Staphylococcus aureus (SA) causes more infections worldwide than any other bacterial pathogen (DeLeo et al., 2010). 20–50% of healthy adult populations around the world are colonized with SA, including >89 million Americans (30% of the population). Of these, 2.3 million people are colonized with methicillin-resistant strains (Kuehnert et al., 2006; Defres et al., 2009). This opportunistic gram-positive bacterium can colonize epithelia such as the mucocutaneous nasopharynx, but clinical infection can occur upon a break in epidermal or mucosal integrity and/or a decline in immune defenses (Graham et al., 2006). In an immunocompromised host, severe life-threatening infections such as meningitis, endocarditis, and pneumonia can occur, causing significant morbidity and mortality (Klevens et al., 2007). Methicillin-resistant SA (MRSA) is no longer limited to hospitals and is found in 1–3% of the general population (Klevens et al., 2007).

The lung epithelium provides the first line of immune defense against pulmonary pathogens because of its structural barrier and mucociliary clearance mechanisms. In addition, the lung epithelium secretes various chemokines, cytokines, and antimicrobial proteins in response to bacterial pathogens (Bals and Hiemstra, 2004). Although adaptive immunity is important to clear MRSA in the skin of mice (Cho et al., 2010), B, T, and NK cells do not seem to be important in the lung because the bacteria are largely cleared by 48 h after infection and Rag2⁻/⁻ Il2r−/− mice are able to clear infection efficiently (Small et al., 2008; von Köckritz-Blickwede et al., 2008). Nevertheless, patients with inherited (autosomal dominant) hyper-IgE syndrome (HIES) are susceptible to recurrent SA pulmonary infections of the skin and lung as the result of loss-of-function mutations in STAT3.
Innate Stat3-induced Reg3γ in MRSA pneumonia | Choi et al.

Similar to prior published findings (Small et al., 2008; von Köckritz-Blickwede et al., 2008), we found that clearance of MRSA (USA300) in a mouse model of SA pneumonia was independent of B, T, and NK cells. However, clearance was defective in mice in which Stat3 was inhibited or deleted in lung epithelial cells. These data demonstrate that nonhematopoietic Stat3 is critical for host defense in MRSA lung infection. We found that Stat3 regulated the expression of Reg3γ (regenerating islet-derived 3γ), which is highly expressed in the lung epithelium during MRSA infection. Moreover, Reg3γ induction was independent of B, T, and NK cells but dependent on Gp130-induced Stat3 signaling. We detected both full-length and cleaved forms of Reg3γ in the bronchoalveolar lavage fluid (BALF) after MRSA pneumonia, and recombinant full-length Reg3γ showed dose-dependent bacteriostatic activity against USA300, whereas the cleaved form showed bactericidal activity. Neutralization of Reg3γ in vivo perturbed clearance of MRSA in the lung. Furthermore, in vivo administration of recombinant Reg3γ restored mucosal immunity against MRSA. Collectively, we have shown that MRSA initiates induction of antimicrobial Reg3γ by pulmonary epithelial cells in a Stat3-dependent manner and epithelial Stat3 is critical for clearance of MRSA in the lung. Recombinant Reg3γ may be a novel way to augment mucosal immunity to this infection.

**RESULTS**

Gp130 ligands IL-6 and leukemia inhibitory factor (Lif) are induced during MRSA pneumonia

We developed a model of MRSA pneumonia and conducted time course experiments to assess bacterial clearance and potential...
activators of the Stat3 signaling pathway. Oropharyngeal aspiration (OA) of C57BL/6 (WTB6) mice with $2 \times 10^7$ CFU of USA300 showed time-dependent clearance of the organism with a clearance of $99\%$ of the bacteria by 24 h after infection (Fig. 1 A). To determine the host immune defenses against MRSA, lung mRNA and protein were obtained and analyzed for levels of cytokines and chemokines. Transcripts for the Gp130 ligands Il6, Lif, and Osm were increased in the lung and were highest at 4 h after infection (Fig. 1, B–D). IL-6 and Lif protein levels were also increased in lung homogenates 4 h after infection (Fig. 1, H and I). This induction was not true for all Gp130 ligands, as transcripts for Cntf and Cmtr were not increased by MRSA pneumonia (Fig. 1, F and G).

### T, B, and NK cells are dispensable for pulmonary MRSA clearance

To localize the cells of Stat3 activation and determine which cells are important for MRSA clearance, USA300 infection was performed in various immunological KO mice. In agreement with previous studies (Small et al., 2008; von Köckritz-Blickwede et al., 2008), pulmonary MRSA clearance was not dependent on T, B, and NK cells, as Rag2$^{−/−}$/Il2r$^{−/−}$ mice cleared USA300 similarly to WTB6 mice at 20 h after infection (Fig. 2 A). As expected, the induction of Il17a and Il22 was absent in Rag2$^{−/−}$/Il2r$^{−/−}$ mice (not depicted); however, the induction of Il6, the primary Gp130 ligand, was unaffected in Rag2$^{−/−}$/Il2r$^{−/−}$ mice (Fig. 2 B). Moreover, clearance of USA300 was unaffected in Il17a$^{−/−}$ and Il22$^{−/−}$ mice (Fig. 2 C). In support of a role of innate immunity in the pulmonary clearance of USA300, macrophages or neutrophils were depleted in WTB6 mice by clodronate or 1A8 antibody, respectively. OA administration of clodronate depleted $\geq 90\%$ of CD11c$^{+}$ cells (not depicted) and resulted in a 2-log increase in lung CFU compared with mice receiving vehicle control (Fig. 2 D). Using FACS and intracellular staining for IL-6, the bulk of IL-6 at 4 h after USA300 infection was in F480$^{+}$ macrophages (Fig. 2 E). Consistent with this, the induction of IL-6 was dependent on macrophages as clodronate administration decreased the BALF levels of IL-6 at 4 h after infection (Fig. 2 F). Depletion of neutrophils also exacerbated MRSA clearance by one-half log but was not as significant as macrophage depletion (Fig. 2 D).

### MRSA clearance is dependent on Gp130 and Stat3 signaling

Autosomal-dominant HIES is caused by mutations in STAT3 that prevent the activation of STAT3-inducible genes, leading to recurrent staphylococcal skin and pulmonary infections. Inhibition of Gp130 or Stat3 activation during MRSA pneumonia increased bacterial burden (Fig. 3). Local administration of an anti–mouse Gp130 neutralizing antibody immediately before MRSA infection significantly increased CFU at 18 h after infection (Fig. 3 A). To confirm the importance of Stat3 signaling in this model, we administered specific Stat3 inhibitors, JSI-124 or S3I-201, 1 h before MRSA infection. Both inhibitors increased MRSA lung burden 16 h after infection (Fig. 3, C and D). Moreover, both systemic (i.p.) and local (OA) routes of administration of S3I-201 resulted in increased bacterial burden at 16 h and 24 h, respectively (Fig. 3, B and C). Finally, JSI-124 inhibition of Stat3 signaling in Rag2$^{−/−}$/Il2r$^{−/−}$ mice also increased pulmonary MRSA burden at 16 h after infection (Fig. 3 E), demonstrating the critical role of Stat3 in WT mice as well as in mice that lack T, B, and NK cells.

### Stat3 activation in airway epithelium is necessary for MRSA clearance

We detected basal P-Stat3 in naïve mouse whole lung; however, there was a significant increase with MRSA infection (Fig. 4 A). To examine where Stat3 is activated during MRSA infection, we performed immunohistochemistry for P-Stat3 and noted that the lung epithelium was a major site of Stat3 activation upon MRSA infection (Fig. 4 B). Moreover, OA administration of the Stat3 inhibitor S3I-201 significantly...
reduced P-Stat3 staining with MRSA infection (Fig. 4, B and C). Because the lung epithelium was a major site of Stat3 activation, we next investigated whether epithelial Stat3 signaling was necessary for MRSA clearance. Adenoviral Cre (Ad-cre) or adenoviral luciferase (Ad-luc) was administered into the lung of Stat3fl/fl mice 6 d before MRSA challenge to deplete pulmonary epithelium of Stat3. Ad-cre targeting of lung epithelial cells significantly increased MRSA lung burden at 16 h after infection compared with control Ad-luc–treated control mice (Fig. 4 D). It is possible that epithelial Stat3 regulates antimicrobial factors in the lung that control clearance of USA300. To explore this possibility, we analyzed gene expression of known antimicrobial genes in a microarray dataset from Stat3fl/fl × SPC-cre mice (GEO accession no. GSE6846). Although calprotectin (S100A8/A9) is induced in the lung during MRSA infection (Fig. 5, A and B), analysis of microarray showed an increase of S100A8/A9 in Stat3–deficient mice, a phenotype which we also observed in USA300–infected mice administered a Stat3 inhibitor (Fig. 5, C and D). These data suggest that the increased expression of S100A8/A9 is not sufficient to compensate for Stat3 inhibition in clearance of USA300 in the lung. Moreover, USA300 failed to induce the expression of mouse β defensins 2 or 3 (not depicted), suggesting that these genes play a limited role in this model.

Reg3γ is induced in the lung during MRSA and limits MRSA growth in vitro

Xu et al. (2007) has reported that deletion of Stat3 in pulmonary epithelial cells changes the expression of several growth factors and cytokines. Among these, Reg3γ was highly repressed after Stat3 deletion in surfactant protein C–positive cells (Matsuzaki et al., 2006; Xu et al., 2007). Consistent with Stat3 activation in the epithelium during pulmonary SA infection (Fig. 4), we found that Reg3γ was expressed at a basal level followed by induction in whole lung tissue as early as 16 h after MRSA infection (Fig. 6 A). Reg3γ protein was induced in the lung as early as 4 h after USA300 infection (Fig. 6 B). Secreted Reg3γ was also detectable by Western blot on BALF taken at 20 h after infection (Fig. 6 C). Of note, protein levels of both IL-6 and Reg3γ were increased in naive Rag2/Il2rg−/− mice compared with WTB6 mice (Fig. 6, D and E), demonstrating that pulmonary Reg3γ expression is not dependent on cytokine products of T, B, or NK cells and may explain normal clearance of USA300 in this mouse strain.

To determine whether Reg3γ has antibacterial activity against USA300, a recombinant fusion protein consisting of mouse Reg3γ and mouse IgG2a (Reg3γ–Fc; Zheng et al., 2008) was expressed in 293T cells and purified over protein G column. Reg3γ–Fc was capable of binding to live MRSA organisms (Fig. 6 F) and inhibited MRSA growth in nutrient-rich tryptic soy broth (TSB) media in a dose-dependent manner (Fig. 6 G). This protein had minimal binding to Streptococcus pneumoniae and minimal detectable binding to gram-negative bacteria including Klebsiella pneumoniae, Haemophilus influenzae, or Moraxella catarrhalis (Fig. 7 A). Reg3γ antibody neutralized the inhibition of MRSA growth by Reg3γ–Fc (Fig. 7 B). As we detected both full-length and cleaved forms of Reg3γ during MRSA infection and N-terminal cleaved Reg3γ has bactericidal activity against Listeria monocytogenes (Mukherjee et al., 2009),
IL-6–stimulated P-Stat3 directly activates transcription of Reg3g in MLE12 cells

Because Gp130 signaling is required for MRSA clearance and the lung epithelium was a prominent site of Stat3 phosphorylation during USA300 infection, we analyzed IL-6 regulation of Reg3g expression in lung epithelium using MLE12 cells. IL-6 stimulation of MLE12 cells increased P-Stat3 protein and induced functional Stat3-mediated transcription of a Stat3 luciferase reporter P-Stat3–luciferase MLE12 cell reporter line (Fig. 8, A and B). IL-6 also increased the level of Reg3g transcripts in these cells (Fig. 8 C). To determine whether P-Stat3 directly activates transcription of Reg3g, chromatin immunoprecipitation (ChIP) assays were performed on chromatin from IL-6–stimulated MLE12 cells using a P-Stat3 antibody. Stimulation with 50 ng/ml IL-22 was performed as a positive control as IL-22 stimulation of Reg3g is known to be Stat3 dependent (Zheng et al., 2008). Although there is a basal level of P-Stat3 binding to the Reg3g promoter, P-Stat3 binding was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D). Therefore, IL-6–stimulated P-Stat3 binding to the Reg3g promoter was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D). Therefore, IL-6–stimulated P-Stat3 binding to the Reg3g promoter was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D).

IL-6–stimulated P-Stat3 directly activates transcription of Reg3g in MLE12 cells

Because Gp130 signaling is required for MRSA clearance and the lung epithelium was a prominent site of Stat3 phosphorylation during USA300 infection, we analyzed IL-6 regulation of Reg3g expression in lung epithelium using MLE12 cells. IL-6 stimulation of MLE12 cells increased P-Stat3 protein and induced functional Stat3–mediated transcription of a Stat3 luciferase reporter P-Stat3–luciferase MLE12 cell reporter line (Fig. 8, A and B). IL-6 also increased the level of Reg3g transcripts in these cells (Fig. 8 C). To determine whether P-Stat3 directly activates transcription of Reg3g, chromatin immunoprecipitation (ChIP) assays were performed on chromatin from IL-6–stimulated MLE12 cells using a P-Stat3 antibody. Stimulation with 50 ng/ml IL-22 was performed as a positive control as IL-22 stimulation of Reg3g is known to be Stat3 dependent (Zheng et al., 2008). Although there is a basal level of P-Stat3 binding to the Reg3g promoter, P-Stat3 binding was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D). Therefore, IL-6–stimulated P-Stat3 binding to the Reg3g promoter was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D). Therefore, IL-6–stimulated P-Stat3 binding to the Reg3g promoter was significantly enhanced by IL-6 and IL-22 stimulation (Fig. 8 D).

Reg3g is induced in lung epithelium in response to MRSA USA300 in vivo, and recombinant Reg3g rescues the defect in MRSA clearance with Stat3 inhibition

Consistent with these in vitro findings, Reg3g transcripts were localized to the lung epithelium during MRSA infection by in situ hybridization (Fig. 9 A). Moreover, Reg3g mRNA

Figure 4. Stat3 activation in airway epithelium is necessary for MRSA clearance. (A) Phosphorylated Stat3 was detected in lung homogenates, naive and 20 h after USA300 infection by Luminex fluorescence intensity (FI). (B) P-Stat3 is localized to lung epithelium by immunohistochemistry. Bars, 200 µm. (C) The number of P-Stat3–positive cells from six random fields was counted in relation to the total number of epithelial cells in immunohistochemistry lung slides from naive, USA300–infected, and Stat3–inhibited–before–USA300–infection mice. n = 5 in each group. (D) Ad-cre was OA administered 6 d before USA300 challenge to deplete pulmonary epithelium of Stat3. CFU of USA300 lung burden was determined 16 h after infection. n = 9 in Ad-loc and n = 11 in Ad-cre groups, independently performed three times. P-values by Mann-Whitney test are as indicated. Error bars represent the SE.

Figure 5. Expression of S100A8/A9. (A and B) Both S100A8 (A) and S100A9 (B) are induced in the lung during USA300 by real-time qPCR. (C and D) Consistent with genetic deletion of Stat3 in the epithelium, administration of JSI-124 increased expression of both S100A8 (C) and S100A9 (D) at 4 h after USA300 infection. n = 5 for each group. Error bars represent the SEM.
expression during MRSA infection was attenuated by inhibition of Gp130/Stat3 signaling (Fig. 9, B–D). Because of the fact that Reg3γ−/− mice have abnormal gut microflora (Vaishnava et al., 2011), which alters this strain’s pulmonary immune response (unpublished observations), we chose to neutralize Reg3γ in vivo with intratracheal administration of anti-Reg3γ antibody. Anti-Reg3γ significantly increased lung CFUs of USA300 at 16 h after challenge (Fig. 9 E). This result was also confirmed by real-time quantitative PCR (qPCR) for SA normalized to GAPDH with a ΔCt of 1.24 ± 0.26 in the control antibody group versus a ΔCt of 14.12 ± 1.64 (P < 0.005 by Mann-Whitney test) in the anti-Reg3γ antibody group. Finally, local administration of recombinant Reg3γ-Fc 4 h after USA300 infection rescued the defect in MRSA clearance by Stat3 inhibition (Fig. 9 F), demonstrating a therapeutic role for Reg3γ. Collectively, Stat3 induces epithelial Reg3γ in vivo, leading to the clearance of MRSA.

**DISCUSSION**

Although Reg3γ has been well described in the gastrointestinal system, to our knowledge this is the first study of functional Reg3γ protein in lung epithelium and its regulation in response to microbial challenge. Although multiple factors responsible for the induction of Reg3γ transcription have been studied, this report of Gp130/Stat3 signaling in MLE12 cells as well as in the respiratory epithelium in mice suggests that Reg3γ is a direct target of Stat3 in vitro and in vivo. The Reg3 family of lectins consists of Reg3α, Reg3β, and Reg3δ in addition to Reg3γ. Reg3β and Reg3γ are very similar in protein structure and are both induced during our model. However, in the in vitro OD600 growth curve experiment, recombinant Reg3β had no bacteriostatic activity against USA300 (unpublished data).

During SA skin infection, both IL-1r1 signaling and IL-17 production by T cells are necessary for host defense against SA (Miller et al., 2007; Cho et al., 2010). In this model, γδ T cells were an essential source of IL-17 and required for clearance of SA (Cho et al., 2010). In contrast, clearance of SA in the lung is fundamentally different, as Rag2−/−/Il2rγ−/− mice clear SA similarly to WTB6 mice. Moreover, IL-17RA KO mice have similar SA lung burdens as WTB6 mice at 16 h after infection (Fig. 2 C). Therefore, different innate immune mechanisms are in play in lung and skin during SA infections.
neutrophils. Indeed, these cells are critical effector cells against SA. In fact, macrophages are a critical source of early IL-6 to activate Stat3 in this model. However, administration of Ad-cre to Stat3fl/fl mice, which deletes Stat3 in pulmonary epithelial cells, was sufficient to disrupt clearance of USA300. Although IL-22 is capable of mediating Stat3 in lung epithelium despite the lack of requirement for T, B, or NK cells, consistent with HIES patients, Stat3 was essential for optimal clearance of MRSA. Immunohistochemistry revealed that a major site of Stat3 phosphorylation is in the epithelium. Our present experiments do not exclude roles of Stat3 signaling in nonepithelial innate immune cells such as macrophages or neutrophils. Indeed, these cells are critical effector cells against SA. In fact, macrophages are a critical source of early IL-6 to activate Stat3 in this model. However, administration of Ad-cre to Stat3fl/fl mice, which deletes Stat3 in pulmonary epithelial cells, was sufficient to disrupt clearance of USA300. Although IL-22 is capable of mediating Stat3 in lung epithelium

**Figure 7.** Reg3γ binding and bactericidal activity against USA300. (A) Reg3γ binding. Bacteria were incubated with recombinant Reg3γ-mouse IgG2a-Fc fusion protein (Reg3γ-Fc) or isotype control, followed by incubation with a secondary anti–mouse IgG–Alexa Fluor 488 antibody. (B and C) Anti-Reg3γ antibody neutralizes the bacteriostatic effect of Reg3γ-Fc, and cleaved Reg3γ is bactericidal against USA300. (B) USA300 was incubated with recombinant Reg3γ-mouse IgG2a-Fc fusion protein (Reg3γ-Fc), Reg3γ-Fc with Reg3γ antibody, or isotype control, and OD600 readings were taken at hourly intervals. (C) USA300 was incubated with varying concentrations of Reg3γ-Fc or cleaved Reg3γ for 2 h in 10 mM MES and 25 mM NaCl, pH 5.5, and plated for CFU analysis. Errors bars represent the SE.

**Figure 8.** IL-6 stimulation induces P-Stat3 activation and direct transcriptional activation of Reg3g expression in MLE12 cells. (A) MLE12 cells respond to IL-6 stimulation and activate Stat3 as determined by increase in fluorescence intensity (FI) in Luminex specific to P-Stat3. (B) IL-6 stimulation of Stat3 signaling is verified by luminescence increase in Stat3-luciferase construct–transduced MLE12 cells 24 h after stimulation. n = 3 for each group, independently performed three times. (C) MLE12 cell induction of Reg3g in response to IL-6 stimulation is seen by real-time PCR normalized to Hprt. n = 6 for each group, independently performed twice. (D) Increased P-Stat3 binding to upstream regions of Reg3g chromatin of MLE12 cells after 1-h stimulation of 10 ng/ml IL-6 or 50 ng/ml IL-22 is detected by PCR. The second through fourth lanes are PCR of input DNA from nonstimulated or IL-6– or IL-22–stimulated cells, and the fifth through seventh lanes are PCR of DNA after chromatin precipitation with P-Stat3 antibody. (E) MLE12 cells (n = 3) were incubated with TNF, IFN-γ, IL-6, or IL-22 for 2 h followed by RNA extraction and assessment of Reg3g expression by real-time PCR normalized to Hprt. (F) IFN-γ was measured during the time course of US300 in lung homogenate of mice (n = 4–5 per time point). All levels were <50 pg/mg during this course of infection. P-values by Mann-Whitney test are as indicated. Error bars represent the SE.
neutrophil recruitment in response to cells was required to control early bacteria replication and pathogen challenge, MyD88 expression by nonhematopoietic neutrophil recruitment (Bals and Hiemstra, 2004). During live oxygen species, inflammatory cytokines, and chemokines for pulmonary epithelium produces antimicrobial peptides, reactive clearance to eliminate microbes from the respiratory tract, pulmonary epithelium employs multiple immune defense strategies and has been under-studied in response to MRSA pneumonia. In addition to providing a physical barrier and using mucociliary clearance to eliminate microbes from the respiratory tract, pulmonary epithelium produces antimicrobial peptides, reactive oxygen species, inflammatory cytokines, and chemokines for neutrophil recruitment (Bals and Hiemstra, 2004). During live pathogen challenge, MyD88 expression by nonhematopoietic cells was required to control early bacteria replication and neutrophil recruitment in response to Pseudomonas aeruginosa, whereas hematopoietic MyD88 expression was required at later time points (Skerrett et al., 2004). Our findings suggest that the pulmonary epithelium plays an undervalued role in early stages of inflammation by expressing the antimicrobial protein Reg3γ.

The role of myeloid versus nonmyeloid cell Stat3 in HIES remains controversial. One study of bone marrow transplantation in a 7-yr-old girl failed to improve the disease despite achieving chimerism in peripheral blood (Gennery et al., 2000). However, successful use of bone marrow transplantation in HIES has been recently reported in two patients, suggesting that some patients with HIES may benefit from bone marrow transplantation (Goussetis et al., 2010). It is important to note that in the MRSA model reported in this paper, the lack of requirement of T, B, or NK cells may be specific to this primary infection model as opposed to secondary lung infections that can occur after colonization of the skin or nasopharynx with SA. However, it is important to understand the contributions of intrinsic Stat3 versus myeloid Stat3 as some HIES patients develop severe enough lung disease to be listed for lung transplantation. Thus, it is critical to determine whether reconstituting intrinsic Stat3 in lung parenchymal cells with a lung transplant is sufficient to correct the pulmonary phenotype. Moreover, most patients with HIES develop infection with methicillin-sensitive SA (MSSA); however, we found that the USA300 strain caused similar induction of IL-6 as MSSA strains. Both MSSA and MRSA are well-known complications of influenza infection (Randolph et al., 2011; Parker and Prince, 2012), and in this setting, Th17 cytokines IL-17 and IL-22 are necessary for host defense against MSSA pneumonia during coinfection with influenza A (Kudva et al., 2011). We hypothesize that the requirement of IL-17 and IL-22 in this model may be caused by compromised epithelial host defenses, and thus epithelial antimicrobial factors may not be available to contain SA.

It is interesting to note that we detected both full-length and cleaved Reg3γ in this model (Fig. 6 C). Full-length Reg3γ protein had bacteriostatic activity, whereas the cleaved protein was bactericidal as has been reported for L. monocytogenes (Mukherjee et al., 2009). For these assays, we replicated similar assays as reported for Listeria at pH 5.5 (Mukherjee et al., 2009). The airway surface liquid does not become acidic, and thus future work will be needed to determine how Reg3γ is cleaved in the lung and whether the full-length protein can opsonize USA300 and could possibly be activated in an acidic compartment such as the phagolysosome. In addition to Stat3, we observed induction of Reg3γ by IFN-γ in MLE12 cells, and it has been reported that Stat6 can also regulate Reg3γ expression in the lung (Kuperman et al., 2005). Thus, it appears that other signaling pathways can clearly regulate Reg3γ in vivo, and these alternative signaling pathways could be induced by other components of the lung microbiota. Thus, in HIES patients that may have coinfection, these alternative signaling pathways could potentially compensate for Reg3γ expression. Future studies will need to determine the regulation of Reg3γ
in more complex co-infection models and determine the contribution of Stat3 in regulating Reg3γ expression under these types of conditions.

Deregulated Stat3 activation is linked to various cancers and poor prognosis, and many Stat3 inhibitors (i.e., JAK2 tyrosine kinase inhibitors and Sunitinib tyrosine kinase inhibitors) are in clinical trials (Yu et al., 2009). Although the use of these therapies for cancer is necessary if proven beneficial, the potential for decreased host immune defense to microbes in these patients has not been determined. In conclusion, Stat3-dependent epithelial production of antimicrobial protein Reg3γ is an essential host defense mechanism against MRSA pneumonia. Furthermore, Reg3γ could be developed to complement antibiotics against multi-drug–resistant SA.

MATERIALS AND METHODS

Mice. Male C57BL/6 (WTB6) 6–8-wk-old mice were obtained from Charles River. Reg3γ−/−, Il22−/−, β1γ−/−, and IL22−/− mice from C.C. Shive (University of California, Stanford) were used. MLE12 cells were obtained from ATCC (CCL-245). Male C57BL/6 mice (M.H. Kaplan all on C57BL/6 background) were housed in specific pathogen–free rooms within animal facilities at the Louisiana State University (LSU) Health Sciences Center. Controls for each experiment were sex and age matched from the same vendor, WTB6s from Charles River and various KO mice from Taconic or Intramurine controls. WTB6 mice were depleted of macrophages or neutrophils by OA 75 µl clodronate (Enscalpa) administration 48 h before infection or i.p. 500 µg IgG antibody (Bio-XCell) administration 24 h before infection, respectively. Lung burden by CFU counts was determined at 24 h after infection. Gp130 receptor was inhibited by OA 50 µg anti-mGp130 antibody (R&D Systems) immediately before USA300 infection. Stat3 signaling was inhibited by i.p. 1.5 mg/kg JSH-124 (Sigma–Aldrich) or i.p. or OA 5 mg/kg S1I-201 (EMD Millipore) 1 h before USA300 infection. All protocols were approved by the Institutional Animal Care and Use Committee at the LSU Health Sciences Center.

OA of MRSA in mice. A single colony of MRSA USA300 strain (from F. DeLeo, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT) was grown for 18 h at 37°C in 5 ml BBL Trypticase Soy Broth (BD). After this period, fresh TSB media was inoculated with the bacteria at 1:100 dilution and incubated at 37°C for 2 h with shaking to obtain bacteria during log phase of growth. These bacteria were washed with PBS, resuspended in 1 ml PBS, and diluted 5× to obtain 4 × 108 CFU/ml of working stock. 50 µl of this working stock was administered with tongue pull method at a dose of ~2 × 107 CFU of USA300 in the lung. Working stock was serially diluted and plated on Luria broth plates to determine the exact concentration. For Reg3γ rescue of Stat3 inhibition, recombinant Reg3γ-Fc at a dose of 60 µg was given OA to USA300–administered mice 4 h after infection. For Reg3γ neutralization in vivo, mice received 50 µg of rabbit IgG or Reg3γ antibody OA immediately before USA300.

Lung bacterial burden, mRNA, and protein analysis. Lungs were harvested in PBS, homogenized with a glass homogenizer, and plated on Luria broth in three serial dilutions. Colonies were counted after overnight incubation at 37°C. Gene expression was determined by mRNA extraction with TRIzol (Invitrogen), reverse transcription with Script (Bio-Rad Laboratories), and qPCR with TaqMan probes (Applied Biosystems) on an iCycler thermal cycler (Bio-Rad Laboratories). All CT values were normalized to Hprt. Lung homogenates were analyzed using bead-based Lumines assay (EMD Millipore) for both cytokine and P-Stat3 levels. BALF IL-6 was measured with ELISA (eBioscience). Reg3γ was detected in whole lung homogenate by sandwich ELISA (USCN Life Sciences) in accordance with the protocol provided with the kit.

Immunohistochemistry. The left lung was inflated with 10% neutral buffered formalin for 24 h before paraffin embedding. Slides were stained with P-Stat3 antibodies (Cell Signaling Technology). The number of P-Stat3-positive cells was counted in relation to the total number of epithelial cells in immunohistochemistry lung slides from naive, USA300-infected, and Stat3-inhibited before–USA300-infection mice.

Reg3γ binding and growth inhibition of MRSA. For binding, overnight cultures of SA USA300 were resuspended in PBS containing recombinant mouse Reg3γ-Fc (provided by W. Ouyang, Genentech, South San Francisco, CA; Zheng et al., 2008) or IgG2a isotype control (BD) for 1 h, followed by goat anti–mouse IgG2a–Alexa Fluor 488 (Invitrogen) for 30 min. Bacteria were fixed with 2% formaldehyde, and flow cytometry was conducted on a FACS Calibur (BD). To assess growth kinetics, overnight cultures were diluted 1:500 in TSB containing isotype control, Reg3γ-Fc, or Reg3γ-Fc with Reg3γ antibody (Abgent), and OD600 readings were measured at hourly intervals.

Reg3γ in BAL. 10 ml BALF was obtained at 20 h after OA administration of USA300 (2 × 107 CFU). BALF was concentrated (centrifugal filter; EMD Millipore), and Reg3γ was detected by Reg3γ antisera (Cash et al., 2006) on an SDS–PAGE Western blot (4–12%). Recombinant Reg3γ-Fc protein was used as positive control.

MLE12 cell culture and IL-6 stimulation. MLE12 (CRL–2110; American Type Culture Collection) was regularly passaged in DMEM/F12 medium supplemented with 1.94 mM l-glutamine, 10 mM Hepes, 5 µg/ml insulin, 10 µg/ml transferrin, 10 nM hydrocortisone, 10 nM β-estradiol, penicillin, streptomycin, and 2% fetal bovine serum. Cells were seeded in 2 × 104 cells/ml in 24–well plates 1 d before stimulation. Cells were stimulated with 10 ng/ml IL-6 (R&D Systems) for 15 min for P-Stat3 binding. MLE12 were seeded stably transduced with P-Stat3–Luciferase by lentiviral construct (SA–Biosciences). Luminescence reading (Promega) was taken at 24 h after stimulation with 10 ng/ml IL-6.

ChIP. MLE12 cells were stimulated with 10 ng/ml IL-6 for 1 h before cross-linking with 35% formaldehyde. ChIP assay was performed with P-Stat3 antibody (Cell Signaling Technology) at 1:50 dilution along the EZ-ChIP kit (EMD Millipore) protocol. Normal rabbit IgG antibody (Cell Signaling Technology) was used as negative control for polyclonal rabbit P-Stat3 antibody. 50–cycle standard PCR was performed with 5′-ATGCTCATG-CAGTCCAGGGATGA-3′ and 5′-CACGAGGAAAGCAGGTCCTT-3′ primers (IDT), flanking the 577-bp area. –650 to –80 bp upstream of the Reg3γ start site.

In situ hybridization. The mouse Reg3γ cDNA was amplified by PCR using gene-specific primers designed based on mouse sequences available in GenBank (accession no. NM_011260). The primer sequences were SQ_mReg3g_F1, 5′-GATGCTTCCCCGTATAACCATCACA-3′; and SQ_mReg3g_R1, 5′-CTAGGCTCTTGAATTTGCCAGACATGGGT-3′. The template used for amplification was a plasmid containing a mouse Reg3γ-Fc construct. The amplified products were ligated into pGEMT vector (Promega) and DNA sequenced. The Vector NTI Advance software package (Invitrogen) was used for analysis of the resulting DNA sequences. We performed stringent in situ hybridization (21-d exposures) as described previously (Reinhardt et al., 2002).

Reg3γ ELISA. Lung homogenates from naive WTB6 and Reg3γ−/− mice were diluted in 0.1 M carbonate coating buffer and plated in 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific) overnight at 4°C. The next day, plates were washed with PBS containing 0.05% Tween, blocked with PBS containing 5% milk, and incubated with rabbit anti–Reg3γ serum for 2 h (provided by L. Hooper, University of Texas Southwestern Medical Center, Dallas, TX; Cash et al., 2006) Plates were then washed, incubated with anti–rabbit IgG-HRP (Cell Signaling Technology) for 1 h, washed, and developed using 1× TMB substrate (eBioscience). Recombinant mouse
Reg3γ-Fc was used as a standard. Reg3γ expression was also confirmed and quantified using a commercial sandwich ELISA (USCN Life Sciences)

**Bactericidal assays.** Bactericidal assays were performed as previously described (Cash et al., 2006). In brief, SA was grown for 8 h in brain heart infusion broth (Sigma-Aldrich) and diluted 1:100 into buffer containing recombinant mouse Reg3γ-Fc or Reg3γ-cleaved (10 mM MES and 25 mM NaCl, pH 5.5). Bacteria were incubated with shaking at 37°C for 2 h, and then serial dilutions were plated on brain heart infusion agar for CFU analysis.

We would like to thank Dr. Lora Hooper for the Reg3γ antisera and cleaved Reg3γ, Dr. Frank DeLeo for the MRSA USA300 strain, and Dr. Wenjun Ouyang for the Reg3γ-Fc construct and protein.

This work was supported by the Public Health Service grant R37-HL079142. The authors have no competing financial interests.

Submitted: 4 June 2012
Accepted: 14 January 2013

**REFERENCES**


