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## Immunological Priming Requires Tregs and Interleukin-10-Producing Macrophages to Accelerate Resolution from Severe Lung Inflammation

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### Abstract

Overwhelming lung inflammation frequently occurs following exposure to both direct infectious and non-infectious agents, and is a leading cause of mortality world-wide. In that context, immunomodulatory strategies may be utilized to limit severity of impending organ damage. We sought to determine whether priming the lung by activating the immune system, or immunological priming, could accelerate resolution of severe lung inflammation. We assessed the importance of alveolar macrophages, regulatory T cells, and their potential interaction during immunological priming. We demonstrate that oropharyngeal delivery of low-dose lipopolysaccharide can immunologically prime the lung to augment alveolar macrophage production of interleukin-10 and enhance resolution of lung inflammation induced by a lethal dose of lipopolysaccharide or by pseudomonas bacterial pneumonia. Interleukin-10 deficient mice did not achieve priming and were unable to accelerate lung injury resolution. Depletion of lung macrophages or regulatory T cells during the priming response completely abrogated the positive effect of immunological priming on resolution of lung inflammation and significantly reduced alveolar macrophage interleukin-10 production. Finally, we demonstrated that oropharyngeal delivery of synthetic CpG-oligonucleotides elicited minimal lung inflammation compared to low-dose lipopolysaccharide, but nonetheless primed the lung to accelerate resolution of lung injury following subsequent lethal lipopolysaccharide exposure. Immunological priming is a viable immunomodulatory strategy used to enhance resolution in an experimental acute lung injury model with the potential for therapeutic benefit against a wide array of injurious exposures.

### INTRODUCTION

Robust lung inflammation induced by infectious and non-infectious stimuli can lead to severe pathological states including acute respiratory distress syndrome (ARDS) and multi-organ failure with devastating, often lethal consequences. Adaptive immunomodulatory

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strategies to protect humans against severe injury represent potentially attractive options in susceptible hosts or during outbreaks of virulent disease (1), yet the possibility of maladaptive immune responses necessitates more thorough understanding of involved cellular mechanisms (2). In the lung, resident alveolar macrophages are crucial to the immune response, uniquely positioned as “first responders” designed to recognize and combat foreign antigen in the airspaces of the lower respiratory tract. Upon activation by toll-like receptor (TLR) or other pattern recognition receptor signaling, macrophages recruit additional pro-inflammatory immune cells including neutrophils, exudative macrophages, and Th1 lymphocytes to the lung and alveolar space as a critical part of the innate immune response (3-6). Once the pro-inflammatory milieu has recognized and removed foreign substances, resident and recruited macrophages undergo an active transition to a pro-resolution phenotype to dampen inflammation, initiate repair and restore the immune barrier (6-8).

Lipopolysaccharide (LPS; endotoxin), a component of the gram-negative bacteria cell wall, is recognized by mammalian Toll-Like Receptor 4-Lymphocyte Antigen 96 (TLR4-MD2) complex, and is abundantly expressed on the cell surface of macrophages and other antigen presenting cells (APCs) (9, 10). Often regarded as the prototypical danger signal - pattern recognition receptor response, LPS binding to membrane TLR4-MD2 activates transcription factors NF- $\kappa$ B and STAT1 by signaling through MyD88 and TRIF-dependent pathways respectively (11), leading to a myriad of pro-inflammatory signaling cascades. Repeated TLR4 stimulation by LPS results in a state of reduced inflammatory capacity known as endotoxin (LPS) tolerance (ET). ET has generally been ascribed to clinical scenarios involving sepsis and other sepsis-like states, in which circulating monocytes can be persistently exposed to endotoxin, resulting in ineffective TNF- $\alpha$  or other pro-inflammatory cytokine production with repeated LPS exposure (12-15). As a result, ET has been associated with worse outcomes including increased mortality and secondary infections (11, 15). In contrast to the systemic effects of sepsis where the majority of immune cells are exposed to the same circulating stimuli, recruited inflammatory macrophages may respond differently than resident macrophages to successive lung stimuli (16, 17).

Macrophages are an important source of interleukin-10 (IL-10), a potent immunomodulatory cytokine with diverse cellular production (18, 19). The primary biological function of IL-10 is to dampen inflammation, but other functions include modulating the proliferation and differentiation of immune cells including T cells, antigen presenting cells, and neutrophils (20), as well as non-immune cells such as keratinocytes, endothelial cells, and epithelial cells (10, 20, 21). IL-10 is produced by the TRIF-dependent TLR4 signaling pathway that dampens inflammation by induction of p50 NF- $\kappa$ B, STAT3, and SOCS3, all negative regulators of the TLR4 signaling cascade (11). Although IL-10 regulates several aspects of the macrophage response to LPS including type, magnitude, and duration, it is dispensable for development of endotoxin tolerance (22, 23). We and others have demonstrated that IL-10 contributes to resolution of inflammation in experimental acute lung injury models including bacterial pneumonia by limiting neutrophil recruitment and aiding in neutrophil removal (24-27), both critical for resolution. Phagocytosis of apoptotic neutrophils by both monocytes and macrophages further augments IL-10 macrophage production (6, 28). Independent of phagocytosis, ligation of the macrophage Fc $\gamma$  receptor can also induce early

IL-10 production with LPS co-stimulation (29). The role of IL-10 in immunological priming as an adaptive immunomodulatory mechanism against severe lung inflammation has not yet been defined.

Regulatory T cells (Tregs) are critical for active resolution of lung inflammation and repair. Tregs modulate innate immune cellular responses (30) including effects on macrophage phenotype and function in animals and humans (31-33). Specifically, Tregs blunt macrophage pro-inflammatory cytokine production, enhance their efferocytosis of apoptotic neutrophils, and transition them to an alternatively-activated phenotype (25, 32). Conversely, macrophages and circulating monocytes are critical for Treg induction, expansion, and suppressive function (34-37), but mechanisms mediating this interaction remain incompletely understood. We surmise that macrophages and Tregs are both necessary, and may act in concert, to regulate the immunological priming response and accelerate resolution of severe lung inflammation.

Our results demonstrate that activation of distinct pattern recognition receptors to induce minimal lung inflammation is sufficient to immunologically prime the lung and accelerate resolution of lung injury resulting from a severe injurious exposure. We found that both macrophages that produce IL-10 and Tregs are critical for immunological priming. Our results suggest the importance of immunological priming as a mechanism to improve host defense against a variety of direct insults to the lung, with significant therapeutic potential.

## METHODS

### Animals

Male C57BL/6 wild type (WT) mice (8-10 weeks old) and *IL-10*<sup>-/-</sup> mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME). *Foxp3*<sup>gfp</sup> and *Foxp3*<sup>DTR</sup> mice (B6.129(Cg)-*Foxp3*<sup>tm3Ayr/J</sup>) were gifts from Dr. Alexander Y. Rudensky (Memorial Sloan-Kettering Institute). *Foxp3*<sup>gfp</sup> reporter mice express an N-terminal GFP-Foxp3 fusion protein to further identify Foxp3<sup>+</sup> T<sub>reg</sub> cells (38). *Foxp3*<sup>DTR</sup> mice express the human diphtheria toxin receptor (DTR) along with GFP, which have been fused to the 3' untranslated region of the *Foxp3* locus, and specific elimination of Foxp3<sup>+</sup> T<sub>reg</sub> cells *in vivo* occurs through intraperitoneal (i.p.) administration of diphtheria toxin (DT) (39). Mice were housed at the Johns Hopkins University Asthma and Allergy Center, and experiments conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee.

### Animal Preparation

For oropharyngeal (o.p.) delivery, mice were deprived of chow for 1-2 hours, anesthetized using inhaled isoflurane, followed by instillation of 1 mg/kg *Escherichia coli* lipopolysaccharide (O55:B5 Sigma L2880, diluted in 50 µL sterile water), 35 µg of class C CpG oligonucleotide (ODN 2395 Invivogen, diluted in 50 µL PBS), or respective vehicle controls. After witnessed aspiration, mice were returned to their cages and all exhibited immediate recovery. Following a priming period (5 or 7 days), we performed intratracheal (i.t.) delivery as before (25). Briefly, mice were anesthetized with intraperitoneal (i.p.)

ketamine/acetylpromazine (150/2.5 mg/kg) before exposure of the trachea. Lipopolysaccharide (LPS) (3 or 7.5 µg/g mouse weight diluted in sterile water), *Pseudomonas aeruginosa* (PAO1) ( $1 \times 10^6$  CFU, ATCC, in 50 µL PBS) or respective vehicle controls were instilled intratracheally via a 20-gauge catheter. After 1, 3, or 5 days, groups of mice were anesthetized with i.p. ketamine/acetylpromazine and euthanized by exsanguination from the inferior vena cava. The lungs were perfused with 1 ml of phosphate-buffered saline (PBS), followed by bronchoalveolar lavage (BAL) of the right lung; the left lung was processed for histology. BAL samples were routinely cultured to assess for bacterial infection. For quantitative measures of bacteria, whole lungs were homogenized without prior lavage, and the lysates were diluted in PBS and streaked on agar plates. After 24 hours at 37 °C, colonies were counted.

### Diphtheria toxin and clodronate liposome injections

Diphtheria toxin (List Biological Laboratories, Inc, Lot # 15043A1, diluted in PBS) was administered via i.p. injection on day -2 (50 µg/kg mouse) and day -1 (15 µg/kg) of the priming period prior to i.t. LPS as described (39). Mice harvested on day +5 also received a DT dose (15 µg/kg) on day +2 after i.t. LPS. Clodronate liposomes (Cl<sub>2</sub>MDP) or PBS liposomes (control) were prepared as described (8), followed by o.p. instillation (60 µL) on day -3, and i.p. injection (500 µl) on days -2 and -1 of the priming period.

### Analysis of bronchoalveolar lavage (BAL)

BAL was obtained by cannulating the trachea with a 20-gauge catheter. The right lung was lavaged with two aliquots of 0.7 ml of calcium-free PBS except when noted. BAL was centrifuged at  $700 \times g$  for 10 min at 4°C. The cell-free supernatants were stored at -80°C until further analysis. The cell pellet was diluted in PBS, and total cell number was counted with a hemocytometer using trypan blue exclusion. Cell differentials (300 cells per sample) were counted on cytocentrifuge preparation with Diff-Quik stain (Baxter Diagnostics, McGaw Park, IL). Total protein was measured in the cell-free supernatant by the Lowry method (40).

### Measurement of Cytokines

Tumor necrosis factor (TNF-α) or IL-10 levels were measured in BAL and cell culture supernatants by ELISA (R&D System, Minneapolis, MN).

### Lung Histology and lung injury scoring

Lungs (n=5 per time point) were inflated to a pressure of 25 cmH<sub>2</sub>O using 1% low melting agarose (Invitrogen) for histologic evaluation by hematoxylin and eosin staining (41). For lung injury scoring two blinded investigators analyzed the samples and determined levels of lung injury according to a semi-quantitative scoring system outlined below. All lung fields ( $\times 20$  magnification) were examined for each sample. Quantification of histological lung injury was determined using the following scoring: 1- normal; 2- focal (<50% lung section) interstitial congestion and inflammatory cell infiltration; 3- diffuse (>50% lung section) interstitial congestion and inflammatory cell infiltration; 4- focal (<50% lung section) consolidation and inflammatory cell infiltration; 5- diffuse (>50% lung section)

consolidation and inflammatory cell infiltration. The mean score was used for comparison between groups.

### Flow Cytometry

For surface staining, cells were incubated with Fc Block-2.4G2 (BD Pharmingen) antibody to block Fc $\gamma$  III/II receptors before staining with a specific antibody. The following antibodies were purchased from BD Pharmingen (San Diego, CA) and Biolegend (San Diego, CA): anti-Annexin V-PE, anti-7-AAD, anti-Ly6G-FITC, anti-Gr1-BV570, anti-CD11b-PETR, and anti-F4/80-APC-Cy7, along with relevant isotype antibodies. For our lymphocyte panel we used anti-CD4-Ax700, anti-CD25-APC-Cy7, anti-CD3-Pacblue, anti-CD8-PECF594. For intracellular staining of Foxp3, following Fc block and surface staining, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience, San Diego, CA), then stained with anti-Foxp3-APC mAbs (eBioscience). For intracellular staining of cytokines, following murine BAL (4 aliquots, 0.9 ml PBS + Golgi Plug (GP) (BD, protein transport inhibitor)), cells were isolated and resuspended ( $0.5 \times 10^6$  cells/ml) in RPMI/FCS/Pen/Strep/GP (unstimulated) or with additional Leukocyte Activation Cocktail (BD; PMA + Ionomycin + Brefeldin A; 2  $\mu$ l/ml, stimulated, to enhance intracellular cytokine signal) for 4 hours. Live-dead discrimination was performed with Fixable UV-excitable Blue Dead Cell Stain (Invitrogen). Cells were Fc blocked, surface stained for macrophage, neutrophil, and lymphocyte markers, and fixed/permeabilized (cytofix/cytoperm, BD pharmingen) and intracellular staining  $\times$  30 min for cytokines including anti-TNF- $\alpha$ -Percep, anti-IL-10-PE, anti-IL-6-APC, and anti-IFN $\gamma$ -PECy7. Monocytes, alveolar macrophages, neutrophils, and lymphocytes were gated with characteristic forward scatter/side scatter using a FACSAria instrument, and CellDiva for data acquisition (Becton Dickinson, San Jose, CA), and FlowJo for analysis (Tree Star Inc, San Carlos, CA).

### Statistical analysis

All values are reported as mean  $\pm$  SEM. Parametric or nonparametric testing was performed as indicated. Markers of injury were compared using the Student *t* test or Mann-Whitney rank sum test. Pairwise comparisons were performed using the Student *t* test with Bonferroni correction. Baseline and pre- and post-treatment data within a group were compared using repeated measure one-way analysis of variance (Fisher's protected least significant difference test). The survival curve was established with Kaplan-Meier survival analysis. A  $p < 0.05$  was used as the cut-off point for significance.

## RESULTS

### LPS priming accelerates lung injury resolution

To begin to examine mechanisms by which the immune system can be reprogrammed or primed in preparation for severe and potentially lethal injury, we needed a route of delivery which safely and quickly activates the immune system. Using oropharyngeal instillation, we effectively delivered intrapulmonary agents in a reproducible manner without surgery or prolonged anesthesia as demonstrated using trypan blue dye (Supp. Fig. 1A) (42). We compared the response of WT mice to lipopolysaccharide (LPS, 1 mg/kg) administered by oropharyngeal (o.p.) versus intratracheal (i.t.) routes, the latter a more established method of

inducing experimental lung inflammation (25, 40, 43), and followed mice for 7 days. Both routes of delivery induced significant weight loss (Supp. Fig. 1B) and signs of systemic injury with huddling and piloerection, significant increases in bronchoalveolar (BAL) protein (Supp. Fig. 1C), BAL total cell counts (Supp. Fig. 1D), and BAL neutrophils (>80% of total alveolar cells, not shown). WT mice challenged with o.p. LPS returned to baseline weight, BAL protein, and BAL cell count values by day 7. In contrast, WT mice challenged with i.t. LPS had persistent weight loss, persistent elevation of BAL protein and total cells at day 7. Notably, the alveolar cell profile of mice treated with o.p. LPS at day 7 was predominantly macrophages (85%), with significantly increased lymphocytes (10-15%) and fewer neutrophils (0-5%) compared to mice treated with i.t. LPS (not shown). These data suggest that o.p. delivery of LPS is a viable method to induce modest, self-limiting lung inflammation.

We sought to determine if immunological priming could protect mice from a subsequent more severe injury, in this case high dose i.t. LPS. We primed WT mice with o.p. LPS (1 mg/kg) or water (non-primed group). To determine whether immunological priming conferred mortality benefit, we then challenged LPS primed and non-primed mice with high dose i.t. LPS (7.5 mg/kg), more than twice our usual acute lung injury (ALI) LPS dose (25, 42), 7 days after priming. After high dose LPS, nearly 70% of the non-primed mice died, and surviving mice appeared ill even after 10 days (Fig. 1A). In marked contrast, none of LPS primed mice died, and all mice appeared healthy and recovered their baseline body weight by day 10 (not shown). Because survival was markedly reduced after high dose LPS in the non-primed group, we elected to use a lower i.t. LPS dose (3 mg/kg) to better discriminate the effects of priming on lung injury and resolution patterns as shown in the rest of Figure 1. After 7 days of “priming phase”, LPS (primed) and water (non-primed) treated animals received i.t. LPS (3 mg/kg), and were followed up to 5 days (Supp. Fig. 1E). In addition to reduced mortality at the 3 mg/kg LPS dose (Fig. 1A), mice in the primed group appeared healthier and had recovered their baseline body weight 5 days after i.t. LPS despite significant weight loss at days 1 and 3. Comparatively, mice in the non-primed group appeared ill, were less mobile, and at day 5 had a significant 20% weight loss from baseline (Fig. 1B). BAL protein, a marker of lung injury, was increased to similar levels in the non-primed and primed groups 1 and 3 days after i.t. LPS, but remained significantly elevated only in the non-primed group by day 5. Total alveolar cells (Fig. 1D) and alveolar neutrophils (Fig. 1E) were higher in the primed mice at day 1 after i.t. LPS, but significantly lower at days 3 and 5 when compared to non-primed mice. The pattern of histological changes in the lung was consistent with the pattern for BAL cells (Fig. 1F). Although interstitial thickening, cellular infiltration, and lung injury score (Fig. 1G) were worse in primed WT mice compared to non-primed mice at day 1, by day 5 only primed mice achieved resolution of histologic injury while non-primed mice remained severely injured.

Next, we examined the effects of immunological priming on lung injury resolution in a live bacterial pneumonia model. WT mice were treated with i.t. *Pseudomonas aeruginosa* (PAO1,  $1 \times 10^6$  CFU) 7 days after being treated with o.p. 1 mg/kg LPS (primed) or water (non-primed), and assessed for parameters of lung injury resolution and bacterial clearance. Primed mice had significantly reduced total alveolar cell counts (Fig. 2A) and reduced histologic injury (Fig. 2B) at day 4, as well as significantly lower bacterial burden in whole



lung at days 1 and 2 after i.t. PAO1 (Fig. 2C). Our studies suggest that priming animals with LPS accelerates resolution after non-infectious and infectious experimental lung injury.

### Lung priming modulates the alveolar inflammatory milieu

Alveolar neutrophils were significantly decreased in primed mice at days 3 and 5 after i.t. LPS. Given that apoptosis is a *sine qua non* for neutrophil removal and central to resolution of inflammation (44-47), we measured alveolar neutrophil apoptosis by annexin V/7-AAD staining. Neutrophil apoptosis was nearly two-fold higher at all measured intervals after i.t. LPS in the primed group compared to the non-primed group (Fig. 3A).

To begin to understand potential mechanisms by which immunological priming could impact alveolar neutrophil abundance and accelerate lung injury resolution, we measured select BAL cytokines TNF- $\alpha$ , IL-10, and active TGF- $\beta_1$ . High BAL levels of TNF- $\alpha$ , a pro-inflammatory cytokine, can promote neutrophil apoptosis in experimental and human ARDS (48-50), and when present, denote a distinctly different phenotype than that which is induced by endotoxin tolerance (11). IL-10 is an anti-inflammatory, pro-repair cytokine, but can also reduce neutrophil burden at sites of inflammation by multiple mechanisms (6, 28, 51). BAL TNF- $\alpha$  was higher at 1 day after i.t. LPS in the primed group compared to the non-primed group (Fig. 3B), similar to the pattern observed with BAL protein and cell counts. As with the rapid decrease in BAL protein and cells after day 1 in primed mice, BAL TNF- $\alpha$  was significantly lower in primed mice at day 3 after i.t. LPS, but further elevated in non-primed mice. BAL IL-10 was 3.5 fold higher on day 1 after i.t. LPS in the primed group and remained significantly elevated compared to the non-primed group at day 3 (Fig. 3C). BAL TGF- $\beta_1$  was significantly higher in primed mice before i.t. LPS (day 0) and after recovery from i.t. LPS (day 5) (Fig. 3D).

### IL-10<sup>-/-</sup> mice do not benefit from immunological priming

To determine if IL-10 plays a role in immunological priming, we attempted to prime IL-10<sup>-/-</sup> mice using o.p. LPS (1 mg/kg) compared to sterile water (non-primed) on day -7. IL-10<sup>-/-</sup> mice treated with o.p. LPS had returned to baseline weight by day 0 (we designated this group as primed), after which they were exposed to i.t. LPS (3 mg/kg) and compared to non-primed IL-10<sup>-/-</sup> mice exposed to i.t. LPS. In marked contrast to our results in WT mice (Fig. 1A), primed IL-10<sup>-/-</sup> mice did not have a survival benefit compared to non-primed IL-10<sup>-/-</sup> mice, with 40-50% mortality in each group (Fig. 4A). Surviving mice from the primed IL-10<sup>-/-</sup> group were ill-appearing with persistent weight loss (Fig. 4B), sustained elevation of total BAL cells (Fig. 4C), BAL neutrophils (Fig. 4D), and persistent histologic injury (Figs. 4E, F). The pattern of these responses was similar to that seen in non-primed IL-10<sup>-/-</sup> mice and distinct from the pattern we observed in primed WT mice. These studies support an important role for IL-10 in the immunological priming response.

### Macrophages are a significant IL-10 source in the priming response

We sought to determine prominent cellular sources of IL-10 after lung priming with LPS. We focused on day 1 after i.t. LPS, at which point BAL IL-10 levels in primed mice were highest. BAL cells were collected from primed and non-primed WT mice 1 day following exposure to i.t. LPS, stimulated *in vitro* in the presence of a Golgi inhibitor, and stained for

intracellular IL-10 expression. When assessed by flow cytometry, alveolar macrophages (F4-80<sup>+</sup>) isolated from primed WT mice produced significantly more IL-10 compared to macrophages isolated from non-primed WT mice (Fig. 5A); a representative histogram flow plot is shown and includes macrophages from primed *IL-10*<sup>-/-</sup> mice for comparison. CD4<sup>+</sup> T lymphocytes were an additional cellular source of IL-10, but CD8<sup>+</sup> T lymphocytes, CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs, and neutrophils were not a significant source of IL-10 (not shown). Alveolar macrophages from primed WT mice on day 1 after i.t. LPS also produced significantly more TNF- $\alpha$  compared to macrophages from non-primed WT mice (Fig. 5B). To further characterize differences between primed and non-primed macrophages, we assessed cytokine production among F4-80<sup>+</sup>CD11c<sup>+</sup> or F4-80<sup>+</sup>CD11b<sup>+</sup> alveolar macrophage subsets. We and others have determined that macrophage CD11b to be an acceptable marker of recruited or exudative macrophages (16, 52, 53). In contrast, CD11c expression among macrophages generally designates resident alveolar macrophages (54), but recruited macrophages also express CD11c at later time points with resolution of inflammation (53). In contrast to similar IL-10 and TNF- $\alpha$  production by CD11c<sup>+</sup> cells in each group (not shown), CD11b<sup>+</sup> macrophages from primed WT mice expressed more IL-10 and TNF- $\alpha$  than CD11b<sup>+</sup> macrophages from non-primed WT mice (Fig. 5C).

To determine whether macrophages present in the alveolar space during the priming response (either recruited during priming or “resident” from before the priming response) could be a prominent source of IL-10, we isolated alveolar macrophages from mice on day 0 (7 days after exposure to o.p. LPS (primed) or water (non-primed)) and stimulated them with LPS (100 ng/mL) in culture. Alveolar macrophages from primed mice secreted nearly five-fold more IL-10 after 18 hours compared to alveolar macrophages from non-primed mice (Fig. 5D).

### IL-10-producing alveolar macrophages are critical for immunological priming

We were interested in defining macrophage contributions to the priming response. Specific tissue and systemic macrophage depletion is readily achieved using clodronate liposomes (42, 54). Mice were primed with o.p. LPS (1 mg/kg) on day -7 followed by o.p. clodronate liposomes (Cl<sub>2</sub>MDP) or PBS-liposomes (control) on day -3, and intraperitoneal (i.p.) Cl<sub>2</sub>MDP or PBS liposome on days -2 and -1 (Supp. Fig. 2A). Following i.t. LPS (3 mg/kg) on day 0, we assess lung injury parameters on days 1 and 5. We confirmed that alveolar macrophages were decreased by >90% at day 0 in the Cl<sub>2</sub>MDP-liposomes group compared to the PBS-liposomes group (not shown). Primed mice treated with PBS liposomes (control) had returned to baseline weight by day 5 after i.t. LPS and were significantly different from primed mice treated with Cl<sub>2</sub>MDP liposomes (macrophage depleted) that had persistent weight loss at day 5 (Fig. 6A). BAL protein (Fig. 6B) and BAL neutrophils (Fig. 6C) were similarly elevated in both groups on day 1 after i.t. LPS, but by day 5, only the control mice had reduced, near-normal levels of BAL protein and BAL neutrophils. Primed macrophage-depleted mice had persistent elevation of BAL protein and neutrophils at day 5. We observed a similar profile of lung injury by histological changes, where primed macrophage-depleted mice had sustained inflammatory cell infiltration and interstitial thickening compared to the primed control mice at day 5 (Fig. 6D). Day 1 BAL IL-10 (Fig. 6E) and TNF- $\alpha$  (Fig. 6F) increases were markedly abrogated in the primed macrophage-depleted



mice, reaffirming that alveolar macrophages are a significant source of IL-10 and TNF- $\alpha$  in the priming response. Day 5 BAL TNF- $\alpha$  levels remained elevated in the macrophage-depleted group, correlating with other phenotypic markers that demonstrate persistent lung inflammation and injury. In macrophage-depleted mice, possible sources of alveolar TNF- $\alpha$  include neutrophils, CD4+ lymphocytes, and residual macrophages. In summary, macrophages that make IL-10 are critical for resolution of lung inflammation mediated by immunological priming.

### Tregs are necessary for immunologic priming and alveolar macrophage IL-10 production

Regulatory T cells (Tregs) are critical for resolution of lung inflammation (25), in part through macrophage interaction (6). Alveolar Tregs are present after o.p. LPS most prominently in the latter half of the priming phase (not shown). We sought to determine whether Tregs are critical for immunological priming in our lung injury model. We primed *Foxp3<sup>DTR</sup>* and *Foxp3<sup>gfp</sup>* mice with 1 mg/kg o.p. LPS (Supp. Fig. 2B). On days -2, -1, and +2, LPS primed *Foxp3<sup>DTR</sup>* (Treg depleted) mice and LPS primed *Foxp3<sup>gfp</sup>* (control) mice were treated with Diphtheria toxin (DT) which successfully depleted >90% Tregs only in *Foxp3<sup>DTR</sup>* mice (Treg depleted, not shown). When we challenged mice with i.t. LPS (3 mg/kg) on day 0, both primed groups displayed similar systemic injury at day 1, but by day 5, only control mice were gaining weight back towards baseline (Fig. 7A). We assessed lung injury parameters on days 1 and 5 to focus on acute injury and resolution time points in this model. At day 1, mice in both primed groups were injured, with similar elevation of BAL protein (Fig. 7B) and BAL neutrophils (Fig. 7C). However, at day 5, only Treg depleted mice had significant lung injury manifest as increased BAL protein and BAL neutrophils, as well as severe histological damage (Fig. 7D) in comparison to primed control mice. To determine whether Treg depletion altered alveolar macrophage IL-10 production, we isolated and stimulated *in vitro* BAL cells from primed Treg depleted mice and primed control mice on day+1 after i.t. LPS and assessed intracellular IL-10 production by flow cytometry. The percentage and number of BAL macrophages (F4-80+) between groups was similar (not shown). BAL macrophages from primed control mice expressed significantly more IL-10 than macrophages from primed Treg depleted mice (Fig. 7E). In contrast, macrophage TNF- $\alpha$ , IL-6 and IFN- $\gamma$  intracellular expression was similar between groups (not shown). In addition, the percentage of macrophages with dual expression of IL-10 and TNF- $\alpha$  was higher in the primed control group compared to the primed Treg depleted group (not shown), a pattern similar to what we observed previously in primed WT mice compared to non-primed WT mice in figure 5. Collectively, these data reinforce the importance of Tregs in resolution of lung injury after immunological priming and suggest that Tregs enhance macrophage IL-10 production.

### Mild inflammation by CpG can induce immunological priming

We sought to determine whether immunological priming could be achieved using a priming agent which induced a milder lung inflammatory response compared to LPS. We used unmethylated CpG dinucleotides, a hallmark of microbial DNA sensed by TLR9, and mimicked by synthetic oligonucleotide containing CpG motifs (CpG). By activating an immune response sufficient to defend against a variety of bacterial, viral and parasitic pathogens (55), CpG has been used as a vaccine adjuvant in infectious disease and as an

immunotherapeutic agent for oncologic illnesses. We treated WT mice with o.p. CpG (or an equal volume of o.p. PBS), followed their weights, and measured parameters of lung injury at peak weight loss (d3 after CpG) (Supp. Fig. 2). Mice primed with CpG had minimal weight change, and behaved similarly to mice treated with PBS (Supp. Fig. 2C). Furthermore, mice primed with CpG had only mild elevations of BAL protein (Supp. Fig. 2D) and BAL total cell count (Supp. Fig. 2E) compared to mice treated with PBS and much less than WT mice primed with LPS at comparable time points (Supp. Fig. 1B-D). Collectively, these data suggest that o.p. CpG induces only mild lung inflammation.

We then challenged WT mice with i.t. LPS (3 mg/kg) after o.p. CpG (Supp. Fig. 2F) and compared their response to o.p. PBS-treated mice (non-primed) for parameters of systemic and lung injury at days 1 and 5. WT mice primed with CpG had similar weight loss (Fig. 8A) and severity of lung injury to non-primed WT mice on day 1 after i.t. LPS (3 mg/kg) based on BAL protein (Fig. 8B) and total cell counts (Fig. 8C). By day 5, CpG primed mice were nearing baseline weight, and had significantly less BAL protein and lower total BAL cell counts than non-primed mice. Amongst BAL cells at day 5, we observed a marked four-fold decrease in neutrophils in the CpG primed group (Fig. 8D). In contrast to WT mice, *IL-10*<sup>-/-</sup> mice treated with CpG followed by i.t. LPS did not achieve accelerated lung injury resolution compared to non-primed *IL-10*<sup>-/-</sup> mice (not shown).

## DISCUSSION

Our findings demonstrate that immunological priming renders the lung capable of robustly responding to a subsequent non-infectious or infectious severe exposure to improve survival and accelerate resolution from severe lung inflammation. IL-10 is critical for immunological priming in our model, and alveolar macrophages appear to be an important source of IL-10. Furthermore, macrophage production of IL-10 requires the presence of alveolar Tregs during the priming response, a finding from which we can infer the importance of cellular cross-talk to achieve resolution of lung inflammation and injury (25). Without either macrophages or Tregs, the benefits of immunological priming disappear.

In experimental acute lung injury models, IL-10 is reported to have somewhat diverse effects. IL-10 was protective at early time points after i.t. LPS-induced lung injury (56), during carrageenan-induced pleuritis (57), and in lung injury secondary to shock (58, 59). However, IL-10 was harmful in a *Pseudomonas aeruginosa* pneumonia model when overexpressed in the lung (60), and following sub-lethal influenza infection where antibody-mediated blockade of IL-10 improved susceptibility to a secondary pneumococcal pneumonia (61). While IL-10 is likely not the only mediator of immunological priming, the divergent conclusions between our work and that of others' suggests that IL-10 may be most beneficial when its production is tightly regulated to counteract pro-inflammatory cascades. With its ability to dampen neutrophil recruitment and enhance neutrophil apoptosis, unopposed IL-10 may be harmful especially early in the inflammatory response when neutrophil presence is most critical. In our model, despite early increases in macrophage-derived IL-10 in primed mice, alveolar neutrophil numbers and overall lung inflammation were similar in both primed and non-primed groups at early time points. Concurrent increases in macrophage-derived TNF- $\alpha$  and potentially other unmeasured pro-

inflammatory cytokines may oppose IL-10 effects to help preserve the early inflammatory response and may help explain why priming accelerated bacterial clearance and resolution of lung inflammation following pseudomonas infection in our model.

Among a subset of alveolar macrophages (F4-80<sup>+</sup> CD11b<sup>+</sup>; Fig. 5C) derived from primed mice, the production of significantly more TNF- $\alpha$  and IL-10 on day 1 after re-challenge with i.t LPS suggests that distinct alveolar macrophage sub-populations may be present during the priming response. Recent work phenotyping lung macrophages during inflammation outlines complicated schema to fully characterize resident versus recruited lung macrophages (54, 62, 63). The current construct of our experimental model does not lend itself to definitive declaration of dynamic macrophage or dendritic cell subpopulations during the priming and post-injury resolution response. However, it is likely that our priming model induces sufficient inflammation to recruit bone-marrow derived macrophages to the alveolar space during the priming phase. As inflammation during the priming phase subsides, a percentage of recruited macrophages remain as new “resident” alveolar macrophages as others have shown (53). We hypothesize that the ability of this new “resident” alveolar macrophage population to produce significant levels of both TNF- $\alpha$  and IL-10 when challenged with a second inflammatory stimulus such as LPS may distinguish them from old “resident” macrophages that may exhibit endotoxin tolerance and produce less TNF- $\alpha$  after repeat LPS challenge (11, 23, 28). The new “resident” population may be distinguishable from additional inflammatory macrophages recruited to the alveolar space within the first day after i.t. LPS in that they produce higher levels of TNF- $\alpha$  but lower levels of IL-10. The concurrent production of TNF- $\alpha$  and IL-10 among primed macrophages may be an important and representative example of adaptive cellular reprogramming by alveolar macrophages that may not be fully recapitulated by exogenous delivery of anti-inflammatory cytokines such as IL-10 or TGF- $\beta$ . In response to a subsequent severe insult, therefore, primed macrophages may simultaneously produce pro-inflammatory mediators critical for the initial immune response, and produce anti-inflammatory mediators necessary to modulate lung inflammation and hasten resolution.

We elected to focus our assessment of immune responses in the alveolar compartment of the lung, but cannot exclude the biological importance of the lung interstitium. When compared to our typical patterns of experimental lung injury and resolution (25), a distinguishing feature of the priming response was the increase in alveolar neutrophil apoptosis at all measured time points. Apoptosis and removal of neutrophils are critical for resolution of lung inflammation and intimately tied to the cytokine and cellular make-up of the alveolar space (44-46, 64), and thus provided us with additional rationale to focus our assessment within the alveolar compartment. We also cannot disregard the possible contribution of other immune cells such as IL-10-producing dendritic cells that can stimulate Tregs (65) or lung MDSC-like cells that can make IL-10 (66) in lung inflammatory models. With lavage, we typically do not recover significant numbers of dendritic cells from the bronchoalveolar spaces in our experimental lung injury models. It is conceivable that lung MDSC-like cells that express F4-80, CD11b, and Gr1 (low) are phenotypically similar to the CD11b subset of alveolar macrophages (F4-80<sup>+</sup>) we define to be a source of significant IL-10 production in our priming model. Lastly, our use of clodronate to specifically deplete macrophages, and

not dendritic cells or neutrophils as others have shown (54), added specificity to the importance of macrophages in the priming response and as a critical source of IL-10.

Prior exposure of immune cells to endotoxin can induce a significantly diminished pro-inflammatory response to subsequent LPS exposure, known as endotoxin tolerance (ET) (67-71). The hallmark of ET is a marked downregulation of pro-inflammatory mediators such as TNF- $\alpha$ , IL-6, IL-12, IL-1 $\beta$  (72, 73) with concurrent increase in anti-inflammatory cytokines including IL-10, TGF- $\beta$  and IL-1ra (74). Although priming with two interval exposures to LPS could be considered an example of *in vivo* endotoxin tolerance, there are major distinctions that suggest the observed phenotype might not be explained primarily by ET. First, following i.t. LPS in primed animals, we observed an initial robust lung inflammatory response notable for significant TNF- $\alpha$  secretion, prominent alveolar neutrophil influx, and increased histologic injury. Second, although cross-tolerance or heterotolerance among TLR ligands can occur (75-77), the immunological priming response to accelerate lung injury resolution was also observed using a distinct TLR agonist, CpG, to prime the mice, and demonstrated similar early inflammatory lung injury patterns as with LPS priming. Third, the priming response evolved over a period of several days, not the 24-48 hour period usually associated with endotoxin tolerance (78, 79).

Our study generates several questions which we are actively pursuing in order to better understand lung priming-mediated protection. One, how do macrophages and Tregs communicate, if at all, to contribute to the priming response and hasten lung injury resolution? We are working to create an *in vitro* priming modeling system. We have shown that cell-cell contact was required for Tregs to modulate macrophage TNF- $\alpha$  production in an *in vitro* co-culture system (25), but did not determine whether Tregs modulate macrophage IL-10 production. We do not yet understand mechanisms mediating lung Treg recruitment during priming, or whether monocyte/macrophage populations contribute specifically to Treg recruitment. Elssner demonstrated the importance of caspase 3-regulated IL-16 secretion by blood monocytes (80). IL-16 is a potent Treg chemoattractant (81) and may be critical in our model for Treg recruitment during the priming response. Two, are there specific cellular proteins or pathways responsible for the priming effect? Our CpG data demonstrates that neither significant lung inflammation nor primary TLR4-based priming is required to accelerate lung injury resolution, but we have not yet compared downstream macrophage TLR signaling in each model. Three, does priming protect against other infectious models of lung injury? We are actively exploring the effects of priming on other prevalent pathogens such as pneumococcus and influenza. Four, do epigenetic, phenotypic, and functional differences exist between primed and non-primed macrophages? It is conceivable that macrophages from primed mice are able to produce other anti-inflammatory mediators such as TGF- $\beta$ , IL-1ra, lipid mediators, or reprogram more effectively from M1 classically activated to M2 alternative activated or regulatory macrophages. Five, do macrophages from primed mice co-signal lymphocytes to modulate their proliferation and skewing more effectively? Lastly, are there additional cellular communications, for instance alveolar macrophage-epithelial or Treg-epithelial, which could contribute to the priming response? We have shown the importance of Th1 lymphocytes for prevention of fibrosis in a bleomycin model (82), from which we infer possible lymphocyte-

epithelial interactions (83). Furthermore, the increase in BAL TGF- $\beta$  we observed in primed mice prior to i.t. LPS may signify enhanced communication with airway and alveolar epithelial cells mediated by  $\alpha\psi\beta6$  integrin (84).

We have developed immunological priming as a strategy to nonspecifically enhance the lung's mucosal immune responses and accelerate clearance of a wide range of pathogens to promote resolution and repair. This unconventional approach could be implemented rapidly in a large population, particularly in areas with high incidence of endemic infections such as influenza or in cases of new epidemics/ pandemics where a lack of sufficient or appropriate medicines and vaccines may preclude timely control of the epidemiological burden. Additionally, if longer lasting priming mediated protection can occur, supplementing current seasonal vaccines may be another area of potential clinical use. Using CpG, we have demonstrated induction of mild lung inflammation to be sufficient to elicit protection against a robust secondary insult, and anticipate translational applicability (85, 86).

Therapy for acute lung injury remains largely supportive. Measures to prevent high-risk patients from developing this often fatal syndrome have been largely disappointing. Moreover, emerging respiratory pathogen epidemics remain a huge concern and protecting individuals at risk is a tremendous challenge. Here we demonstrate a role for priming of alveolar macrophages leading to accelerated resolution of lung inflammation by non-infectious and infectious agents; applicability is enhanced by our ability to use a priming agent that induces only minimal lung inflammation. Understanding the cellular and molecular mechanisms of primed immune cells could lead to novel targets useful for resolution of lung inflammation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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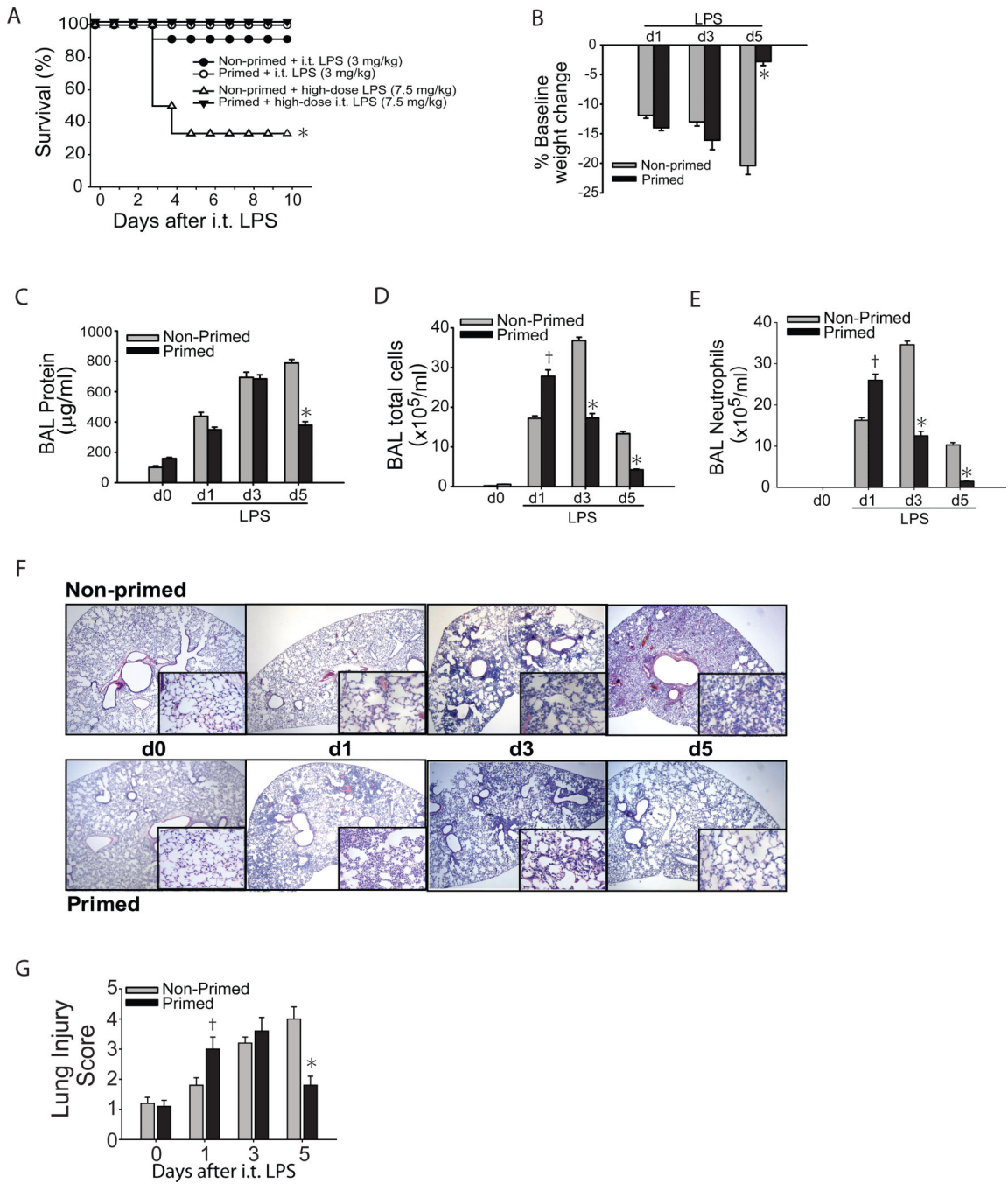


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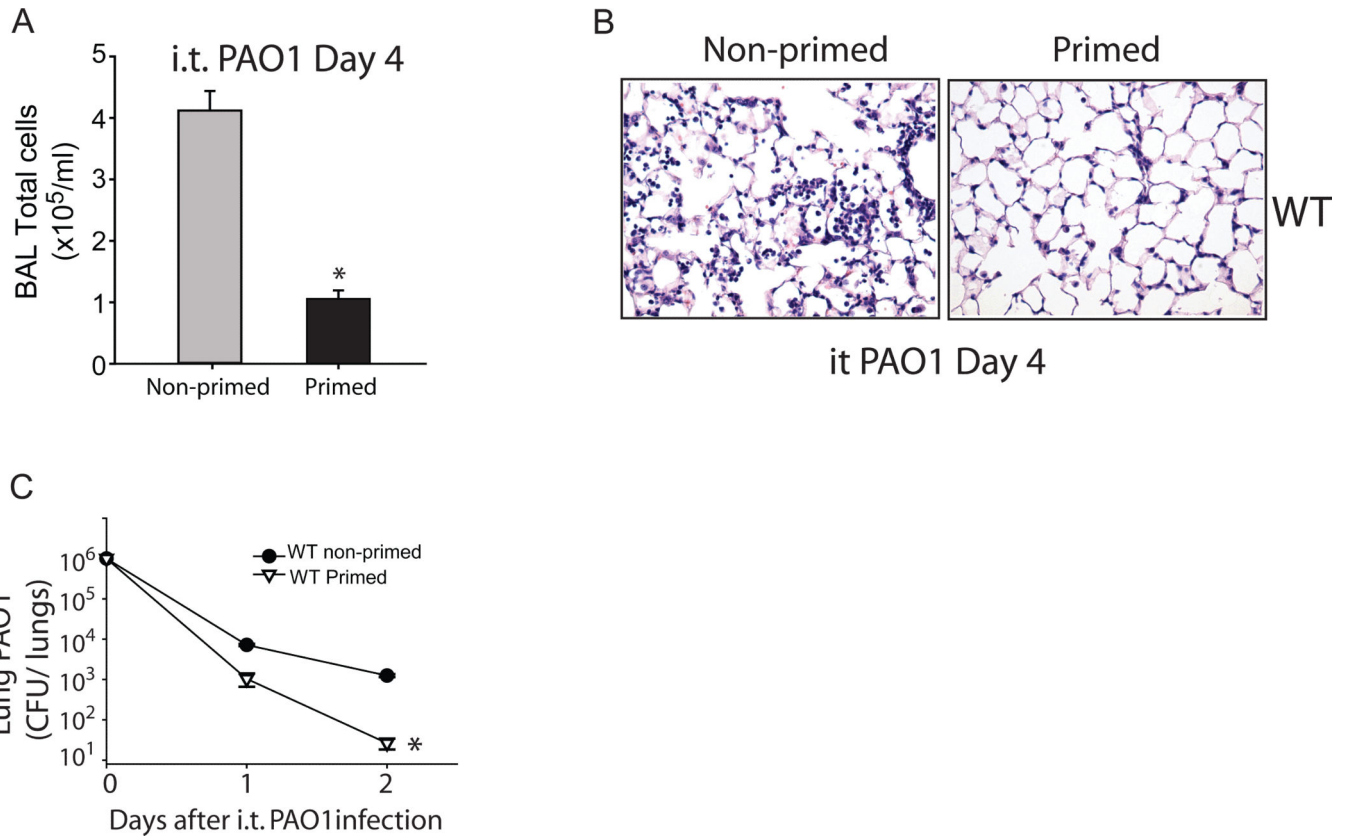
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**Fig. 1. Immunological priming accelerates lung injury resolution**

Following a 7-day priming period, primed and non-primed WT mice were assessed for survival after either 3 mg/kg or 7.5 mg/kg i.t. LPS (A). After either dose of i.t. LPS, survival over 10 days was determined in primed and non-primed WT mice (n=8-10 per time point). Primed and non-primed WT mice were assessed for body weight relative to baseline (B), bronchoalveolar (BAL) protein (C), BAL total cell counts (D) and BAL neutrophils (E) at intervals after i.t. LPS injury. (F) Histological sections were stained with H & E in primed and non-primed WT mice. Original magnifications x20; x100 (inserts). (G) Histopathological mean lung injury scores from x20 sections (n=4-6 animals per group per time point). Values expressed as mean ± SEM; \* or † paired t-test against other group at same time point, p<0.05, \* log-rank test for survival curve.

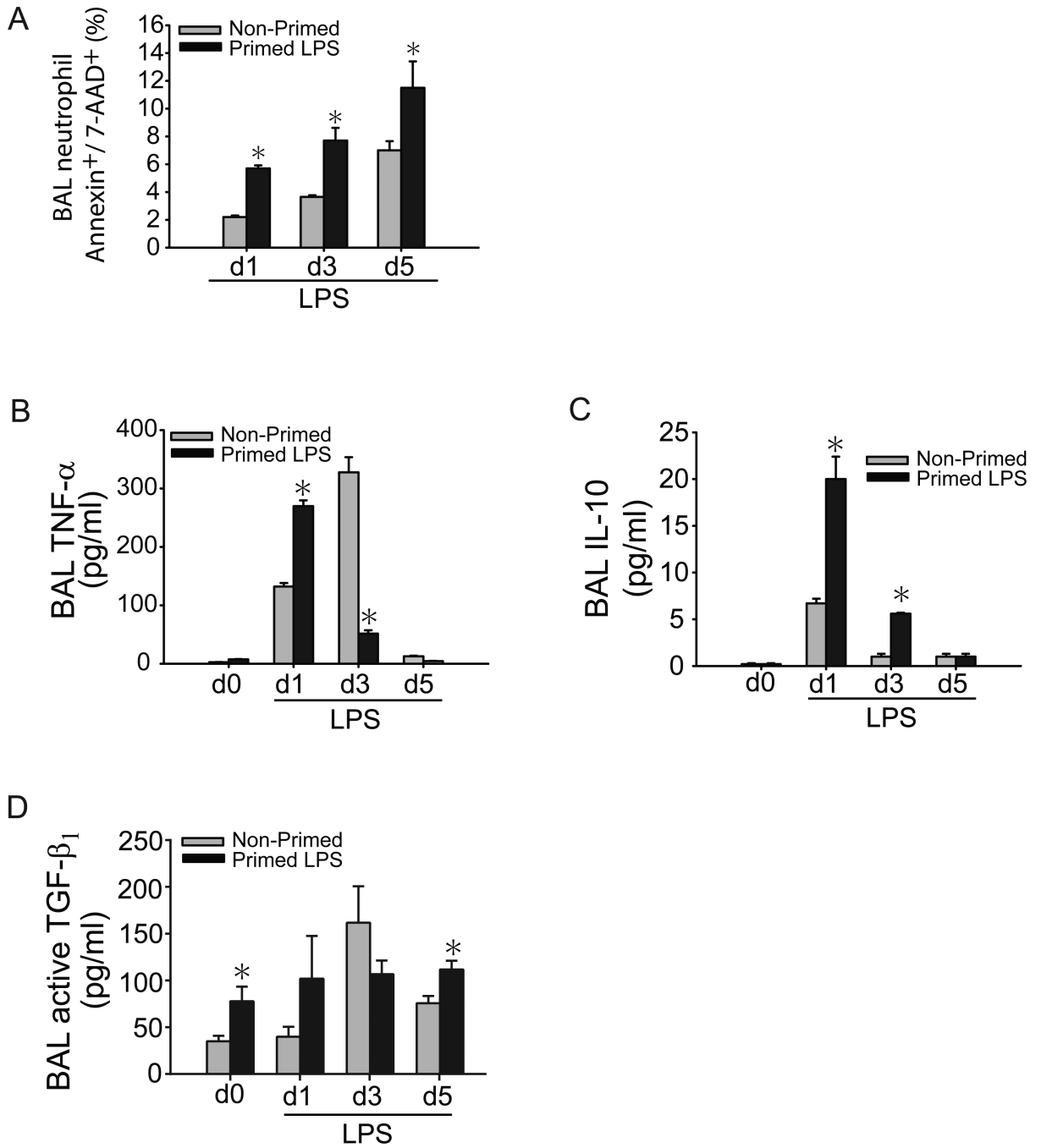




**Fig. 2. Immunological priming accelerates resolution from bacterial pneumonia**

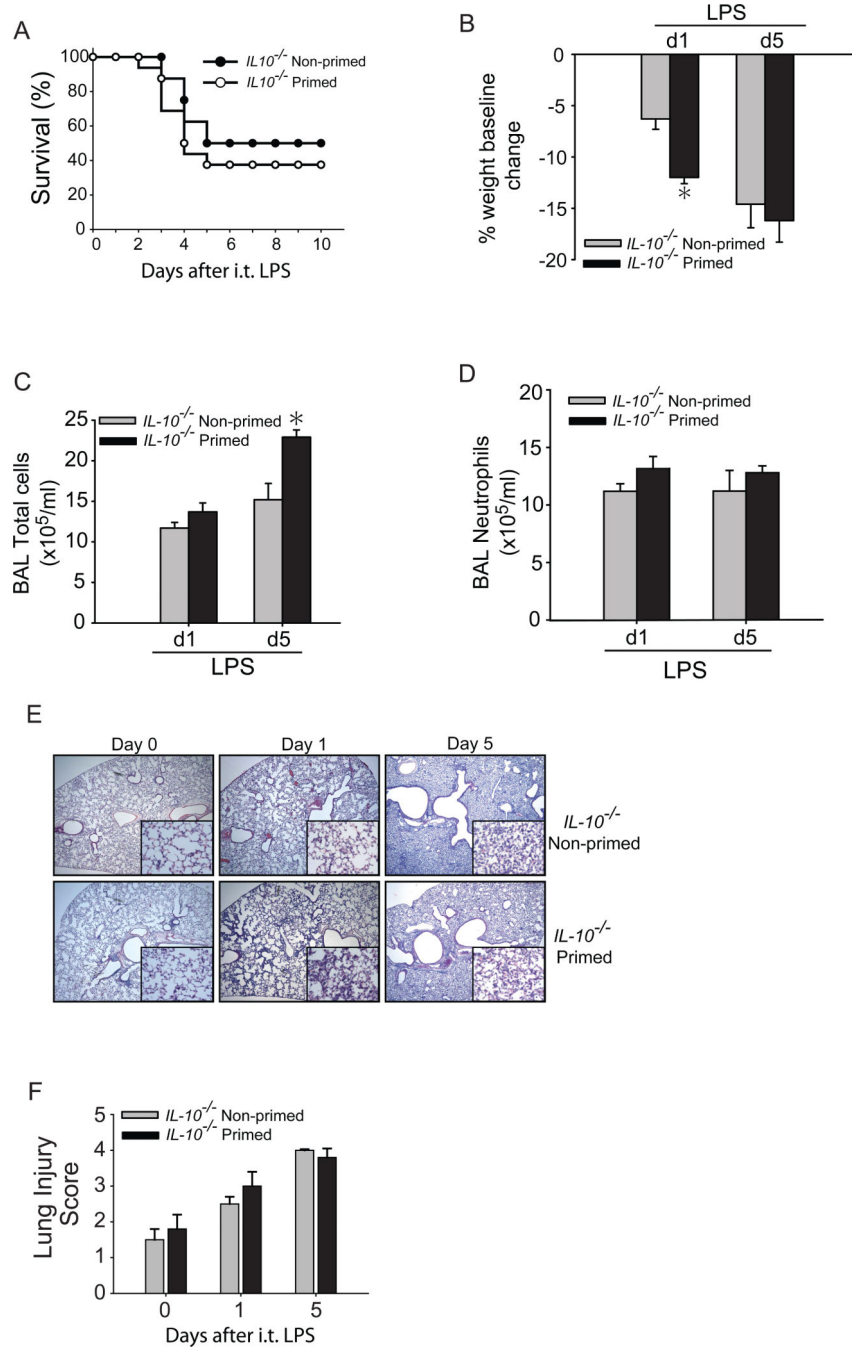
Following an 7-day priming period, BAL total cells (A) and lung histology by H&E staining (B) were assessed in primed and non-primed WT mice at day 4 after i.t. PAO1 ( $1 \times 10^6$  CFU). Bacterial clearance was determined by measurement of whole lung PAO1 colony-forming units (CFU) in WT primed and non-primed mice on days 1 and 2 after i.t. PAO1 (C). Values expressed as mean  $\pm$  SEM; \*paired t-test against other group at same time point,  $p < 0.05$ . (n=4-6 animals per group per time point)





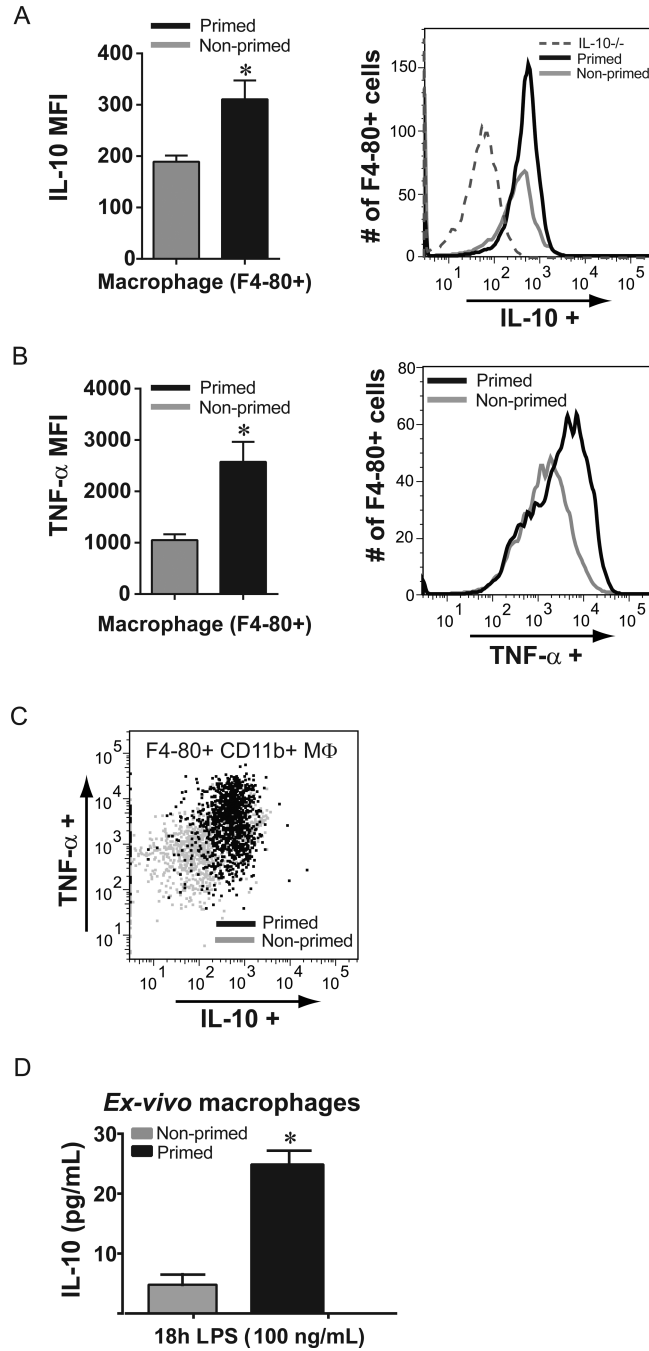
**Fig. 3. Lung priming modulates the alveolar inflammatory milieu**

(A) Apoptosis (Annexin V<sup>+</sup>/7-AAD<sup>+</sup>) of BAL neutrophils from primed and non-primed WT mice on days 1, 3, or 5 after i.t. LPS was assessed by flow cytometry. BAL neutrophils were gated by characteristic granulocyte forward and side scatter, sub-gated for Gr-1+ to identify neutrophils, and then for AnnexinV/7-AAD, percentages for which are quantified in (A). BAL TNF-α (B), IL-10 (C), and active TGF-β<sub>1</sub> (D) cytokine secretion were assessed at designated time points after i.t. LPS in primed and non-primed WT mice. Values expressed as mean ± SEM; \*paired t-test against other group at same time point, p<0.05. (n=4-6 animals per group per time point)



**Fig. 4. *IL-10*<sup>-/-</sup> mice do not benefit from immunological priming**

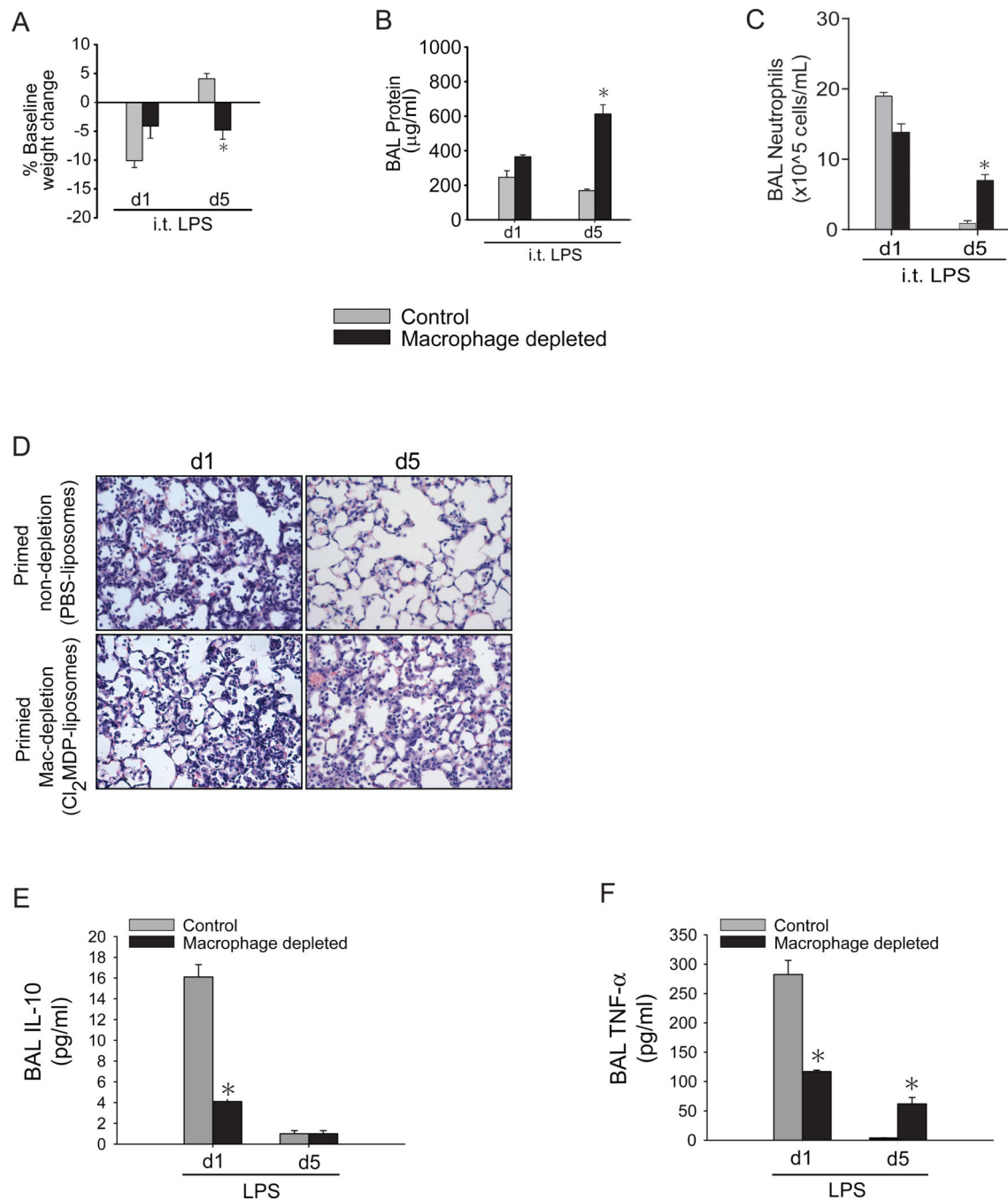
(A) Survival was determined in primed and non-primed *IL-10*<sup>-/-</sup> mice. Primed and non-primed *IL-10*<sup>-/-</sup> mice were assessed for body weight relative to baseline (B), BAL total cell counts (C) or BAL neutrophils (D) at days 1 or 5 after i.t. LPS injury. (E) Histological sections were stained with H & E in primed and non-primed WT mice. Original magnifications x20; x100 (inserts). (F) Histopathological mean lung injury scores from x20 sections. Values expressed as mean ± SEM; \*paired t-test against other group at same time point, p<0.05, (n=4-6 animals per group per time point). log-rank test for survival curve, n=8-10 in primed and non-primed groups.



**Fig. 5. Macrophages are a significant IL-10 source after lung priming**

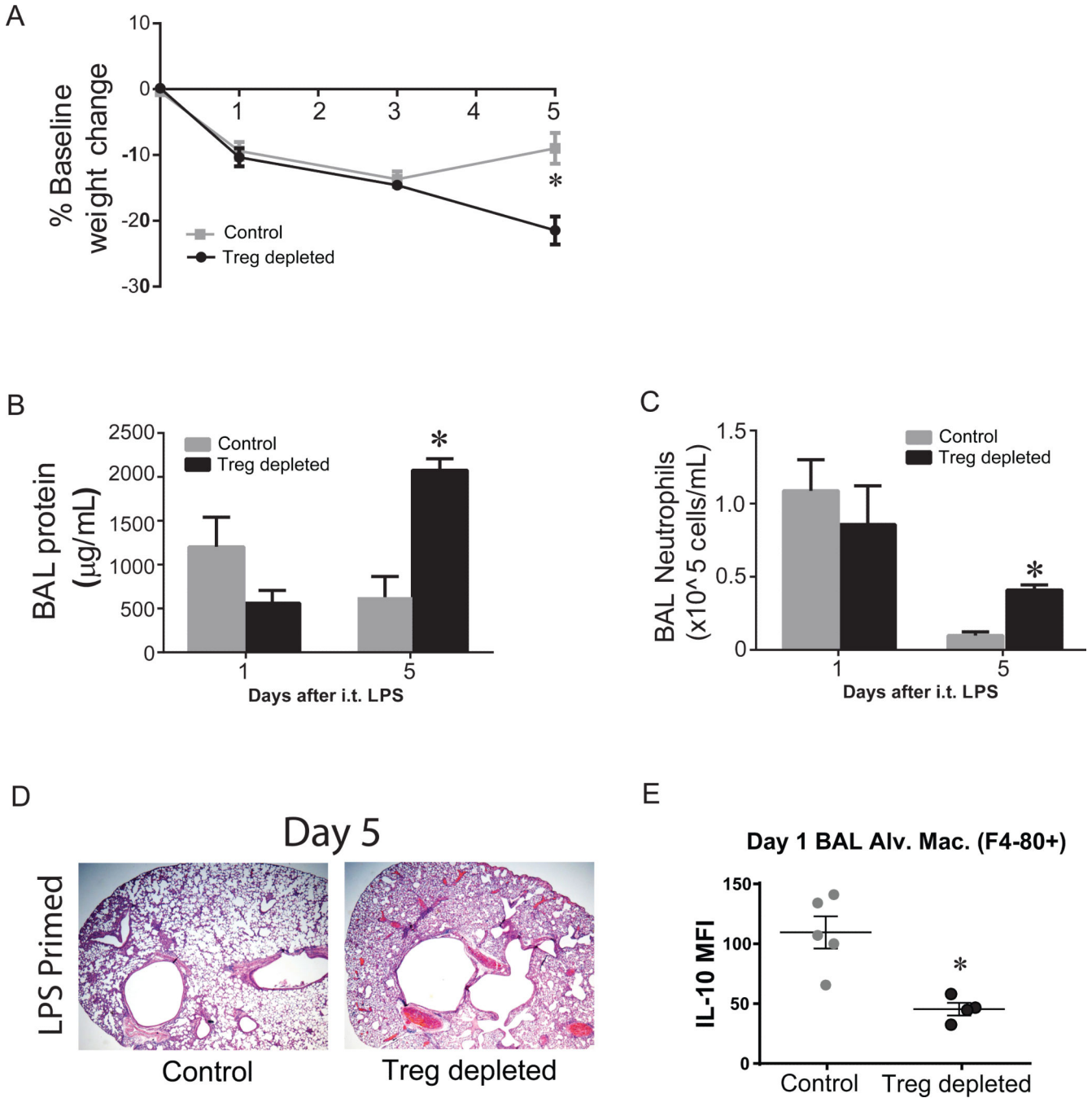
Alveolar cells from primed and non-primed WT mice were isolated at day +1 after i.t. LPS (3 mg/kg) challenge, and then restimulated, and stained for macrophage, neutrophil, and lymphocyte flow markers as well as intracellular cytokine production. IC IL-10 production (A) and IC TNF-α production (B) were quantified by mean fluorescence intensity (MFI) in F4-80+ alveolar macrophages collected from primed and non-primed WT mice; a representative flow cytometry histogram is shown for each, and for IL-10 includes alveolar macrophages from primed *IL-10*<sup>-/-</sup> mice (dashed line). (C) Among F4-80+ CD11b+ alveolar cells from primed (black) and non-primed (gray) mice, a dot plot demonstrating individual cell IC production demonstrates a predominant increase in dual cytokine production from primed alveolar cells. (D) Alveolar macrophages were isolated 7 days

after o.p. LPS or o.p. water (control), and stimulated with LPS (100 ng/mL); IL-10 secretion was quantified by ELISA after 18 hours of stimulation. Values expressed as mean  $\pm$  SEM; \*paired t-test against other group at same time point,  $p < 0.05$ . (n=4-5 animals or wells per group per time point)



**Fig. 6. IL-10 producing alveolar macrophages are critical for immunological priming**

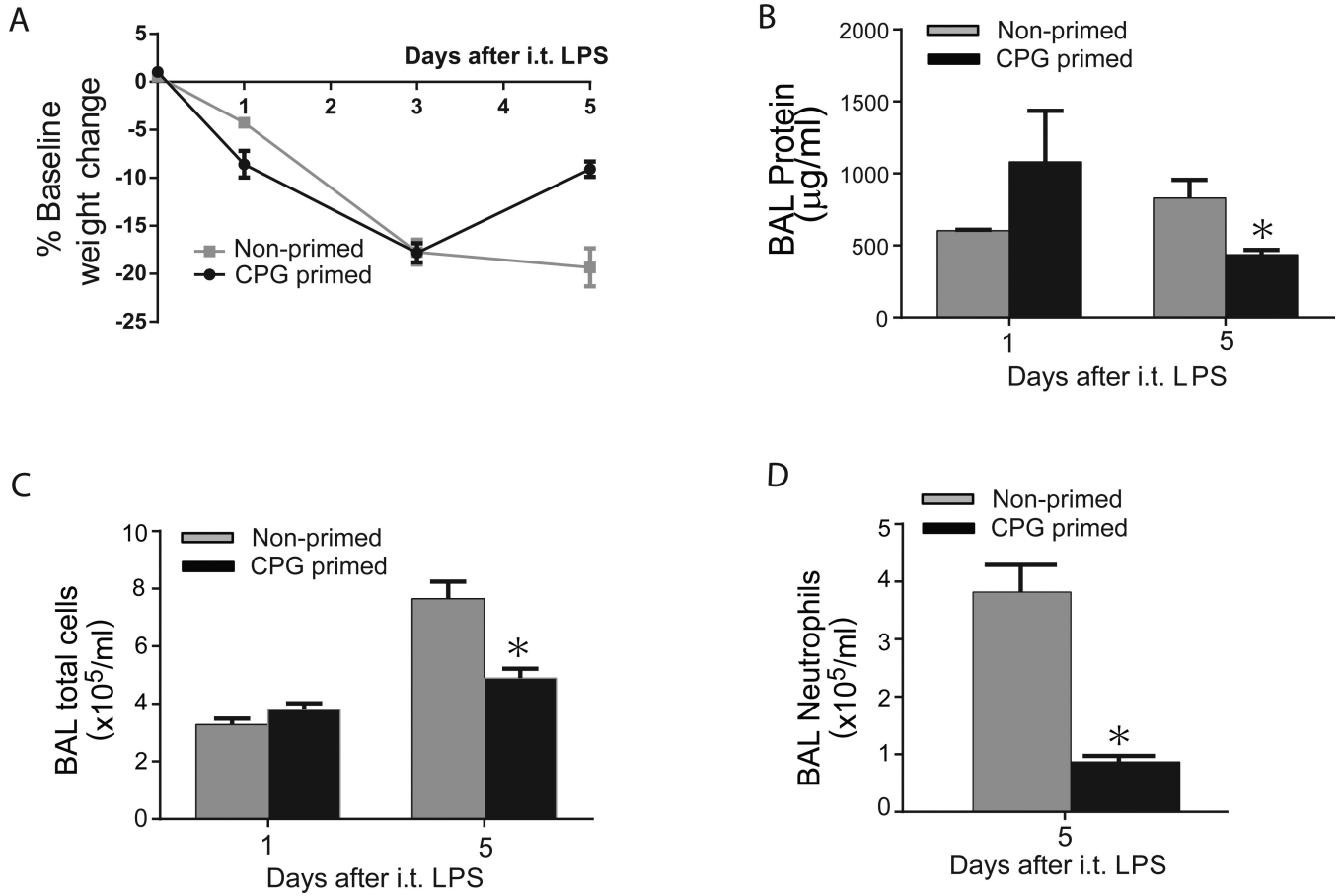
Primed mice treated with PBS-liposomes (control) and primed mice treated with  $\text{CL}_2$ -MDP liposomes (macrophage depleted) were assessed for body weight relative to baseline (A), BAL total protein (B), BAL neutrophils (C) or histological damage (D) by H&E staining ( $\times 100$  magnification) at days 1 or 5 after i.t. LPS injury. BAL IL-10 (E) and TNF- $\alpha$  from primed control and primed macrophage-depleted were measured at days 1 or 5 after i.t. LPS. Values expressed as mean  $\pm$  SEM; \*one-way ANOVA (A-C) or paired t-test (E-F) against other groups at same time point,  $p < 0.05$ , ( $n = 4-6$  animals per group per time point)



**Fig. 7. Tregs are necessary for priming and alveolar macrophage IL-10 production**

Primed *Foxp3<sup>flp</sup>* mice (control) and primed *Foxp3<sup>DTR</sup>* (Treg depleted) were assessed for body weight relative to baseline (A), BAL total protein (B), BAL neutrophils (C), or histological damage (D) by H&E staining (x2x magnification) at days 1 or 5 after i.t. LPS injury. All mice received i.p. DT injections (15 µg/kg) on days -2, -1, and +2 when harvested at day 5. (E) At day 1 after i.t. LPS, we determined intracellular production of IL-10 among F4-80+ BAL macrophages by flow cytometry. Values expressed as mean ± SEM, as well as individual values in E; \*paired t-test against other primed group at same time point,  $p < 0.05$ , (n=4-6 animals per group per time point)





**Fig. 8. Immunological priming is achieved with minimal inflammation**

WT mice were treated with o.p. PBS (Non-primed) or o.p. CpG (primed). 5 days later, mice were challenged with i.t. LPS (3 mg/kg), and were assessed for body weight relative to baseline (A), BAL total protein (B), BAL total cells (C), or BAL neutrophils (D) at days 1 or 5 after i.t. LPS injury. Values expressed as mean ± SEM; \*paired t-test against other primed group at same time point,  $p < 0.05$ , (n=4-6 animals per group per time point)