The Journal of Immunology

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J Immunol 2013; 190:5731-5738; Prepublished online 29 April 2013;
doi: 10.4049/jimmunol.1203362
http://www.jimmunol.org/content/190/11/5731

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

TGF-β1 Limits the Onset of Innate Lung Inflammation by Promoting Mast Cell–Derived IL-6

Kirthana Ganeshan,[1] Laura K. Johnston, and Paul J. Bryce

TGF-β1 is an important suppressive mediator of inflammation, but it can also drive fibrosis and remodeling in the lung. In response to intratracheal LPS, neutrophils migrate into the lung, and TGF-β1 was suggested to protect against the ensuing injury. However, the mechanisms for this protective role remain unknown. Using a model of acute lung injury, we demonstrate that TGF-β1 decreases neutrophil numbers during the onset of injury. This was due to increased apoptosis rather than reduced migration. We demonstrate that TGF-β1 does not directly regulate neutrophil apoptosis but instead functions through IL-6 to promote neutrophil clearance. rIL-6 is sufficient to promote neutrophil apoptosis and reduce neutrophilia in bronchoalveolar lavage fluid, while IL-6 increases rapidly following LPS-induced injury. Mast cells are a critical source of IL-6, because mast cell–deficient mice exhibit increased neutrophil numbers that are reduced by reconstitution with wild-type, but not IL-6$^{-/-}$, mast cells. Although IL-6 diminishes neutrophilia in mast cell–deficient mice, TGF-β1 is ineffective, suggesting that these effects were mast cell dependent. Taken together, our findings establish a novel pathway through which TGF-β1, likely derived from resident regulatory T cells, controls the severity and magnitude of early innate inflammation by promoting IL-6 from mast cells. ***The Journal of Immunology*****, 2013, 190: 5731–5738.**

Acute lung injury (ALI), or acute respiratory distress syndrome (ARDS) in its most severe form, is an edematous inflammatory response in the lung that occurs during direct (e.g., pneumonia) or indirect (e.g., sepsis) insult. Approximately 40% of ALI patients die, despite intensive clinical care (1, 2). Immunologically, a hallmark of most ALI responses is an acute phase that consists of the rapid recruitment of neutrophils to the lung and their activation (3). This is followed by a resolution phase, whereby neutrophils enter apoptosis and are cleared by infiltrating mononuclear cells (4). In animal models, failure of this apoptosis, due to deficiency in either TRAIL or caspase-1, leads to an enhanced inflammatory response (5, 6), and strategies to pharmacologically induce apoptosis promote enhanced resolution of ALI responses (7). Neutrophils from patients with ARDS exhibit lower levels of apoptosis than normal (8), and their numbers were lower in patients who survived ARDS compared with those who did not (9). However, the processes that regulate the numbers of neutrophils or induce their apoptotic clearance remain largely unknown.

TGF-β plays a well-established role in promoting lung fibrosis and remodeling in many inflammatory disorders, including asthma (10) and chronic obstructive pulmonary disease (11). TGF-β1 promotes epithelial–mesenchymal transition of alveolar epithelial cells via a functional complex of its receptor with α3 integrin that initiates β-catenin activation (12). In contrast to this pathogenic effect of TGF-β1 on the chronic responses of the lung, it can also exert potent immunoregulatory influences during the onset and progression of inflammation; however, the mechanisms behind this role are largely unclear. In the context of ALI, TGF-β1 was shown to diminish the magnitude of the early acute response upon intranasal LPS challenge (13). More recently, TGF-β–expressing Foxp3^{+} regulatory T cells (Tregs) were also shown to augment the later resolution phase of ALI (14). However, although ALI-associated inflammation is highly neutrophilic, the direct effect of TGF-β1 on neutrophils themselves seems to be relatively modest. Depending on the experimental approach taken, TGF-β1 was reported to both directly stimulate and inhibit neutrophil migration and degranulation (15–17), but these effects were mild compared with other stimuli. Recent work showed that blocking TGF-β–activated kinase 1 reduced the production of neutrophil-derived cytokines and resistance to apoptosis (18), suggesting that TGF-β–driven pathways might actually enhance neutrophil functions and survival. Consequently, the mechanisms through which TGF-β diminishes ALI responses are undefined.

We recently demonstrated that rTGF-β1– and TGF-β–expressing Tregs modulate the responsiveness of mast cells to inflammatory stimuli, including LPS (19). In addition to their established role in allergy, mast cells are being increasingly recognized for their roles in innate immune responses and in shaping the nature of the ensuing inflammatory response (20). Indeed, our study demonstrated that Tregs and TGF-β1 actively increased mast cell–derived IL-6 and that this enhanced the development of Th17 cells and intestinal homeostasis in a food allergy model (19). In vitro production of IL-6 by mast cells occurs rapidly upon LPS-driven activation and is independent of the degranulation response that underlies the established role of mast cells in IgE-mediated immediate hypersensitivity (21). Although the physiological roles of mast cell–derived IL-6 remain largely unknown, recent studies showed that it is important in protection against *Klebsiella* pneumonia infection (22) and in limiting tumor growth (23), implying a role in innate immune responses.

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Received for publication December 7, 2012. Accepted for publication March 29, 2013.

This work was supported by National Institutes of Health Grant R01 AI-076456 (to P.J.B.) and a predoctoral award (AHA#10PRE2630137) from the American Heart Association (to K.G.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BMMC, bone marrow–derived mast cell; sIL-6R, soluble IL-6R; Treg, regulatory T cell; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1203362

In a similar way to TGF-β1, IL-6 is most commonly studied for its proinflammatory activities and role in chronic inflammatory diseases, such as rheumatoid arthritis (24). However, during the initiation of inflammation, a relatively small number of cells, including neutrophils, is actually capable of directly responding to IL-6 because of the restricted expression of membrane-bound IL-6R. Instead, most cells are stimulated via *trans*-signaling by IL-6 in complex with soluble IL-6R (sIL-6R) via binding to gp130, the common IL-6 family signaling receptor subunit (25). Neutrophils express high levels of IL-6R, and the liberation of sIL-6R upon cell death was shown to be essential in regulating chemokine production from other cells and the subsequent recruitment of mononuclear cells during peritoneal infection (26). Regulation of neutrophil numbers themselves by IL-6 during acute peritoneal inflammation was shown to be dependent on STAT3 signaling from gp130 (27), although this study did not determine whether the alteration of neutrophil frequency was due to changes in recruitment or survival. Interestingly, although IL-6 *trans*-signaling is antiapoptotic for many cells, direct IL-6 signaling on neutrophils was shown to promote their apoptotic death (28), although the mechanistic differences in this unique response remain unclear.

Based on our recent study demonstrating the priming influence of TGF-β1 on mast cell production of IL-6, including in response to LPS (19), we hypothesized that TGF-β1 might actually diminish neutrophilic inflammation during ALI by altering the IL-6 production by mast cells. Our data demonstrate that TGF-β1 dose dependently reduces the numbers of neutrophils observed after LPS administration and that this is associated with a significant increase in neutrophil apoptosis. Importantly, our data demonstrate that LPS-induced ALI is regulated by mast cell–derived IL-6 and that TGF-β1 functions indirectly via this mechanism to enhance neutrophilic apoptosis and limit the severity of the early neutrophilic inflammation. Consequently, mast cells and IL-6 represent a novel mechanism through which the magnitude of the developing inflammatory response is controlled in the lung and may be a pathway for therapeutic intervention in the treatment of ALI.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic Farms (Cambridge City, IN). IL-$6^{-/-}$, WBB6F_1Kit$^{W/W\text{-}v}$ (W/Wv) and their littermate controls, WBB6F_1 (WBB6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Kit$^{W\text{-}sh}$ (Sash) mice were bred and housed under specific pathogen–free conditions at Northwestern University. All experiments were approved by the Northwestern University Animal Care and Use Committee.

LPS-induced lung injury

Mice were lightly anesthetized with isoflurane (Abbott Laboratories). Intratracheal delivery of 10 μg *Escherichia coli* LPS (0127:B8 Sigma L4516), with or without rIL-6 or rTGF-β1 (R&D Systems), at a final volume of 100 μl in sterile 1× PBS, was performed. Sterile PBS was administered as a control.

Bronchoalveolar lavage fluid analysis

Bronchoalveolar lavage (BAL) was performed by lavage through the trachea with 1 ml BAL fluid (1× PBS, 10% FCS, 1 mM EDTA). Total cell counts were performed using a hemacytometer. Differential cell counts were assessed as previously described (29). These were assessed by cytologic preparation (Cytospin; Shandon Scientific) and stained with Diff-Quik stain (Siemens). Differential cell numbers were calculated based on the percentage of differential cells of total cells. BAL fluid was centrifuged at 10,000 × *g* for 5 min, and supernatants were stored at −80°C for analysis, whereas cells were stained for analysis by flow cytometry. BAL fluid was analyzed for IL-6 by sandwich ELISA (BD Biosciences), and KC, MIP-2α, and sIL-6R were analyzed by DuoSet Sandwich ELISA (R&D Systems), following the manufacturers' instructions.

Flow cytometry

BAL cells were counted and resuspended at 1×10^6 cells/ml, and 100 μl was stained with allophycocyanin-conjugated anti–Gr-1 (BD Biosciences). Samples were then washed and stained with FITC-labeled Annexin V (Invitrogen), following the manufacturer's recommended protocol. Briefly, Annexin V was added to cells in Annexin V binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated for 15 min at room temperature. Pacific Blue–SYTOX (Invitrogen) was added (1 μl/sample) 5 min prior to flow cytometric analysis. Samples were gated on Gr-1^+ cells and analyzed using FlowJo software.

Bone marrow–derived mast cell generation and reconstitution

Bone marrow was isolated from the femurs of female, age-matched C57BL/6 or IL-$6^{-/-}$ mice and cultured as described (19). After 4 wk, bone marrow–derived mast cells (BMMCs) were analyzed for surface expression of FcεRI and c-kit by flow cytometry. Cultures with >95% FcεRI$^+$ and c-kit$^+$ BMMCs were used to reconstitute 6-wk-old W/W^v mice by i.v. injection of 5×10^6 WT or IL-$6^{-/-}$ BMMCs. Eight weeks after reconstitution, mast cell reconstitution was confirmed as previously described (30), and hematocrit levels were measured to ensure that anemia was unaltered by reconstitution.

Results

TGF-β1 promotes neutrophil apoptosis during LPS-induced lung injury

To determine whether rTGF-β1 was sufficient to alter neutrophilia during the onset of ALI responses, C57BL/6 mice (WT) received 10 μg LPS via intratracheal injection in combination with rTGF-β1 (0.5, 5, or 50 ng). Twenty-four hours later, bronchoalveolar lavage (BAL) was performed, and samples were analyzed for cellular infiltration. Although rTGF-β1 alone did not elicit any cellular infiltration in the BAL (data not shown), rTGF-β1 dose dependently reduced the total numbers of cells (Fig. 1A). The cellular infiltration in all groups remained dominated by neutrophils (~85%) (Supplemental Fig. 1A), suggesting that TGF-β1 did not alter the cellular composition of the inflammatory response. However, there was a significant decrease in the total numbers of neutrophils in the BAL fluid, whereas macrophages, the only other cells seen, were unaltered (Fig. 1B). The reduction in BAL neutrophils by TGF-β1 was most likely not due to reduced neutrophil chemoattraction, because levels of KC and MIP-2α, two critical neutrophil chemoattractants in the lung (31, 32), were unaltered in the BAL fluid (Supplemental Fig. 1B, 1C). Because neutrophil apoptosis is a key step in the late resolution of ALI inflammation, we questioned whether it might also be occurring within this early initiation period. As represented in Fig. 1C, we observed a dose-dependent increase in late apoptotic/dead neutrophils (Gr-1^+/Annexin V$^+$/SYTOX$^+$) within the BAL fluid in response to rTGF-β1 24 h after treatment that was statistically different from untreated mice over multiple animals and experiments (Fig. 1D).

Reduction of lung neutrophilia by TGF-β1 is IL-6 dependent

Because IL-6 has been implicated in neutrophil apoptosis, and IL-$6^{-/-}$ mice have a significantly exacerbated inflammatory response in the LPS-induced ALI model (33), we postulated that TGF-β1 might alter ALI via an IL-6–dependent mechanism. To test this, WT and IL-$6^{-/-}$ mice received LPS (10 μg), with or without 5 ng rTGF-β1, and the inhibitory influence of rTGF-β1 was determined. As seen previously, the total numbers of inflammatory cells (Fig. 2A) and neutrophils (Fig. 2B) in WT mice were significantly reduced by rTGF-β1 treatment. In contrast, IL-$6^{-/-}$ mice, which exhibited a heightened responsiveness to LPS alone, were unaffected by rTGF-β1 treatment, suggesting that IL-6 is required for the protective effects of rTGF-β1.

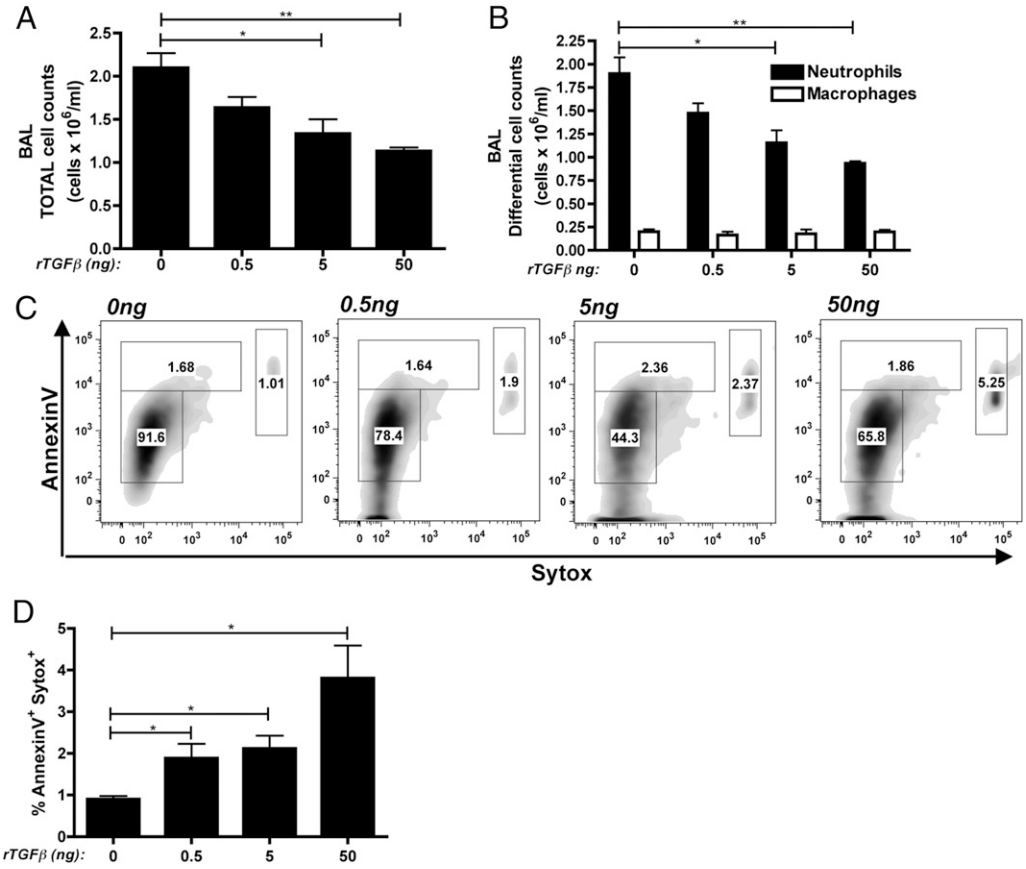

FIGURE 1. rTGF-β1 is sufficient to promote neutrophil apoptosis in LPS-induced lung injury. . WT mice received 0, 0.5, 5, or 50 ng rTGF-β1 with 10 μg LPS intratracheally. Total cell counts (**A**) and differential cell counts (**B**) were performed on BAL samples 24 h posttreatment. (**C**) BAL cells were gated on Gr-1$^+$ and analyzed for the percentage of neutrophil death by flow cytometry. (**D**) Representative flow plots with quantification of dead neutrophils. Data represent mean ± SEM (n = 4–5 mice/group from two independent experiments). *$p < 0.05$, **$p < 0.01$.

IL-6 is sufficient to promote neutrophil apoptosis

To determine whether IL-6 was sufficient to promote neutrophil apoptosis during LPS-induced lung injury, WT mice were administered intratracheal rIL-6, with or without concurrent LPS treatment. As demonstrated previously (34), the intratracheal administration of rIL-6 alone did not elicit any cellular infiltration. However, mice receiving rIL-6 plus LPS exhibited significantly reduced total and neutrophil cell counts compared with those receiving LPS alone (Fig. 3A, 3B). Furthermore, analysis of the BAL cells for neutrophil apoptosis demonstrated that rIL-6 led to a significant increase in late apoptotic neutrophils within the BAL (Fig. 3C, 3D).

IL-6 production precedes neutrophil infiltration and apoptosis in LPS-induced lung injury

IL-6 production was shown to occur during the resolution of acute inflammation and in the key transition from innate to adaptive immunity (26, 33), but it is unknown whether IL-6 is actively produced during the earlier initiation period of neutrophilic inflammation. Therefore, we examined levels of IL-6 in the lung at 2, 12, 18, and 24 h after challenge with 10 μg LPS. Interestingly, IL-6 levels in the BAL fluid peaked within 2 h and declined over time, reaching their lowest level at 24 h (Fig. 4A). In contrast, the total (Fig. 4B) and neutrophil (Fig. 4C) cell counts rose at 12 h post-challenge and increased with time, suggesting that the inflammatory cells enter into an already IL-6–rich environment in the lung.

We then examined neutrophil apoptosis in the BAL samples at 12, 18, and 24 h post-LPS challenge. We observed increases in Annexin V$^+$/SYTOX$^+$ neutrophils over time (Fig. 4D, 4E). Apoptotic neutrophils were shown to shed sIL-6R (35), and we also observed increased sIL-6R over time (Fig. 4F). Taken together, our data demonstrate that IL-6 production precedes neutrophil infiltration and that neutrophil apoptosis is an early event during ALI responses. Finally, to determine whether IL-6 levels were specifically altered by rTGF-β1 treatment, we examined IL-6 levels 2 h after administration of LPS, with or without rTGF-β1. As predicted, rTGF-β1 led to a significant increase in BAL IL-6 levels (Fig. 4G).

Neutrophil clearance is regulated by mast cell–derived IL-6

We next asked what role mast cells and, in particular, mast cell–derived IL-6 play in LPS-induced lung injury. Despite possessing an intrinsic neutropenia (36), mast cell–deficient W/Wv mice displayed significantly greater total and neutrophil cell counts 24 h after intratracheal LPS than did their littermate controls (WBB6) (Fig. 5A, 5B). Reconstituting W/Wv mice with WT BMMCs (W/Wv+WT) reduced the exaggerated inflammatory response back to control levels. Although we observed a modest increase in MIP-2α levels in W/Wv mice (Supplemental Fig. 2A), there was no difference in KC levels (Supplemental Fig. 2B), suggesting that the expression of neutrophil chemoattractants was not significantly affected. However, W/Wv mice exhibited a significantly lower percentage of dead neutrophils compared with controls (Supplemental Fig. 2C, 2D), suggesting that mast cells regulate neutrophil cell death processes. To specifically assess the role of mast cell–derived IL-6, W/Wv mice were reconstituted with mast

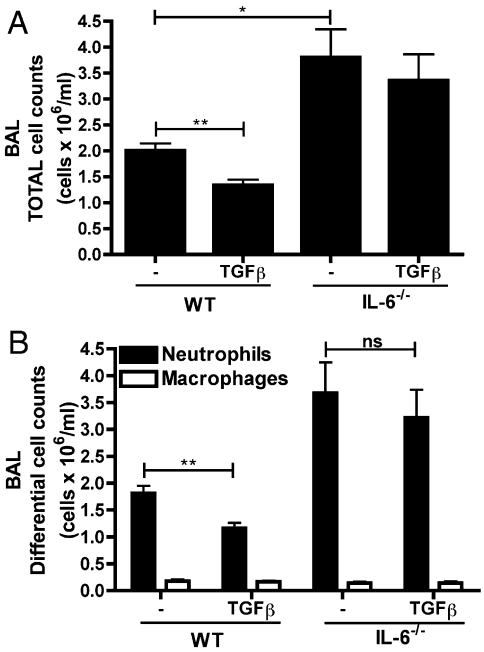

FIGURE 2. TGF-β1 functions through an IL-6–dependent mechanism to promote neutrophil clearance. WT or IL-$6^{-/-}$ mice were challenged with 10 μg LPS alone or LPS with 5 ng rTGF-β1. Twenty-four hours postchallenge, total (**A**) and differential (**B**) cell counts were performed on BAL samples. Data represent mean ± SEM (n = 3–7 mice/group from two independent experiments). *$p < 0.05$, **$p < 0.01$. NS, Not significant.

cells derived from IL-$6^{-/-}$ mice (W/Wv+IL-$6^{-/-}$). W/Wv+IL-$6^{-/-}$ mice exhibited significantly increased numbers of total cells (Fig. 5A) and neutrophils (Fig. 5B) compared with those seen after reconstitution with WT mast cells, and no statistically significant differences were seen compared with nonreconstituted W/Wv, although there was a trend toward fewer cells.

TGF-β1 functions through a mast cell–dependent mechanism to promote neutrophil clearance

Because these data suggested a role for mast cells in limiting the early inflammatory response to LPS, we asked whether TGF-β1 might exert its effects via mast cells. For these experiments we chose to use Sash mice, another mast cell–deficient strain that lacks the neutropenia seen in W/Wv mice (37). Sash or WT controls were challenged intratracheally with 10 μg LPS, with or without rTGF-β1. rIL-6 was also used, because, according to our hypothesis, this would act downstream of the role for mast cells. Similar to the W/Wv strain, Sash mice exhibited significantly increased numbers of total cells (Fig. 6A) and neutrophils (Fig. 6B) in response to LPS challenge alone, although the magnitude of the increase was even greater than that seen with W/Wv mice. The administration of rTGF-β1 led to a significant reduction in the numbers of total cells and neutrophils in the BAL of WT mice, but it had no effect in Sash mice. Conversely, rIL-6 reduced the inflammatory response in both WT and Sash mice. These data demonstrate that the protective effects of TGF-β1 on lung neutrophilia during ALI function via a mast cell–dependent mechanism.

Tregs limit neutrophilic inflammation during the early stages of ALI

Because our previous work demonstrated that Tregs are capable of providing TGF-β to mast cells and enhancing their ability to generate IL-6 (19), and mast cells and Tregs were shown to colocalize within tissues (38), we postulated that these cells might also participate in limiting the number of neutrophils. Although D'Alessio et al. (14) demonstrated that Tregs are necessary for the resolution of ALI at 72 h post-LPS challenge, we examined the effects of depleting Tregs prior to the initiation of ALI. Treatment with an anti-CD25 Ab (PC61) led to a significant increase in the numbers of total cells and neutrophils within the BAL at 24 h post-LPS challenge (Fig. 7), suggesting that Tregs are also likely to be important in limiting the early neutrophilic inflammatory response during the initiation of ALI responses.

Discussion

TGF-β1 has long been recognized for its pivotal role in promoting the fibrotic remodeling of the lung during inflammation (39), but its role during the initiation of inflammation was unclear. In this study, we found that TGF-β1 enhances neutrophil apoptosis in a model of ALI. We demonstrate that this is mediated via an indirect pathway that is dependent on IL-6 and mast cells. Indeed,

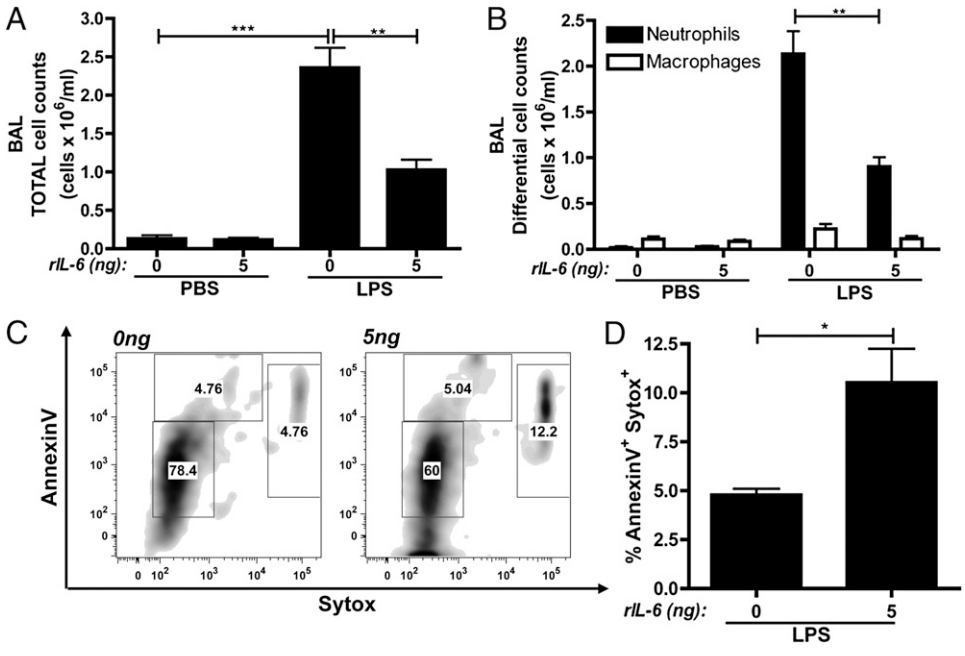

FIGURE 3. rIL-6 is sufficient to promote neutrophil apoptosis in LPS-induced lung injury. WT mice were challenged with 5 ng rIL-6 in combination with PBS or 10 μg LPS via intratracheal administration. Total (**A**) and differential (**B**) cell counts were performed on BAL samples 24 h postchallenge. Representative flow plots of BAL cells (gating on Gr-1^+ cells) (**C**) with quantification of dead neutrophils (**D**). Data represent mean ± SEM (n = 5 mice/group from two independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

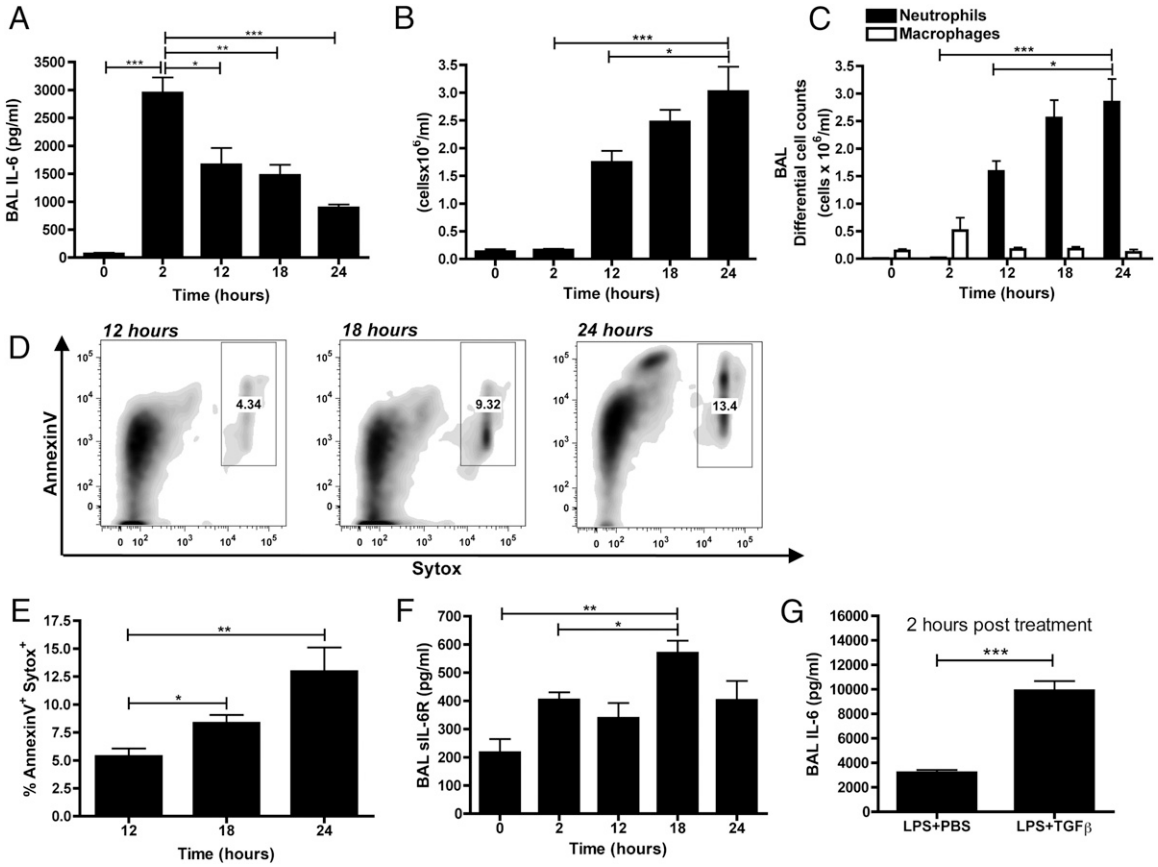

FIGURE 4. IL-6 production peaks at 2 h following LPS challenge. WT mice were challenged via intratracheal administration of 10 μg LPS, and BAL samples were collected at 0, 2, 12, 18, and 24 h. Total (**A**) and differential (**B**) cell counts were performed on BAL samples. (**C**) BAL supernatants were analyzed for IL-6 production by ELISA. Representative flow plots of BAL cells (gating on Gr-1$^+$ cells) (**D**) with quantification of dead neutrophils (**E**). (**F**) sIL-6R levels were measured in BAL supernatants by ELISA. (**G**) BAL supernatants were analyzed for IL-6 production by ELISA, with or without 5 ng rTGF-β1. Data represent mean ± SEM (n = 6 mice/group from two independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the exaggerated neutrophilic inflammation in two different strains of mast cell–deficient mice and in IL-6$^{-/-}$ mice suggests that this mechanism is required for homeostatic regulation over neutrophil numbers in the lung.

The overall concept that IL-6 might serve as a protective factor in inflammation seemed initially contradictory to the well-established proinflammatory effects of IL-6 and the therapeutic benefit of anti–IL-6R Abs in diseases such as rheumatoid arthritis and systemic lupus erythematosus (40). However, IL-6 is capable of exerting several inhibitory influences over innate immune responses, including suppression of CXCL1 and CXCL8 release, induction of IL-1 and TNF antagonists, and neutrophil apoptosis (28, 41). Interestingly, the −174 G/C polymorphism in the IL-6 promoter, which correlates with enhanced IL-6 levels (42), was shown to associate with enhanced disease severity in both rheumatoid arthritis (43–46) and systemic lupus erythematosus (47–49). In sharp contrast, it associates with reduced ARDS, septic shock, and multiple organ dysfunction syndrome in patients with pneumococcal community-acquired pneumonia (50), illustrating that increased IL-6 may be clinically protective in the context of neutrophilic diseases.

We demonstrated previously that Tregs were capable of enhancing mast cell production of IL-6 upon IgE-mediated activation and that this was mediated via TGF-β1 (19). Interestingly, TGF-β1 primed the mast cell for IL-6 production upon stimulation, rather than drove IL-6 production directly. In Supplemental Fig. 3, we now show that this is also evident with LPS-driven activation in vitro. In the context of mast cell activation, LPS-mediated activation was shown to increase IL-6 production independent of degranulation and preformed mediator release (21). This separation of cytokine production from degranulation may be a critical aspect that facilitates seeing this as a regulatory response by the mast cell versus the established inflammatory potential of mast cell activation. In support of this, histamine, which is released upon degranulation, was shown to reduce the suppressive ability of Tregs (51). Consequently, it is intriguing to postulate that the precise role of mast cells and Tregs in regulating inflammation may be highly context specific and influenced by the nature of the stimuli. Supporting this notion, neutrophil recruitment to the lung in response to adenosine, which leads to mast cell degranulation, was significantly lower in W/Wv mice (52), contrasting with our findings using LPS stimulation.

Our data showing that Treg depletion leads to elevated neutrophils 24 h after LPS administration suggests that resident Tregs are regulating the early initiation of inflammation. In allergic airway inflammation, Treg depletion prior to sensitization was shown to lead to exaggerated responsiveness to airway sensitization (53), whereas, more recently, tissue-resident Tregs were shown to be critically important in oral tolerance (54). Consequently, it is becoming increasingly evident that tissue-resident Tregs contribute to controlling a variety of local inflammatory responses. In contrast, D'Alessio et al. (14) demonstrated a requirement for newly recruited

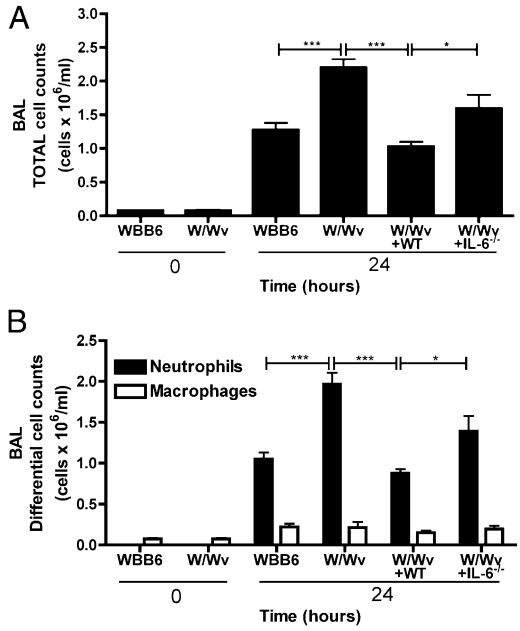

FIGURE 5. Mast cell–derived IL-6 promotes neutrophil clearance in LPS-induced lung injury. Controls (WBB6), mast cell–deficient (W/Wv) mice, and W/Wv mice reconstituted with WT mast cells (W/Wv+WT) or IL-6$^{-/-}$ mast cells (W/Wv+IL-6$^{-/-}$) were challenged intratracheally with 10 μg LPS. At 24 h posttreatment, total (**A**) and differential (**B**) cell counts were performed on BAL samples. Data represent mean ± SEM (n = 5–7 mice/group from three independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

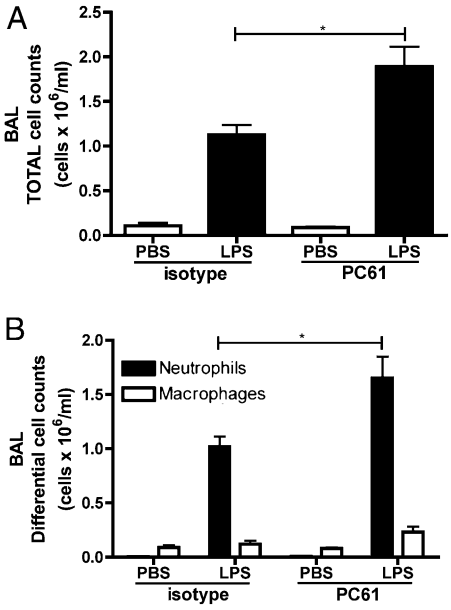

FIGURE 7. Tregs are required for early neutrophil clearance in ALI. WT mice were administered 1 mg anti-CD25 mAb (PC61) or isotype control. Mice were then treated with PBS or 10 μg LPS intratracheally. Total (**A**) and differential (**B**) cell counts were performed on BAL samples 24 h posttreatment. Data represent mean ± SEM (n = 4–6 mice/group from three independent experiments). *$p < 0.05$.

Tregs in the resolution of LPS-induced ALI. This also was mediated via TGF-β1, because a blocking anti–TGF-β1 Ab impaired resolution. It remains to be determined whether the resolution of ALI responses is also dependent on mast cells and IL-6. However, we saw no changes in the Treg numbers within the lung during the initiation time period that we focused on (data not shown), suggesting that tissue-resident cells were the likely population regulating responses at 24 h.

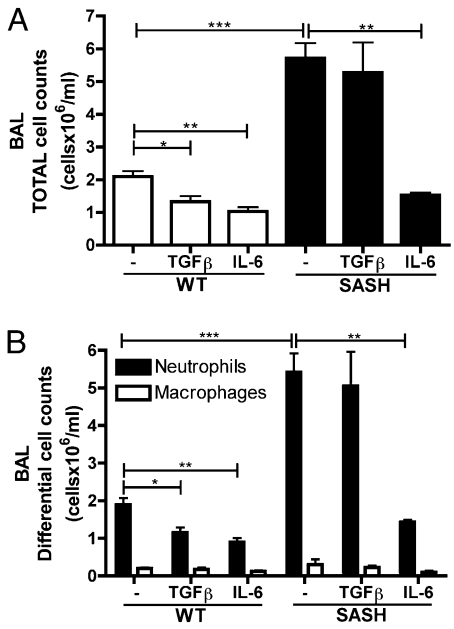

FIGURE 6. TGF-β promotes neutrophil clearance through a mast cell–dependent mechanism. Mast cell–deficient Kit$^{w\text{-}sh}$ (Sash) mice were challenged with 10 μg LPS alone, LPS with 5 ng rIL-6, or LPS with 5 ng rTGF-β1. At 24 h after challenge, total (**A**) and differential (**B**) cell counts were performed on BAL samples. Data represent mean ± SEM (n = 3–7 mice/group from three independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

In considering the biological relevance of the pathway that we defined in this study, there seem to be two potential benefits to regulating neutrophil apoptosis during the initiation of inflammation. In the first, Tregs may be exerting a controlling influence over the strength of the inflammatory response, such that, in the example of a modest infectious stimulus, they prevent overexuberant responses that may lead to tissue damage. In support of this concept, high doses of LPS exhibited higher neutrophilia and overcame the limiting influence of TGF-β1 (data not shown); this may be one explanation for why D'Alessio et al. (14) failed to observe a difference in neutrophilia at 24 h: they used a relatively high LPS dose compared with the one used in our study. Alternatively, the induction of neutrophil apoptosis may be necessary for the dissemination of sIL-6R and, subsequently, the ability of other cells to respond to IL-6 via gp130. Indeed, as we show in Fig. 4, the shedding of sIL-6R seems to coincide with the arrival of neutrophils into the lung. Abrogation of IL-6–mediated gp130 responses was shown to prevent the generation of adaptive immune responses to *Toxoplasma gondii* infection (55), suggesting that signals via gp130 are important for protective memory responses.

In conclusion, our findings describe a previously unappreciated process through which the magnitude of innate inflammation is regulated in the lung. Surprisingly, this occurs via two cytokines, TGF-β1 and IL-6, which have been characterized extensively for their deleterious effects in chronic inflammation. However, as we propose, the priming influence of TGF-β1 on tissue-resident mast cells serves to enhance IL-6 production upon LPS exposure, which drives the infiltrating neutrophil into apoptotic clearance.

Acknowledgments

We thank Miller Scientific Communications for editorial assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Rubenfeld, G. D., E. Caldwell, E. Peabody, J. Weaver, D. P. Martin, M. Neff, E. J. Stern, and L. D. Hudson. 2005. Incidence and outcomes of acute lung injury. *N. Engl. J. Med.* 353: 1685–1693.
2. Wheeler, A. P., and G. R. Bernard. 2007. Acute lung injury and the acute respiratory distress syndrome: a clinical review. *Lancet* 369: 1553–1564.
3. Grommes, J., and O. Soehnlein. 2011. Contribution of neutrophils to acute lung injury. *Mol. Med.* 17: 293–307.
4. Ware, L. B., and M. A. Matthay. 2000. The acute respiratory distress syndrome. *N. Engl. J. Med.* 342: 1334–1349.
5. McGrath, E. E., H. M. Marriott, A. Lawrie, S. E. Francis, I. Sabroe, S. A. Renshaw, D. H. Dockrell, and M. K. Whyte. 2011. TNF-related apoptosis-inducing ligand (TRAIL) regulates inflammatory neutrophil apoptosis and enhances resolution of inflammation. *J. Leukoc. Biol.* 90: 855–865.
6. Rowe, S. J., L. Allen, V. C. Ridger, P. G. Hellewell, and M. K. Whyte. 2002. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J. Immunol.* 169: 6401–6407.
7. Rossi, A. G., D. A. Sawatzky, A. Walker, C. Ward, T. A. Sheldrake, N. A. Riley, A. Caldicott, M. Martinez-Losa, T. R. Walker, R. Duffin, et al. 2006. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. [Published erratum appears in 2006 *Nat. Med.* 12: 1434.] *Nat. Med.* 12: 1056–1064.
8. Fialkow, L., L. Fochesatto Filho, M. C. Bozzetti, A. R. Milani, E. M. Rodrigues Filho, R. M. Ladniuk, P. Pierozan, R. M. de Moura, J. C. Prolla, E. Vachon, and G. P. Downey. 2006. Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome. *Crit. Care* 10: R155.
9. Steinberg, K. P., J. A. Milberg, T. R. Martin, R. J. Maunder, B. A. Cockrill, and L. D. Hudson. 1994. Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 150: 113–122.
10. Halwani, R., S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid. 2011. Role of transforming growth factor-β in airway remodeling in asthma. *Am. J. Respir. Cell Mol. Biol.* 44: 127–133.
11. Morty, R. E., M. Königshoff, and O. Eickelberg. 2009. Transforming growth factor-beta signaling across ages: from distorted lung development to chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* 6: 607–613.
12. Kim, K. K., Y. Wei, C. Szekeres, M. C. Kugler, P. J. Wolters, M. L. Hill, J. A. Frank, A. N. Brumwell, S. E. Wheeler, J. A. Kreidberg, and H. A. Chapman. 2009. Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J. Clin. Invest.* 119: 213–224.
13. Ulich, T. R., S. Yin, K. Guo, E. S. Yi, D. Remick, and J. del Castillo. 1991. Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am. J. Pathol.* 138: 1097–1101.
14. D'Alessio, F. R., K. Tsushima, N. R. Aggarwal, E. E. West, M. H. Willett, M. F. Britos, M. R. Pipeling, R. G. Brower, R. M. Tuder, J. F. McDyer, and L. S. King. 2009. CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *J. Clin. Invest.* 119: 2898–2913.
15. Balazovich, K. J., R. Fernandez, V. Hinkovska-Galcheva, S. J. Suchard, and L. A. Boxer. 1996. Transforming growth factor-beta1 stimulates degranulation and oxidant release by adherent human neutrophils. *J. Leukoc. Biol.* 60: 772–777.
16. Shen, L., J. M. Smith, Z. Shen, M. Eriksson, C. Sentman, and C. R. Wira. 2007. Inhibition of human neutrophil degranulation by transforming growth factor-beta1. *Clin. Exp. Immunol.* 149: 155–161.
17. Ghio, M., L. Ottonello, P. Contini, M. Amelotti, C. Mazzei, F. Indiveri, F. Puppo, and F. Dallegri. 2003. Transforming growth factor-beta1 in supernatants from stored red blood cells inhibits neutrophil locomotion. *Blood* 102: 1100–1107.
18. Ear, T., C. F. Fortin, F. A. Simard, and P. P. McDonald. 2010. Constitutive association of TGF-beta-activated kinase 1 with the IkappaB kinase complex in the nucleus and cytoplasm of human neutrophils and its impact on downstream processes. *J. Immunol.* 184: 3897–3906.
19. Ganeshan, K., and P. J. Bryce. 2012. Regulatory T cells enhance mast cell production of IL-6 via surface-bound TGF-β. *J. Immunol.* 188: 594–603.
20. Palker, T. J., G. Dong, and W. W. Leitner. 2010. Mast cells in innate and adaptive immunity to infection. *Eur. J. Immunol.* 40: 13–18.
21. Leal-Berumen, I., P. Conlon, and J. S. Marshall. 1994. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *J. Immunol.* 152: 5468–5476.
22. Sutherland, R. E., J. S. Olsen, A. McKinstry, S. A. Villalta, and P. J. Wolters. 2008. Mast cell IL-6 improves survival from *Klebsiella* pneumonia and sepsis by enhancing neutrophil killing. *J. Immunol.* 181: 5598–5605.
23. Oldford, S. A., I. D. Haidl, M. A. Howatt, C. A. Leiva, B. Johnston, and J. S. Marshall. 2010. A critical role for mast cells and mast cell-derived IL-6 in TLR2-mediated inhibition of tumor growth. *J. Immunol.* 185: 7067–7076.
24. Nishimoto, N., and T. Kishimoto. 2004. Inhibition of IL-6 for the treatment of inflammatory diseases. *Curr. Opin. Pharmacol.* 4: 386–391.
25. Rose-John, S., J. Scheller, G. Elson, and S. A. Jones. 2006. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J. Leukoc. Biol.* 80: 227–236.
26. Hurst, S. M., T. S. Wilkinson, R. M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, S. Rose-John, G. M. Fuller, N. Topley, and S. A. Jones. 2001. Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity* 14: 705–714.
27. Fielding, C. A., R. M. McLoughlin, L. McLeod, C. S. Colmont, M. Najdovska, D. Grail, M. Ernst, S. A. Jones, N. Topley, and B. J. Jenkins. 2008. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. *J. Immunol.* 181: 2189–2195.
28. McLoughlin, R. M., J. Witowski, R. L. Robson, T. S. Wilkinson, S. M. Hurst, A. S. Williams, J. D. Williams, S. Rose-John, S. A. Jones, and N. Topley. 2003. Interplay between IFN-gamma and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J. Clin. Invest.* 112: 598–607.
29. Bryce, P. J., R. Geha, and H. C. Oettgen. 2003. Desloratadine inhibits allergen-induced airway inflammation and bronchial hyperresponsiveness and alters T-cell responses in murine models of asthma. *J. Allergy Clin. Immunol.* 112: 149–158.
30. Wolters, P. J., J. Mallen-St Clair, C. C. Lewis, S. A. Villalta, P. Baluk, D. J. Erle, and G. H. Caughey. 2005. Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clin. Exp. Allergy* 35: 82–88.
31. Jeyaseelan, S., R. Manzer, S. K. Young, M. Yamamoto, S. Akira, R. J. Mason, and G. S. Worthen. 2005. Induction of CXCL5 during inflammation in the rodent lung involves activation of alveolar epithelium. *Am. J. Respir. Cell Mol. Biol.* 32: 531–539.
32. Jeyaseelan, S., H. W. Chu, S. K. Young, M. W. Freeman, and G. S. Worthen. 2005. Distinct roles of pattern recognition receptors CD14 and Toll-like receptor 4 in acute lung injury. *Infect. Immun.* 73: 1754–1763.
33. Xing, Z., J. Gauldie, G. Cox, H. Baumann, M. Jordana, X. F. Lei, and M. K. Achong. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.* 101: 311–320.
34. Shanley, T. P., J. L. Foreback, D. G. Remick, T. R. Ulich, S. L. Kunkel, and P. A. Ward. 1997. Regulatory effects of interleukin-6 in immunoglobulin G immune-complex-induced lung injury. *Am. J. Pathol.* 151: 193–203.
35. Chalaris, A., B. Rabe, K. Paliga, H. Lange, T. Laskay, C. A. Fielding, S. A. Jones, S. Rose-John, and J. Scheller. 2007. Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils. *Blood* 110: 1748–1755.
36. Chervenick, P. A., and D. R. Boggs. 1969. Decreased neutrophils and megakaryocytes in anemic mice of genotype W/W. *J. Cell. Physiol.* 73: 25–30.
37. Zhou, J. S., W. Xing, D. S. Friend, K. F. Austen, and H. R. Katz. 2007. Mast cell deficiency in Kit(W-sh) mice does not impair antibody-mediated arthritis. *J. Exp. Med.* 204: 2797–2802.
38. Gri, G., S. Piconese, B. Frossi, V. Manfroi, S. Merluzzi, C. Tripodo, A. Viola, S. Odom, J. Rivera, M. P. Colombo, and C. E. Pucillo. 2008. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* 29: 771–781.
39. Wynn, T. A. 2011. Integrating mechanisms of pulmonary fibrosis. *J. Exp. Med.* 208: 1339–1350.
40. Mima, T., and N. Nishimoto. 2009. Clinical value of blocking IL-6 receptor. *Curr. Opin. Rheumatol.* 21: 224–230.
41. Jones, S. A. 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. *J. Immunol.* 175: 3463–3468.
42. Terry, C. F., V. Loukaci, and F. R. Green. 2000. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *J. Biol. Chem.* 275: 18138–18144.
43. Lee, Y. H., S. C. Bae, S. J. Choi, J. D. Ji, and G. G. Song. 2012. The association between interleukin-6 polymorphisms and rheumatoid arthritis: a meta-analysis. *Inflamm. Res.* 61: 665–671.
44. Panoulas, V. F., A. Stavropoulos-Kalinoglou, G. S. Metsios, J. P. Smith, H. J. Milionis, K. M. Douglas, P. Nightingale, and G. D. Kitas. 2009. Association of interleukin-6 (IL-6)-174G/C gene polymorphism with cardiovascular disease in patients with rheumatoid arthritis: the role of obesity and smoking. *Atherosclerosis* 204: 178–183.
45. Pawlik, A., J. Wrzesniewska, M. Florczak, B. Gawronska-Szklarz, and M. Herczynska. 2005. IL-6 promoter polymorphism in patients with rheumatoid arthritis. *Scand. J. Rheumatol.* 34: 109–113.
46. Pascual, M., A. Nieto, L. Matarán, A. Balsa, D. Pascual-Salcedo, and J. Martín. 2000. IL-6 promoter polymorphisms in rheumatoid arthritis. *Genes Immun.* 1: 338–340.
47. Lee, Y. H., H. S. Lee, S. J. Choi, J. D. Ji, and G. G. Song. 2012. The association between interleukin-6 polymorphisms and systemic lupus erythematosus: a meta-analysis. *Lupus* 21: 60–67.
48. Santos, M. J., D. Fernandes, S. Capela, J. C. da Silva, and J. E. Fonseca. 2011. Interleukin-6 promoter polymorphism -174 G/C is associated with nephritis in Portuguese Caucasian systemic lupus erythematosus patients. *Clin. Rheumatol.* 30: 409–413.
49. Schotte, H., B. Schlüter, S. Rust, G. Assmann, W. Domschke, and M. Gaubitz. 2001. Interleukin-6 promoter polymorphism (-174 G/C) in Caucasian German patients with systemic lupus erythematosus. *Rheumatology (Oxford)* 40: 393–400.

50. Martín-Loeches, I., J. Solé-Violán, F. Rodríguez de Castro, M. I. García-Laorden, L. Borderías, J. Blanquer, O. Rajas, M. L. Briones, J. Aspa, E. Herrera-Ramos, et al. 2012. Variants at the promoter of the interleukin-6 gene are associated with severity and outcome of pneumococcal community-acquired pneumonia. *Intensive Care Med.* 38: 256–262.
51. Forward, N. A., S. J. Furlong, Y. Yang, T. J. Lin, and D. W. Hoskin. 2009. Mast cells down-regulate CD4+CD25+ T regulatory cell suppressor function via histamine H1 receptor interaction. *J. Immunol.* 183: 3014–3022.
52. Tilley, S. L., M. Tsai, C. M. Williams, Z. S. Wang, C. J. Erikson, S. J. Galli, and B. H. Koller. 2003. Identification of A3 receptor- and mast cell-dependent and -independent components of adenosine-mediated airway responsiveness in mice. *J. Immunol.* 171: 331–337.
53. Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Köhl, Y. Belkaid, and M. Wills-Karp. 2005. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202: 1549–1561.
54. Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Müller, T. Sparwasser, R. Förster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34: 237–246.
55. Silver, J. S., J. S. Stumhofer, S. Passos, M. Ernst, and C. A. Hunter. 2011. IL-6 mediates the susceptibility of glycoprotein 130 hypermorphs to *Toxoplasma gondii*. *J. Immunol.* 187: 350–360.