MALP-2 increased leukocyte recruitment and cytokine release in IAV-infected murine lungs. Leukocytes and cytokines in BALF samples taken 6 days after IAV infection were quantified to investigate the local inflammatory response in IAV-infected murine lungs after MALP-2 treatment (Fig. 1A and B). In comparison with solvent-treated mice, MALP-2 treatment of sham-infected mice evoked an increase in total leukocyte numbers within 24 h after application, with neutrophils mainly contributing to this increase (Fig. 1A). Furthermore, MALP-2 treatment induced the release of IL-1β, KC, and MIP-2 into the bronchoalveolar space of uninfected mice, whereas levels of TNF-α, IFN-γ, MCP-1, RANTES, and IL-10 remained unchanged (Fig. 1B). As expected, IAV infection resulted in pulmonary leukocyte recruitment and proinflammatory cytokine and chemokine release 6 days after infection. Interestingly, MALP-2 induced further significant accumulations of neutrophils and macrophages in IAV-infected lungs (Fig. 1A) and increased pulmonary production of IL-1β and—by trend—TNF-α, RANTES, and MIP-2, whereas the levels of the other analyzed cytokines were almost unchanged (Fig. 1B).

MALP-2 treatment did not affect the systemic leukocyte response and clinical parameters of IAV-infected mice. To assess potential systemic effects of pulmonary MALP-2 treatment, blood leukocytes (Fig. 2A) and systemic IL-6 levels (see Fig. S1A in the supplemental material) were quantified. Six days after IAV infection, neutrophil numbers and IL-6 levels were highly increased in the blood of solvent-treated mice, while IAV infection did not affect the numbers of macrophages and lymphocytes (Fig. 2A). Notably, the numbers of neutrophils, macrophages, and lymphocytes in the blood, as well as plasma IL-6 levels, were unaltered after pulmonary MALP-2 treatment in both uninfected and infected mice.

In the current study, transnasal application of IAV (100 PFU) resulted in clinically apparent pneumonia with an 80% lethality (Fig. 2B) and with a significant reduction of body weight up to day 8 postinfection ($P < 0.0001$). However, pulmonary MALP-2 stimulation had no significant effect on survival rates and clinical parameters (Fig. 2B; see Fig. S1B). Statistical evaluation of body weights beyond day 8 postinfection failed due to high pneumonia-induced dropout rates.

Pulmonary MALP-2 treatment of IAV-infected mice improved survival after pneumococcal superinfection. To investigate the effect of MALP-2 on the clinical outcome of pneumococcal superinfection in IAV pneumonia, mice were treated with MALP-2 or solvent 5 days after IAV infection and challenged with *S. pneumoniae* on day 6. Survival of MALP-2-treated, IAV- and *S. pneumoniae*-coinfected mice was significantly improved compared to that of solvent-treated coinfected mice (Fig. 3A). Furthermore, MALP-2 considerably reduced the loss of body weight and protected mice from hypothermia within the first 24 h after secondary pneumococcal infection (Fig. 3B), whereas no effect on the overall course of body weight was detected up to day 8 (see Fig. S2 in the supplemental material). Pneumonia-induced dropouts precluded the statistical analysis of body weights beyond day 8 (2 days post-*S. pneumoniae* infection).

MALP-2 modulated the innate immune response in IAV-infected mice without increasing pulmonary inflammation after subsequent pneumococcal superinfection. Leukocytes and cytokines in BALF samples taken at 7 days postinfection were quantified to investigate the effect of MALP-2 treatment on the local inflammatory response in IAV-infected mice after secondary pneumococcal infection (Fig. 4A and B). IAV infection induced neutrophil ($P < 0.01$), macrophage ($P < 0.05$), and lymphocyte ($P < 0.05$) infiltration into the bronchoalveolar space (Fig. 4A) and elevated levels of TNF-α, IFN-γ, IL-10, KC, MCP-1, RANTES, and MIP-2 (Fig. 4B) at 7 days postinfection. Interestingly, after secondary infection with *S. pneumoniae*, no further increase in leukocyte populations and cytokine levels was observed compared to the levels in mice solely infected with IAV (Fig. 4A and B). Compared to the case in solvent-treated mice, pulmonary MALP-2 treatment evoked an increase in the number of neutrophils in BALF and increased the release of IL-1β, MIP-2, and—by trend—TNF-α into the bronchoalveolar space 7 days after IAV infection. MALP-2 stimulation of IAV-infected mice prior to sec-

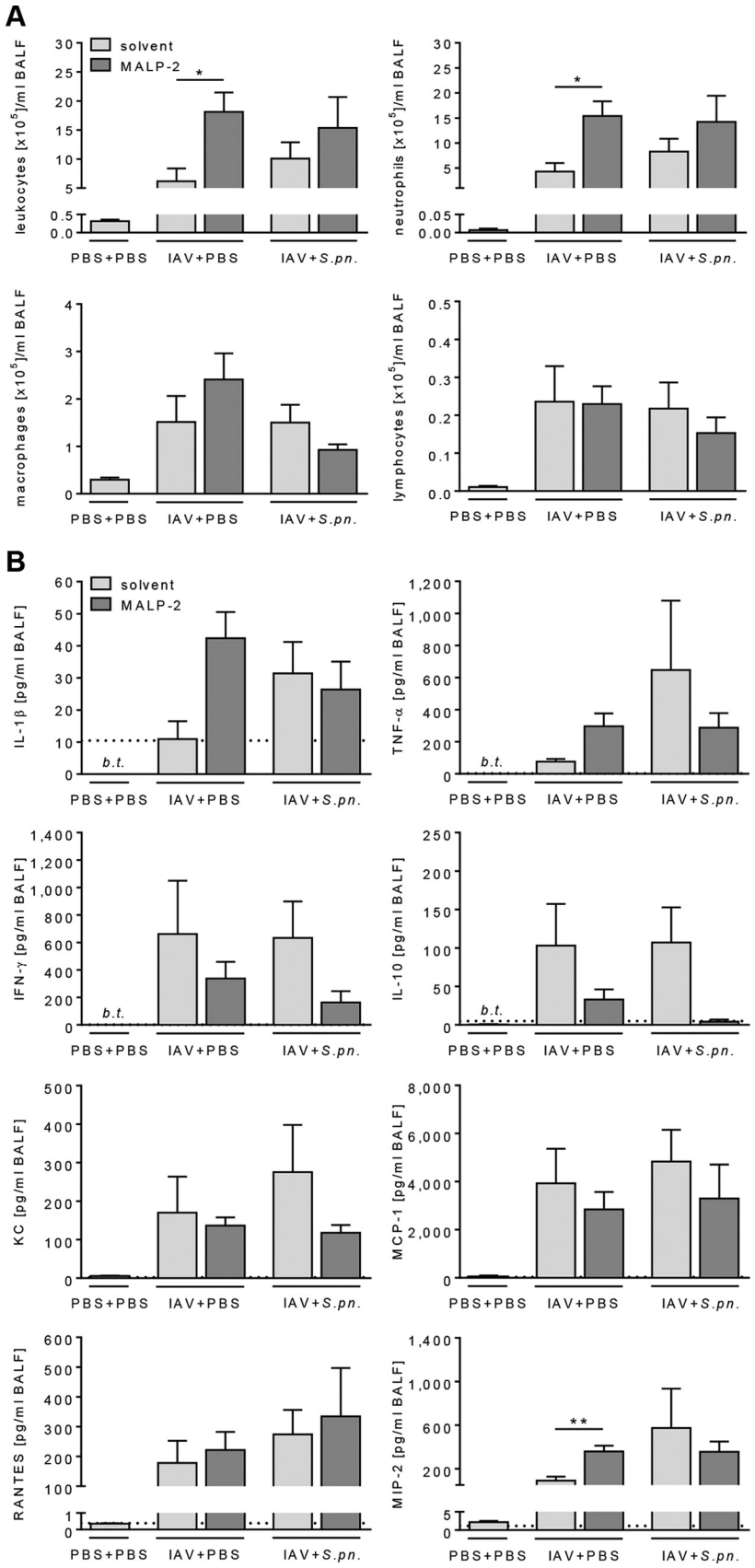

FIG 4 MALP-2 treatment of IAV-infected lungs did not increase pulmonary inflammation after subsequent secondary pneumococcal infection. Mice infected with 10^2 PFU IAV or sham infected with PBS were treated with 0.5 μg MALP-2 or solvent on day 5. Secondary infection with 10^3 CFU *S. pneumoniae* or sham infection with PBS was performed on day 6. Seven days after IAV infection, BAL was performed, and BALF leukocytes (A) and cytokines (B) were quantified. Values are given as means plus SEM ($n = 5$ to 8 [A] or 3 to 8 [B] for each group). Dotted lines indicate the lower limit of the cytokine assay working range and are missing if all values were within the working range. *b.t.*, values below the threshold. *, $P < 0.05$; **, $P < 0.01$ (for the indicated comparisons).

ondary pneumococcal challenge did not alter leukocyte recruitment but decreased IL-10, IFN-γ, and KC levels by trend, whereas all other investigated cytokines were only moderately altered compared to those in solvent-treated coinfected mice (Fig. 4A and B). In the blood, leukocyte numbers and plasma IL-6 levels were unaffected by pulmonary MALP-2 treatment prior to secondary bacterial infection (see Fig. S3A and B in the supplemental material).

MALP-2 altered neutrophil accumulation in IAV-induced bronchointerstitial pneumonia without aggravating suppurative bronchopneumonia after secondary pneumococcal infection. The dissemination and quality of pathological lung alterations were assessed by histopathological analysis. IAV-infected lungs at 7 days postinfection (Fig. 5A and C) showed mild to moderate, multifocal, acutely necrosuppurative bronchointerstitial pneumonia, with accumulations of neutrophils and macrophages mainly at interstitial sites and within bronchi and alveoli. MALP-2 treatment further increased the number of neutrophils within the bronchi and alveoli of IAV-infected lungs (Fig. 5B and D). Secondary pneumococcal infection after IAV infection resulted in suppurative bronchopneumonia without detectable morphological differences between solvent-treated and MALP-2-treated coinfected lungs (Fig. 5E and F). Histopathological analysis of lungs from sham-infected and solvent-treated mice showed minimal to mild, multifocal interstitial infiltration predominantly by macrophages and a few neutrophils, probably resulting from repeated application of sterile sham fluid (Fig. 5G). The total lung area affected by inflammation was determined by semiquantitative microscopic analysis (Fig. 5H; see Fig. S4 in the supplemental material). IAV-induced pathological alterations extended, on average, to 12.5% of lung tissue, whereas secondary pneumococcal infections did not induce further significant dissemination. Furthermore, MALP-2 did not affect the expansion of lung lesions in either IAV-infected or coinfected lungs.

Dissemination of IAV in murine lungs remained unaltered by MALP-2. To examine the effect of MALP-2 on the dissemination of IAV in murine lungs, separate sets of lung sections were immunostained using anti-IAV antibody (Fig. 6A to E). In murine lungs with or without secondary pneumococcal infection, IAV was mainly detected in the bronchial compartment, predominantly allocated to epithelial cells, as well as in the alveolar compartment, predominantly in alveolar macrophages (Fig. 6A and C). MALP-2 treatment had no effect on viral distribution within the lungs of mice after IAV monoinfection (Fig. 6B) or secondary pneumococcal infection (Fig. 6D). No immunostaining was detected in sham-infected, solvent-treated control lungs (Fig. 6E). Furthermore, in the virus plaque assay, no difference in pulmonary viral load was detected between MALP-2-treated and solvent-treated mice infected with *S. pneumoniae* after IAV infection (Fig. 6F).

MALP-2 reduced lung bacterial loads after secondary pneumococcal infection. To analyze the effect of MALP-2 on the dissemination of *S. pneumoniae* in murine lungs, immunolabeling of *S. pneumoniae* was performed on lung sections of IAV-infected mice treated with MALP-2 or solvent and challenged by secondary pneumococcal infection (Fig. 7A to E). In 50% of solvent-treated coinfected mice, pneumococci were observed within bronchi and alveolar spaces (Fig. 7A and C). In contrast, no bacteria were detected in bronchi and alveolar spaces of MALP-2-treated mice infected with IAV and *S. pneumoniae* (Fig. 7B and D). No immunostaining was detected in sham-infected and solvent-treated control lungs (Fig. 7E). Quantification of CFU from lung tissue after flushing of the pulmonary vascular bed revealed that pulmonary bacterial loads were considerably reduced in MALP-2-treated mice compared to those in solvent-treated mice after secondary pneumococcal infection (Fig. 7F). However, bacteremia after secondary pneumococcal infection was detected in only 2 of 11 solvent-treated mice and none of 10 MALP-2-treated mice. Thus, the impact of MALP-2 treatment on the development of bacteremia was not assessable in the current study (see Fig. S5 in the supplemental material).

DISCUSSION

Commonly used antiviral therapy fails to reverse the impairment of the antibacterial host defense during pulmonary influenza virus infection. Local pulmonary immunostimulation may provide a therapeutic perspective to improve innate immunity and therefore the outcome of subsequent bacterial infection.

In this *in vivo* study, we showed that pulmonary immunostimulation by a single intratracheal application of the specific TLR-2/6 agonist MALP-2 increased the release of the proinflammatory cytokine IL-1β and enhanced the recruitment of neutrophils and macrophages into the airways of IAV-infected mice, without detectable systemic or clinical side effects. Consequently, preventive immunostimulation prior to pneumococcal superinfection resulted in enhanced pulmonary bacterial clearance and improved the survival of mice with murine influenza virus pneumonia and subsequent pneumococcal infection.

Consistent with our previous study (28), pulmonary treatment of uninfected mice with MALP-2 stimulated the release of proinflammatory cytokines and chemokines. MALP-2 is known to specifically activate the receptor heterodimer TLR-2/6 (29, 30), followed by MyD88-mediated activation of nuclear factor κB (NF-κB)-dependent gene expression (41). The resultant TLR-2-mediated leukocyte recruitment into the bronchoalveolar space was shown to reach its maximum 24 h after application, followed by a decrease within 2 days (28). Specific TLR-mediated immunostimulation was also demonstrated in previous studies to increase the innate immune response and resistance to lethal influenza virus monoinfection *in vivo*. However, treatment was initiated prior to infection (42, 43) or no later than 24 h after infection (44), although a highly increased susceptibility to secondary pneumococcal invasion associated with impaired bacterial clearance (19) and high mortality rates (45) was detected between days 5 and 7 after influenza virus infection. Notably, in the present study, pulmonary TLR-mediated immunostimulation with MALP-2 effectively increased the level of proinflammatory IL-1β and subsequent immigration of leukocytes, particularly neutrophils and macrophages, into murine airways even 6 days after IAV infection, despite an ongoing antiviral host defense. Consistently, increased numbers of neutrophils within bronchi and alveoli, in addition to virus-induced bronchointerstitial tissue alterations, were detected by histological analysis. The stimulatory effect of MALP-2 was confined to the lungs, as indicated by unaffected blood leukocytes, plasma IL-6 levels, and clinical parameters. The absence of such systemic side effects is consistent with the findings of a previously published *in vivo* safety report which showed that treatment of mice with an aerosolized TLR-9 agonist combined with a TLR-2/6 agonist was well tolerated (46). Pulmonary MALP-2 treatment of IAV-infected mice, however, had no protective impact on the course of IAV monoinfection.

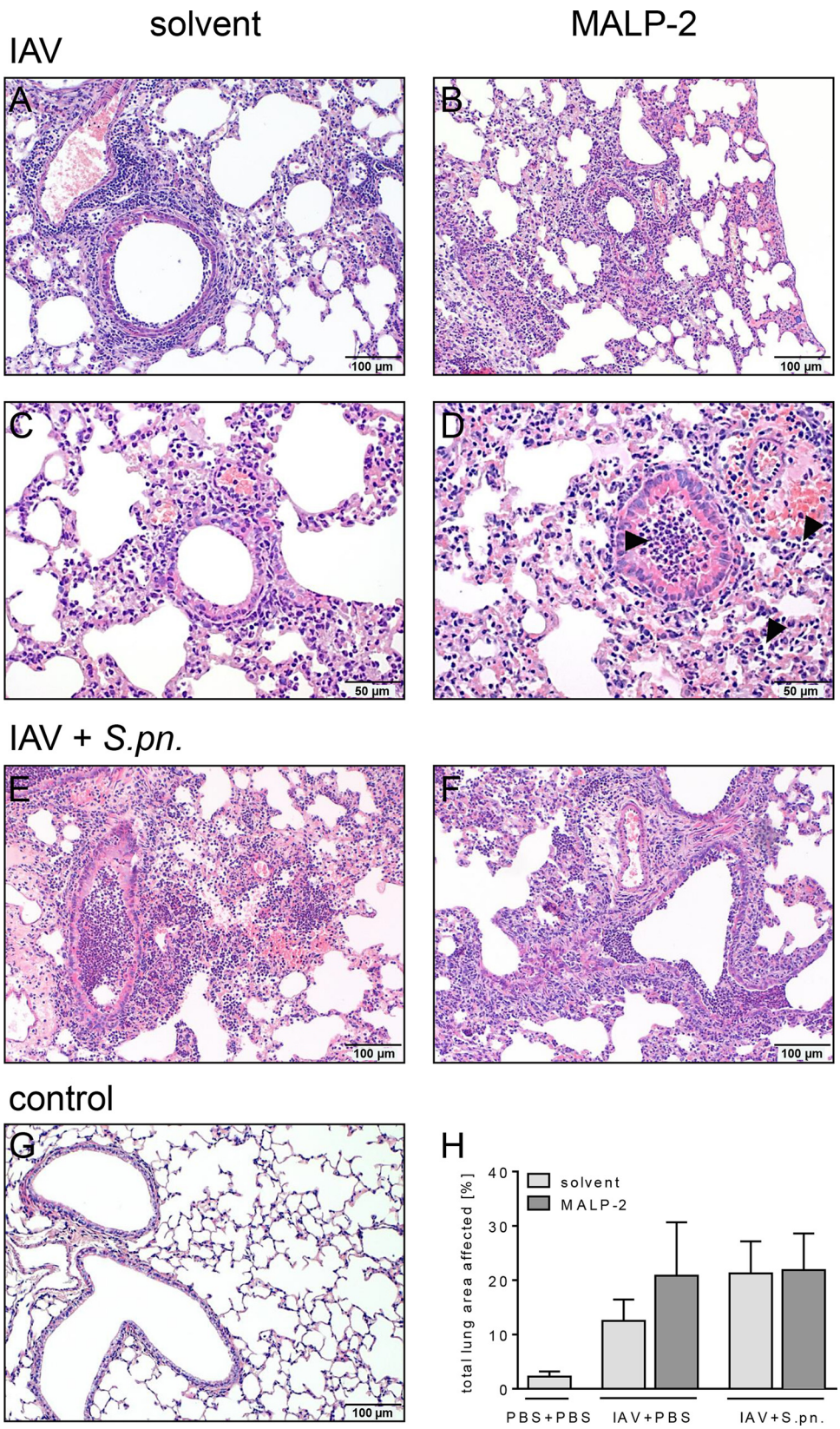

FIG 5 MALP-2 altered neutrophil accumulation in IAV-induced bronchointerstitial pneumonia without aggravating suppurative bronchopneumonia after secondary pneumococcal infection. Mice infected with 10^2 PFU IAV were treated with 0.5 μg MALP-2 or solvent on day 5 and challenged with 10^3 CFU *S. pneumoniae* or sham infected with PBS on day 6. Lungs were harvested on day 7, and formalin-fixed and paraffin-embedded sections were prepared and stained with hematoxylin and eosin for histopathological analyses. (A to D) IAV infection induced bronchointerstitial pneumonia, accompanied by numerous neutrophils within bronchi and alveolar spaces after MALP-2 stimulation (D; black arrowheads). (E and F) Secondary pneumococcal infection resulted in suppurative bronchopneumonia in lungs of both solvent-treated and MALP-2-treated IAV-infected mice. (G) Lung sections from sham-infected and solvent-treated mice served as a negative control. Representative images are shown ($n = 3$ or 4 for each group). (H) Total lung areas affected by inflammation. Values are given as means plus SEM ($n = 3$ or 4 for each group).

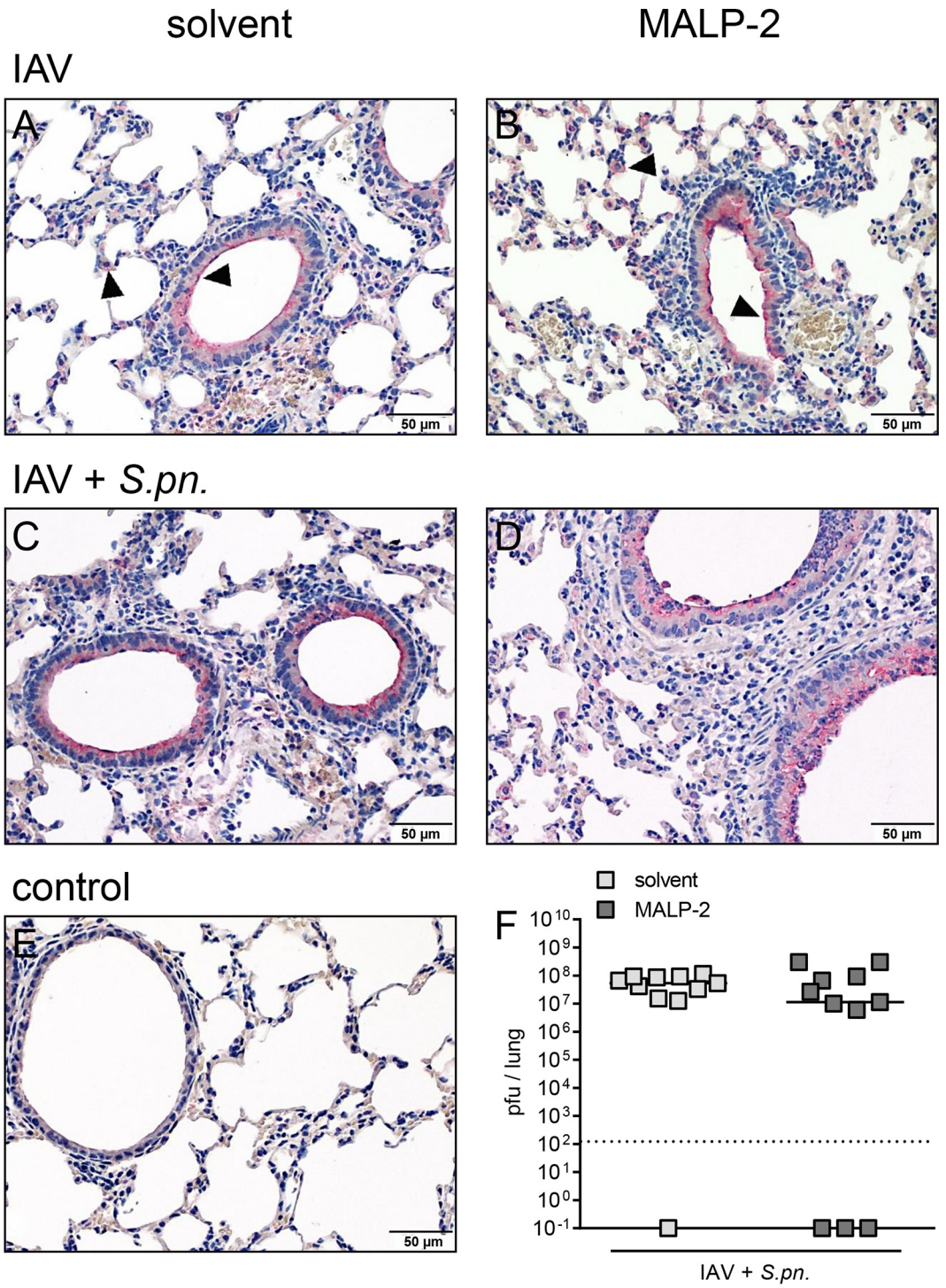

FIG 6 The pulmonary IAV load remained unaltered by MALP-2 stimulation. Mice infected with 10^2 PFU IAV were treated with 0.5 μg MALP-2 or solvent on day 5 and challenged with 10^3 CFU *S. pneumoniae* or PBS on day 6. Seven days after IAV infection, lung sections were prepared, and immunohistochemistry for IAV (red staining) was performed. IAV was observed mainly within bronchi and in alveolar macrophages (black arrowheads) of lungs from both solvent (A and C)- and MALP-2 (B and D)-treated mice. (E) Lung sections from sham-infected and solvent-treated mice served as a negative control. Representative images are shown ($n = 3$ or 4). (F) Lung viral loads after secondary bacterial infection were determined on day 7. Values are given as individual data and means ($n = 11$). The dotted line indicates the lower detection limit.

To investigate the effects of pulmonary TLR-2-mediated immunostimulation on the course of pneumococcal superinfection, IAV-infected mice were intratracheally treated with MALP-2 5 days after infection, in a state of increased susceptibility to secondary invading bacteria, followed by secondary pneumococcal infection 24 h later, when the highest leukocyte recruitment due to MALP-2 was suggested (28). Notably, MALP-2-treated mice showed significantly improved survival compared to solvent-

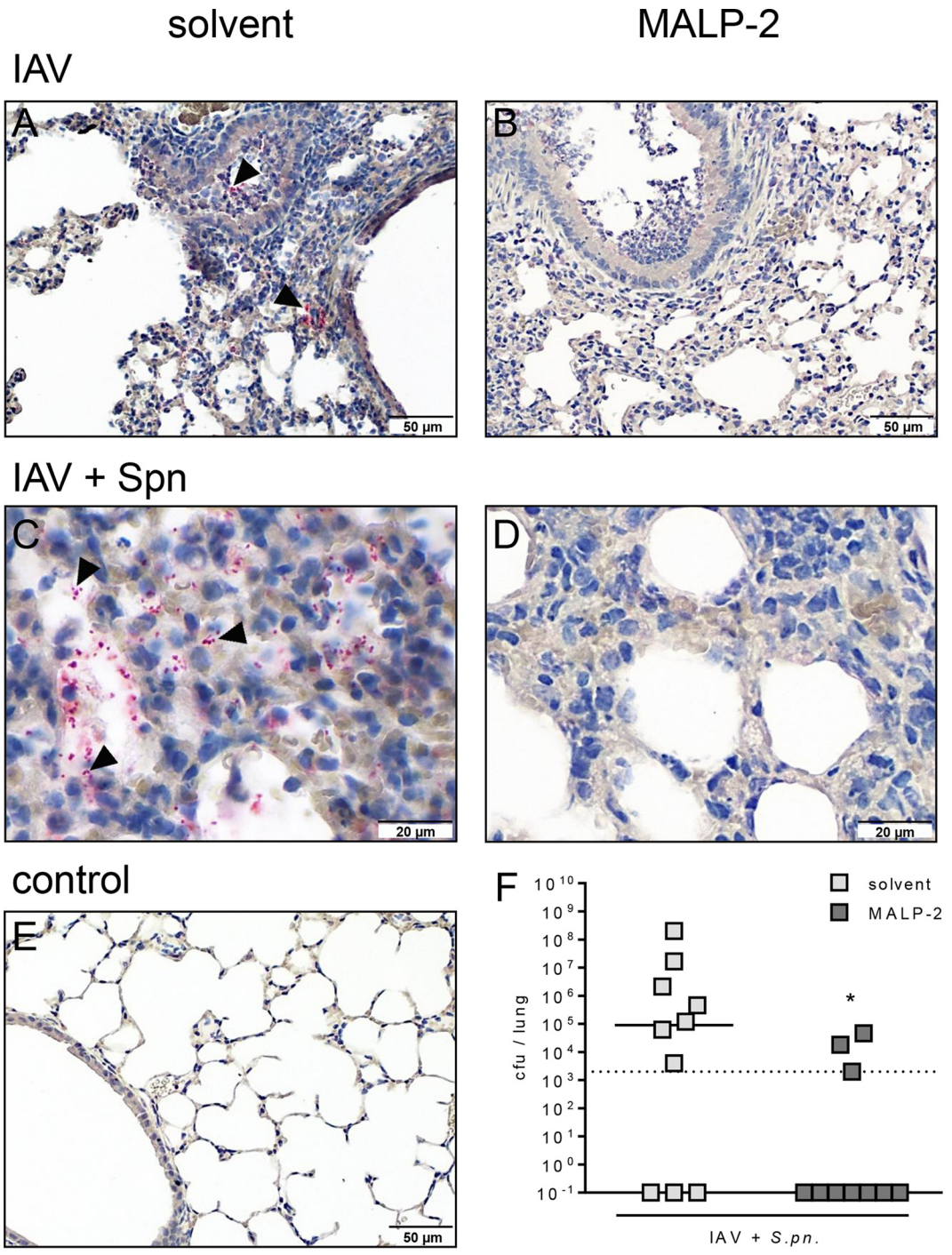

FIG 7 MALP-2 reduced the pulmonary bacterial load after secondary pneumococcal infection. Mice infected with 10^2 PFU IAV were treated with 0.5 μg MALP-2 or solvent on day 5 and challenged with 10^3 CFU *S. pneumoniae* on day 6. (A to E) Bacteria were detected by immunohistochemistry with an anti-*S. pneumoniae* antibody (red staining) on day 7. Pneumococci were observed within bronchi and alveolar spaces (black arrowheads) in 50% of lungs from solvent-treated infected mice (A and C), but not in lung tissue from MALP-2-stimulated infected mice (B and D). (E) Lung sections from sham-infected and solvent-treated mice served as a negative control. Representative images are shown ($n = 4$). (F) Lung bacterial loads after secondary pneumococcal infection were determined on day 7. Values are given as individual data and means ($n = 10$). The dotted line indicates the lower detection limit. *, $P < 0.05$ versus solvent-treated group.

treated mice suffering from combined IAV and *S. pneumoniae* pneumonia. Likewise, pulmonary overexpression of GM-CSF protected mice from fatal secondary *Staphylococcus aureus* pneumonia after sublethal influenza virus infection by increasing the number of macrophages and neutrophils and improving effector cell function within the airways before and after secondary bacterial infection (20). However, TLR-2 has been described to enhance inflammation and virus replication in specific models of viral in-

fection (47, 48). Extensive TLR-2 stimulation by bacterial components may induce abundant neutrophil recruitment in influenza virus-infected murine lungs and may contribute to exaggerated immunopathology despite adequate antibacterial therapy (49). Furthermore, another study provided evidence that innate immune cells reaching the influenza virus-infected lung potentially induce a chemokine-driven explosive feed-forward circuit leading to excessive neutrophil-mediated inflammatory damage and a lethal outcome of influenza pneumonia (50). This suggests the possibility that TLR-2/6-mediated immunostimulation with MALP-2 may initiate protective antibacterial innate immune responses at the expense of an aggravated virus-induced pathology. However, in our study, we did not observe excessive cytokine release or a further increase of neutrophil influx provoked by MALP-2 treatment in influenza virus-infected mice, as indicated by neutrophil numbers and proinflammatory cytokine levels on day 6 (Fig. 1B) compared to those on day 7 (Fig. 4B; see Fig. S6 in the supplemental material). Moreover, MALP-2 specifically activates TLR-2/6 and therefore induces a more selective innate immune response than bacterial lysates stimulating numerous pattern recognition receptors. Thus, we suppose that local MALP-2 treatment of influenza virus-infected mice counterbalances the impaired antibacterial defense without aggravating influenza pathology.

Seki et al. reported higher intrapulmonary levels of inflammatory cytokines and larger airway leukocyte numbers for mice successively infected with influenza virus and *S. pneumoniae* than those for viral monoinfection, which contributed to the progressive pathology of combined pneumonia (51). Note that the current *in vivo* study demonstrated that MALP-2 treatment improved the innate immune response of IAV-infected lungs without inducing exaggerated pulmonary inflammation or systemic side effects after bacterial superinfection. In IAV-infected murine lungs, the secondary pneumococcal challenge induced suppurative bronchopneumonic lung alterations, as commonly described (45); however, levels of proinflammatory mediators and the number of leukocytes, which were elevated by the previous IAV infection, remained unaltered. Interestingly, MALP-2 treatment prior to bacterial superinfection decreased the airway levels of IL-10, IFN-γ, and KC in BALF by trend, whereas the levels of all other investigated cytokines and chemokines, as well as the total leukocyte number and the extent of lung pathology, were unaffected compared to those in solvent-treated coinfected mice.

IL-10 and IFN-γ have been described as being involved in impaired immune responses, mediating increased susceptibility to invading bacteria in murine models of primary pneumococcal pneumonia (52) and *Klebsiella pneumoniae* lung infection (53). Anti-IL-10 treatment before secondary pneumococcal infection resulted in reduced bacterial outgrowth in lungs and prolonged survival of mice with postinfluenza pneumonia (18). Furthermore, neutralization of IFN-γ in influenza virus-infected lungs, which has been reported to inhibit bacterial phagocytosis, considerably decreased bacterial susceptibility and improved survival of mice with murine secondary pneumococcal pneumonia (17). Thus, the reductions of IL-10 and, at least in part, IFN-γ evoked by MALP-2 may have contributed to an improved pulmonary host defense against pneumococcal superinfection.

Immune priming by intranasal administration of recombinant GM-CSF (19) or systemic treatment with specific TLR-4-activating monoclonal antibodies (54) decreased lung bacterial counts of influenza virus-infected mice after pneumococcal superinfection; however, immunostimulation was performed early (24 h before or concurrently with influenza virus infection, respectively). In the current study, pulmonary immune stimulation with MALP-2 5 days after IAV infection (24 h before pneumococcal challenge) also resulted in decreased lung bacterial loads 24 h after bacterial infection. Moreover, specific TLR-mediated immunostimulation prior to or shortly after experimental influenza virus monoinfection has been shown to reduce the viral titer (43, 44). However, MALP-2 treatment performed on day 5 after IAV infection had no effect on viral load or viral distribution in the murine lung tissue. These findings suggest that the improved innate immune response of IAV-infected lungs by MALP-2 enhanced bacterial elimination without affecting the antiviral host defense. Moreover, the reduced bacterial outgrowth in lungs of MALP-2-treated IAV-infected mice presumably provided an attenuated proinflammatory stimulus, thereby contributing to decreased production of cytokines, such as KC and (probably) IFN-γ.

Besides stimulating leukocyte recruitment, TLR-2-mediated immunostimulation has been described as producing an overall improvement in antibacterial activity of phagocytic cells (55, 56) and pulmonary epithelial cells (25), which may counteract the attenuation of bacterium-driven antimicrobial peptide expression by IAV (57). Furthermore, γδ T cells and Th17 cells, which participate in bacterial clearance by IL-17A production (58, 59), may represent promising cellular targets for future mechanistic studies. In the IAV-infected lungs, MALP-2 significantly elevated the levels of IL-1β, which has been described to rescue the induction of IL-17 in IAV- and *S. aureus*-coinfected mice (60). However, the precise mechanisms mediating the protective effect of MALP-2 on pneumococcal superinfection remain elusive. Moreover, the current work was limited by focusing on MALP-2 treatment prior to pneumococcal superinfection and should be extended in future studies to test the ability of MALP-2 to prevent disease when administered after the infection.

In summary, we found an improved pulmonary innate immune response in IAV-infected mice after local application of a synthetic TLR-2/6 agonist, resulting in an adequate host defense against subsequent pneumococcal lung infection and a markedly improved survival of combined influenza virus/pneumococcal pneumonia. Thus, our experimental data suggest that pulmonary immunostimulation may provide a future perspective for patients with pandemic or seasonal influenza to prevent bacterial lung infections.

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