STAT3 mediated IL-17 production by post-septic T cells exacerbates viral immunopathology of the lung

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Abstract

Survivors of severe sepsis exhibit increased morbidity and mortality in response to secondary infections. Although bacterial secondary infections have been widely studied, there remains a paucity of data concerning viral infections post-sepsis. In an experimental mouse model of severe sepsis (cecal ligation and puncture, CLP) followed by respiratory syncytial virus (RSV) infection, exacerbated immunopathology was observed in the lungs of CLP mice compared to RSV-infected sham surgery mice. This virus-associated immunopathology was evidenced by increased mucus production in the lungs of RSV-infected CLP mice and correlated with increased IL-17 production in the lungs. RSV infected CLP mice exhibited increased levels of Th2 cytokines and reduced IFNγ in the lungs and lymph nodes compared to RSV-infected sham mice. In addition, CD4 T cells from CLP mice produced increased IL-17 *in vitro* irrespective of the presence of exogenous cytokines or blocking antibodies. This increased IL-17 production correlated wth increased STAT3 transcription factor binding to the IL-17 promoter in CD4 T cells from CLP mice. Further, in vivo neutralization of IL-17 prior to RSV infection led to a significant reduction in virus induced mucus production and Th2 cytokines. Taken together, these data provide evidence that post septic CD4+T cells are primed toward IL-17 production via increased STAT3-mediated gene transcription, which may contribute to the immunopathology of a secondary viral infection.

Keywords

Inflammation; infection; mucus; cytokines; lymphocytes

INTRODUCTION

Morbidity and mortality associated with severe sepsis occurs during two distinct phases of the septic response. During the acute phase of the disease, unchecked production of proinflammatory mediators by immune cells results in a "cytokine storm" which can result in multi-organ dysfunction and death (1). Additionally, survivors of severe sepsis go on to exhibit profound immune suppression, resulting in increased morbidity (2–4). Following sepsis, lymphocytes exhibit numerous deficiencies in activation and effector function, including poor proliferative responses and aberrant T-helper cytokine production (5, 6). In addition, modulations in peripheral regulatory T cell populations have been observed in both humans and animal models post-sepsis (7, 8), and this increase in suppressive lymphocytes

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correlates in animal models with increased susceptibility to solid tumors (9) and non-lethal bacterial challenge (10). Experimental models of severe sepsis utilize "two-hit" models to investigate the underlying mechanisms of post-septic immunosuppression (11), often using infectious agents that require the innate immune system for protection and clearance (e.g. *Aspergillus fumigatus*) (12). While these models are useful for investigating innate immune dysfunction post-sepsis, they are not as robust for studying lymphocyte responses post-sepsis, as these infectious models are often controlled with relatively little involvement of the adaptive immune system. Therefore, the development of novel "two-hit" models that target adaptive immunity post-sepsis *in vivo* are needed for identifying mechanisms governing lymphocyte dysfunction in the context of post-septic immunosuppression.

Respiratory syncytial virus (RSV) is a negative-sense single-strand RNA virus that is a significant human health concern, especially for infants and immunocompromised patients (13, 14). The pathology of RSV infection is unique among respiratory viral pathogens in that it displays a biphasic T-helper cytokine profile, with T_H1 type cytokines (such as IFNγ) predominating during the early phase of the infection, and a shift towards T_H2 (such as IL-13) (15) and Th17 (IL-17) cytokines at later time points (16). While the shift from T_H1 to T_H2-type inflammation may play a role in the correlation between RSV infection during infancy and increased susceptibility to asthmatic responses later in life (17), the Th17 responses may drive the chronicity of the primary RSV disease and exacerbate an existing allergic condition (16).

Based on the unique nature of RSV immune responses, and the fact that RSV is both a ubiquitous pathogen and a concern for immunocompromised patients, we tested whether survivors of severe sepsis (who are themselves immunocompromised) exhibit modulations in their ability to respond to airway infection with RSV. The present studies were aimed at identifying the possible deleterious outcomes for secondary viral infection in survivors of severe sepsis as well as identifying possible lymphocyte dysfunction following sepsis *in vivo*. The results of this study indicate that post-septic animals exhibit increased susceptibility to RSV infection, evidenced by increased viral burden in the lungs and enhanced production of proinflammatory cytokines (such as IL-17) leading to deleterious immunopathology. In addition, CD4+ T cells from post-septic mice produced increased levels of IL-17. Additionally, blockade of IL-17 in post-septic animals *in vivo* reduced the immunopathology seen following RSV infection. Taken together, these results suggest that as a consequence of severe sepsis, overproduction of IL-17 by CD4+ T cells can participate in viral-induced immunopathology through inhibiting viral clearance and promoting mucus production in the airways.

MATERIALS AND METHODS

Mice

6–8 week old female Balb/c mice were purchased from The Jackson Laboratories. All mice were maintained in specific pathogen free facilities in the Unit for Laboratory Animal Medicine at the University of Michigan and all experiments were approved by the University Committee of Use and Care of Animals (UCUCA).

Cecal Ligation and Puncture and RSV infection

CLP surgery was performed on mice as described previously (5). Briefly, a midline incision was performed on anethesized mice. For CLP. the cecum was ligated and punctured seven times with a 21-gauge needle. For sham surgery mice, the cecum was manipulated without ligation or puncture. Both sham surgery and CLP mice were treated with the antibiotic INVANZ (Ertapenem, Merck & Co., Inc., Whitehouse Station, NJ) administered at 75 mg/

kg via intraperitoneal injection beginning at 6 hours after surgery and re-injected every 24 hours until day 3 (day -11) after surgery. The average mortality rate for mice subjected to CLP in this study was 40–60% by day 4 after surgery. 14 days after the surgery, mice were infected with RSV (Day 0) intratracheally by tongue pull at 1×10^5 plaque-forming units (PFU)(16). The experimental groups are identified in the text and figures as follows: "SNR" – sham surgery, no RSV; "SR" – sham surgery followed by RSV challenge; "CNR" – CLP surgery, no RSV; "CR" – CLP surgery followed by RSV challenge.

Histology and RT-PCR

For histology, right lobes of the lung from infected mice were removed, fixed in 10% formalin, and stained as indicated. For RT-PCR, total RNA was extracted from the tissue using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA. Murine primers for IL4, IL13, IFNγ, IL17, and GAPDH were purchased from Applied Biosytems (Carlsbad, CA). Primers and probes for Muc5ac, Gob5, RSV-F, RSV-N and RSV-G were determined using primer/probe detection sets (PE Biosystems, Foster City, CA) and purchased from Sigma-Aldrich. Fold expression was calculated using the delta-delta Ct method with GAPDH serving as a housekeeping gene.

CD4 T cell skewing and Protein Assays

Spleens from from sham or CLP mice were isolated at day 14 following surgery, and CD4+ T cells were purified using magnetic beads (Miltenyi Biotech, Cambridge, MA). The cells were then plated and skewed as follows, Th0: anti-CD3 (Clone 145-2C11, BD Biosciences, San Diego, CA) (2ug/ml) and anti-CD28 (2ug/ml) (Clone 37.51, BD Biosciences); Th1: anti-CD3, anti-CD28, anti-IL-4 (10ug/ml) (eBioscience, San Diego, CA) and rIL12 (10ng/ml) (R&D Systems, Minneapolis, MN); Th2: anti-CD3, anti- CD28, anti-IL-12 (10ug/ml) (eBioscience), anti-IFNγ (10ug/ml) (eBioscience) and rIL4 (10ng/ml) (R&D Systems); Th17: anti-CD3, anti-CD28, rTGFβ (2ng/ml) (R&D Systems), rIL6 (20ng/ml) (R&D Systems), anti-IL-4 (10ug/ml) and anti-IFNγ (10ug/ml). Four days later the cells were rested for three days and then restimulated for another 48 hours. Supernatants were collected and analyzed for cytokine expression and quantified using a bead-based (Luminex) cytokine assay (Bio-Rad Laboratories, Hercules, CA). For lymph node restimulations, single cell suspensions of lymph nodes isolated from individual mice at the indicated timpoints following experimental surgery and/or RSV infection were seeded in a 96-well plate and restimulated with RSV. Forty-eight hours later the supernatants were harvested and analyzed for cytokines as described above.

Flow cytometry

Collagenase-digested (Sigma-Aldrich, St. Louis, MO) lung lobes from mice were processed into single cell suspensions by passing tissues through sterile 40-mm filters, then ammonium chloride lysis buffer was used to eliminate erythrocytes. Cells were stained with the following fluorescent antibodies in flow cytometry buffer (phosphate buffered saline, 1% w/v bovine serum albumin, 0.05% w/v sodium azide): Purified anti-CD16/32 (Fc Block) (BD Biosciences, San Jose, CA), Pacific Blue-CD3e (eBioscience), PE-Cy7-CD4 (eBioscience), PE-CD8a (BioLegend, San Diego, CA), PerCP-Cy5.5-CD49b (pan-NK) (BioLegend) and FITC-TCRγ/δ (BioLegend). For intracellular cytokine analysis, bead purified splenic CD4+ T cells were cultured in 24-well plates containing plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2.5 μg/ml). After overnight incubation and in the presence of Golgi-Plug (BD Biosciences) for the last 2 hours, the cells were stained using the indicated flurophore-conjugated antibodies, in addition to Fc block (as indicated above): PE-IL17a (Biolegend), PerCP-Cy5.5-CD4 (BioLegend) and APC-Cy7 TCRβ (BioLegend). Cells were fixed in 4% paraformaldehyde and analyzed using a LSR II (BD Biosciences). Flow cytometry data was analyzed using FlowJo 9.0.1 (Tree Star, Ashland, OR, USA).

RSV and Viral Plaque Assay

RSV A strain (line 19) was derived from a clinical isolate at the University of Michigan(16). Supernatants from whole lung dispersions were serially diluted and viral plaques were determined using a RSV-specific polyclonal antibody (Millipore, Billerica, MA), as described previously(16).

Neutralization of IL-17

Neutralizing rabbit anti-mouse IL-17 antibody was generated in our laboratory as previously described (18). Animals were pre-treated via intraperitoneal injection with 2.5 mg per mouse of purified polyclonal anti-mouse IL-17 or an equivalent dose of control IgG antibody (Cab) 2.5 hours before RSV infection on day 0 and then every other day until day 6.

Statistical analysis

Significance was calculated utilizing repeated measures ANOVA when necessary, followed by *post-hoc* Bonferroni tests for significance between experimental groups. For single-group analysis, two-tailed Student's *t* tests were used to determine significance. In all cases, p values equal to or less than p=0.05 were considered statistically significant. Data analysis was performed with GraphPad Prism v5.0a for Macintosh (GraphPad software, San Diego, CA).

RESULTS

Increased mucus production in CLP RSV lungs

To assess the ability of post-septic mice to respond to a viral infection of the airways, sham and CLP mice were infected with RSV at day 14 post-surgery (Fig. 1A). Uninfected animals (both sham and CLP) were used as controls for the analysis of lung inflammation; as exposure to inactivated RSV does not result in lung inflammation in mice, inactivated RSV control groups were not utilized(19). At day 8 post-infection, mice were sacrificed and lungs were subjected to histological analysis. There was no significant difference in inflammatory infiltrate and mucus production in both sham and CLP lungs without RSV challenge (Fig. 1B). In response to RSV infection, sham mice exhibited extensive peribronchial inflammation, along with evidence of mucus production by airway epithelial cells, particularly in the larger conducting airways (Fig. 1B). In comparison, CLP mice infected with RSV exhibited significantly increased peribronchial inflammation as compared to sham RSV mice (Fig. 1B), with evidence of mucus plugging in both small and large conducting airways (Fig. 1B).

Following RSV infection, robust increases in *Gob5* and *Muc5ac* mRNA were observed in lungs of sham infected (SR) animals (Fig. 1C). In comparison, CLP infected (CR) animals exhibited greater and significant increases in the expression of *Gob5* as compared to SR animals (Fig. 1C). No significant differences were observed in the expression level of *Muc5ac* in the lungs of either SR or CR animals (Fig. 1C).

Increased viral burden in CLP RSV lungs

To confirm that the increase in mucus production seen in CLP RSV lungs was due to an increase in viral burden, lung homogenates from sham and CLP animals were analyzed via plaque assay. At day 3 following infection, both SR and CR mice exhibited productive viral replication in the lungs, as indicated by plaque formation in assays of lung homogenates (Fig. 2A). CR lungs exhibited increased plaque formation as compared to SR lungs (Fig. 2A). Additionally, lungs from CR animals exhibited increased expression of RSV surface protein-encoding genes (G and F) as compared to SR lungs (Fig. 2B).

Increased IL-4 production by lymph node cells from CLP RSV mice

Total lymph node cells from sham and CLP mice (either unchallenged or RSV-challenged) were restimulated *in vitro* with RSV for analysis of cytokine production in response to viral antigen stimulus. Supernatants from *in vitro* restimulated lymph node cultures were analyzed via multiplex bead assay (Bioplex) for the production of various T_H1, T_H2 and T_H17-related cytokines (Fig. 3A). While the production of IL-5, IL-17 and IFNγ appeared increased in CR lymph node cultures as compared to SR, and IL-5 in particular, these differences were not statistically significant do to variances in cytokine production by CR lymph node cells (p=0.0572 for CR vs. SR IL-5) (Fig. 3A). However, CR lymph node cultures did exhibit a significant increase in IL-4 production as compared to SR lymph node cultures following RSV challenge *in vitro* (Fig. 3A).

Increased IL-17 mRNA in lungs of CLP RSV mice

Lungs from SR and CR mice were recovered at day 3 and day 8 post-infection with RSV and were analyzed via real-time PCR for the expression of pro-inflammatory cytokine genes. At day 3 post-infection, lungs from CR infected mice exhibited decreased expression of IFNγ mRNA as compared to SR lungs (Data not shown). However, this difference was no longer observed in lungs from mice at day 8 post-infection (Data not shown). CR lungs exhibited significant increases in IL-17 mRNA production at both day 3 and day 8 post infection as compared to SR lungs (Fig. 3B). Lungs were also analyzed for upregulation of IL-17 associated cytokines, such as the T_H17-instructing cytokines IL-6 and TGFβ. While both cytokines were observed in the lungs of RSV-infected animals, there was no significant difference in fold expression between SR and CR groups (day 3, fold expression over SNR; IL-6: 4.31 ± 1.7 for SR vs. 4.42 ± 1.7 for CR, p>0.05; TGFβ: 1.87 ± 0.6 for SR, 8.97 ± 5.0 for CR, p>0.05).

No significant differences in total number of T & NK cells in lungs of sham or CLP mice following RSV infection

One possible explanation for the increase in proinflammatory cytokine production in the lungs of CLP RSV mice (concomitant with increased mucus production) is an increase in immune cell recruitment to the lungs following CLP, in particular T cells and NK cells, both of which are involved with IFN-γ and IL-17 production. To determine if the increase in lung cytokine production was due to increased cell recruitment, lungs from sham and CLP mice were harvested at 2 and 4 days following RSV infection, and analyzed via flow cytometry for the presence of CD4+ and CD8+ T cells, γδ T cells and pan natural killer (NK) cells. While these timepoints are relatively early in the context of RSV pathogenesis, and do not necessarily represent the peak of leukocyte infiltration into the airways (20), these timepoints were chosen in an attempt to correlate the increase in proinflammatory cytokine production in the lungs with increased cell recruitment. Total cell counts were obtained by multiplying the total viable cell count obtained from digested/dispersed lungs by the percentages obtained by flow cytometry.

At day 2 post-infection, the predominant cell type in both sham and CLP RSV lungs was CD8+ T cells (Table 1). However, there were no significant differences observed in the total number of CD8+ T cells between sham and CLP RSV-infected lungs. In addition, no significant differences were observed in total numbers of CD4+ T cells between sham and CLP RSV-infected lungs (Table 1). Total numbers of γδ T and NK cells were also similar between sham and CLP lungs, with no differences apparent in response to RSV infection as shown in Table 1. At day 4 post-infection, CD4+ T cells were the predominant cell type in the lungs of both sham and CLP RSV lungs; however, as with the day 2 analyses, there were no significant differences observed in total numbers of these cells between sham and CLP RSV lungs. Similar results were observed for CD8+ T cells between sham and CLP RSV

lungs (Table 1). In contrast to day 2, the total number of $\gamma\delta$ T cells and natural killer (NK) cells had decreased and increased, respectively; however, these differences were not statistically significant between sham and CLP RSV lungs (Table 1).

Splenic CD4+ T cells from CLP animals produced increased IL-17 as compared to sham in response to in vitro skewing with exogenous cytokines

Based on the flow cytometry data, the increased production of IL-17 in the lungs of CLP RSV lungs was not due to increased numbers of lung lymphocytes. These results suggest that post-septic lymphocytes may produce more IL-17 in inflammatory responses as compared to lymphocytes from sham mice. Previous studies indicate that post-septic CD4+ T cells produce more IL-17 in response to both polyclonal stimulus *in vitro* (6) and in response to antigen stimulation *in vivo* (5), suggesting that the IL-17 production potential of post-septic CD4+ T cells would be observable in these cells prior to viral infection with RSV. Therefore, to determine if CD4+ T cells were the possible source of the increased IL-17 in lungs of CLP-RSV mice, splenic CD4+ T cells from sham and CLP mice (not infected with RSV) were restimulated with polyclonal stimulus *in vitro*. Splenic CD4+ T cells were utilized as a proxy for lung-resident CD4+ T cells, as naïve lungs (in this case, SNR and CNR lungs) contain relatively few CD4+ T cells as compared to peripheral immune organs (e.g. spleen and lymph nodes). Splenic CD4+ T cells from CLP exhibited increased IL-17 production, as evidenced by an increased number of IL-17+ cells following *in vitro* re-stimulation (43.03% ± 0.6% for CLP vs. 32.25 ± 1.1% for sham, p < 0.001) (Fig. 4A). In addition, CLP-T cells exhibited increased production of IL-17 per cell as evidenced by mean fluorescence intensity (877.4 ± 13 for CLP vs. 627.3 ± 43 for sham, p < 0.001).

To determine if the modulations in *in vivo* cytokine expression in CLP mice were related to modulations in CD4+ T cell effector function, CD4+ T cells from the spleens of sham and CLP mice (not infected with RSV) were isolated and skewed *in vitro* using polyclonal stimulus and exogenous cytokines, to determine if the increase in IL-17 production observed in CLP CD4+ T cells could occur in the context of opposing T-helper polarizing conditions (i.e. T_H1 and T_H2). CD4+ T cells from both sham and CLP mice produced similar amounts of IFNγ (Fig.4B) and IL-2 (data not shown) in response to T_H0 re-stimulation conditions. Additionally, both sham and CLP T_H0 cells produced low levels of IL-4 and IL-5 (data not shown). However, CLP T_H0 cells produced significantly less IL-13 and more IL-17 than sham T_H0 cells (Fig. 4B). This increase in IL-17 production by CLP T cells was also observed in T_H1 cultures (Fig. 4B), and was concomitant with a decrease in IFNγ production as compared to sham T cells (Fig. 4B). This increase in IL-17 production by CLP T cells was also seen in T_H2 cultures, along with a decrease in IL-13 production, as compared to sham T cells (Fig. 4B). A similar pattern was observed in T_H17 cultures, with CLP T cells producing increased IL-17 and decreased IL-13 as compared to sham T cells (Fig. 4B).

Splenic CD4+ T cells from post-septic animals exhibit increased binding of STAT3 to regions of the IL-17 promoter

The increased production of IL-17 by CLP T cells suggested a modulation in IL-17 gene regulation in these cells. To test this hypothesis, splenic CD4+ T cells from sham and CLP mice were analyzed *ex vivo* for transcription factor binding to the promoter region of IL-17 via chromatin Immunoprecipition (ChIP) assay. As the transcription factor RORγt is essential for T_H17 lineage commitment, initial ChIP assays were performed to identify RORγt binding to the IL-17 promoter; however, there was no observed increase in RORγt binding to the IL-17 promoter in CLP T cells (data not shown). IL-17 production in CD4+ T cells can also be induced in a STAT3-dependent manner; to test this, ChIP assays were performed with αSTAT3 antibodies and primers directed to putative STAT3 binding sites

within 500 base pairs of the IL-17a transcription start site (Fig. 5A). Increased STAT3 binding was observed in CLP T cells as compared to sham, at two putative STAT3 binding sites proximal to the IL-17a transcription start site (Fig. 5B).

In vivo blockade of IL-17 reduced mucus production and viral burden in lungs of CLP mice following RSV infection

To determine if the increase in IL-17 production observed both in RSV-infected lungs and in activated CD4+ T cells from spleens of CLP mice was involved with the increased mucus production and viral burden observed in these mice, blocking antibodies to IL-17 were used in the CR infection model. SR and CR animals were treated with αIL-17 antibody via intraperitoneal injection at the time of viral infection, as well as two and four days later (Fig. 6A). Two days after the final antibody treatment, mice were sacrificed and lungs were analyzed for mucus production, mucus-associated gene expression, and expression of proinflammatory cytokine genes and viral gene products. In addition, lymph nodes from SR and CR mice were re-stimulated *in vitro* with RSV for analysis of cytokine expression. Treatment with αIL-17 had a significant effect on mucus production and lung inflammation, with a reduction in inflammatory infiltrates and PAS+ epithelial cells (Fig. 6B). These histological changes were also associated with reductions in expression of the mucus-associated genes *Gob5* and *Muc5ac*, with αIL-17 treatment reducing their expression by half in CR lungs (Fig. 6C).

To determine if the reduction in lung inflammation was due to changes in proinflammatory cytokine expression in the lungs of CR mice receiving αIL-17, lymph nodes were analyzed for protein (Fig. 6D) expression following RSV re-stimulation, and lungs were analyzed for cytokine mRNA expression (Fig. 6E), as well as viral gene expression (Fig. 6F). Treatment of CLP+RSV mice with αIL-17 *in vivo* had a slight effect on the production of IFNγ (increased), IL-4 and IL-13 (decreased) in lymph node cells; however these differences were not statistically significant (Fig. 6D). In contrast, αIL-17 treatment had a dramatic effect on expression of IFNγ mRNA in whole lung tissue, with a significant increase as compared to Control ab (Cab)-treated CLP+RSV lungs (Fig. 6E). No significant differences were observed in the expression of IL-4 or IL-13 mRNA in the lungs of CLP+RSV αIL-17 treated mice as compared to Cab treatment (Fig. 6E). In addition, αIL-17 treatment resulted in a significant decrease in the expression of the RSV-G gene in CLP+RSV lungs, as compared to Cab treatment (Fig. 6F). Marked decreases in RSV-F and RSV-N were also observed in CLP+RSV αIL-17 treated animals. (Fig. 6F)

DISCUSSION

Survivors of severe sepsis remain susceptible to secondary and nosocomial infections, especially in regards to opportunistic infections of the lung. The results of this study indicate that following sepsis, animals are more susceptible to airway infection with RSV. The increase in IL-17 production in post-septic mice in the context of RSV infection appears to play a role in the increased viral burden and mucus production seen in the airways. IL-17a mRNA levels are significantly increased in post-septic mice at early timepoints following RSV infection, and remain elevated throughout the progression of the infection. Additionally, IL-17 production is only observed in restimulated lymph node cultures from RSV-infected animals, and IL-17a mRNA levels remain at "wild-type" levels in post-septic lungs in the absence of RSV infection, suggesting that viral infection stimulates increased IL-17 production in post-septic animals. In addition, *in vivo* neutralization of IL-17 results in a reduction in the severity of both disease parameters. Taken together, these results indicate that survivors of severe sepsis develop a propensity to produce IL-17 in response to inflammatory stimuli, and that this increased IL-17 production in response to secondary

infection can result in deleterious immunopathology in certain contexts, i.e. viral infections of the lung.

Sepsis-induced mortality can manifest in two distinct fashions: either as a result of multi-organ dysfunction following the "cytokine storm" of acute sepsis, or as a result of the complex immunosuppression that follows. Experimental models of sepsis often utilize opportunistic lung infections such as *Aspergillus fumigatus* (12) to model post-septic immunosuppression. Viral infections also can have an adverse effect on sepsis severity and post-septic mortality; for example, sepsis can reactivate latent cytomegalovirus (CMV) infections in both humans and animal models (21, 22), and certain studies suggest that CMV reactivation can have a deleterious effect on the survival of septic patients (23). Despite these results, there remains a paucity of experimental models utilizing secondary viral infections following sepsis. Therefore, the use of RSV in a "two-hit" sepsis model is useful, as RSV is a ubiquitous human pathogen that can cause significant pathology in immunosuppressed individuals. Additionally, the use of RSV as a secondary infectious agent is pertinent to the T_H shift observed in survivors of severe sepsis (24), as immune responses to RSV are multiphasic in nature, and proceed through T_H1, T_H2 and T_H17-type cytokine responses (16).

Recent studies in human patients and animal models have identified divergent roles for IL-17 in the pathogenesis of sepsis and sepsis-induced mortality. Originally, it was thought that IL-17 would play a prominent role in the acute phase of sepsis, primarily though its ability to modulate the development and activation of neutrophils (25), innate immune cells that play an important role in mediating the septic response. However, studies of IL-17 levels in peripheral blood of septic patients indicate little correlation between IL-17 production and the severity of the septic response (26). In contrast, modulation of IL-17 function in experimental models of sepsis can have a dramatic effect on sepsis-induced mortality. For example, blockade of IL-17 *in vivo* using antagonist antibody treatment increased survival in mouse models of polymicrobial sepsis (27). In addition, mice lacking the ability to signal through the IL-17 receptor (IL-17R$^{-/-}$) exhibited increased susceptibility to "mild" models of surgically induced sepsis, unlike control animals that did not exhibit mortality (28). Interestingly, increased IL-17 production by post-septic CD4+ T cells has been observed in response to polyclonal stimulus *in vitro* (6) and in the context of both T_H1 and T_H2 inflammation *in vivo* (5). In addition, increases in IL-17 mRNA have been observed in intraepithelial γδ T cells following the onset of experimental sepsis in animal models (29). These results indicate that polymicrobial sepsis can create an environment promoting IL-17 production, and this dysregulated IL-17 production by CD4+ T cells may adversely modulate the immune response to secondary infections in post-septic patients.

Recent studies have indicated a role for IL-17 in mediating reactive airway disease phenotypes, and mucus production in particular. Members of the extended IL-17 family have been implicated in mediating allergic airway disease phenotypes; for example, IL-17E (also known as IL-25) can induce the production of many T_H2 cytokines in the lung, including IL-4, -5 and -13, and can mediate eosinophil accumulation via increased eotaxin (CCL11) production (30). In addition, recent studies have implicated IL-17A as an important factor for mucus production in the airways (16, 18). Interestingly, these studies provide a mechanism whereby viral infections, often considered to generate primarily T_H1-type responses, can mediate T_H2-type reactive airway pathology though the upregulation of IL-17 production. This phenomenon is evidenced in the present study, whereby *in vivo* blockade of IL-17 results in a reduction of RSV-induced airway mucus production in sham surgery animals. These results suggest that the aberrant production of IL-17 in post-septic animals exacerbates RSV immunopathology both through limiting productive immunity (as evidenced by increased viral titers) and exacerbating deleterious responses (such as type 2

cytokines and mucus production). Further, these results suggest that IL-17 may negatively regulate productive viral immunity in the lung, especially when taken in context with previously published reports highlighting links between neutralization of IL-17 and decreased RSV viral burden(16). Future studies into the role of IL-17 in post-septic immune dysfunction may help clarify whether this increase in IL-17 is attributable to *de novo* generation of T_H17 cells, or active suppression of other T-helper subsets via IL-17 dependent mechanisms.

In contrast, the effects of increased IL-17 production on post-septic susceptibility to RSV infection may be due to suppression of IFNγ-mediated immune responses rather than strictly a promotion of T_H2 cytokines. While there remains a paucity of data implicating a role of IL-17 in suppressing T_H1 responses, previous studies of RSV pathogenesis suggest that modulations in IFNγ can have a dramatic effect on the outcome of the infection(31). As post-septic CD4+ T cells exhibit deficiencies in IFNγ production, this decreased T_H1 response coupled with an exacerbated T_H17 response may be driving infection severity in post-septic animals. These results suggest that treatment of post-septic animals (and perhaps human patients) with exogenous IFNγ may help to protect against viral infection following sepsis. However, previous studies suggest that post-septic immunosuppression (evidenced by increased susceptibility to bacterial infection) is not resolved with the administration of IFNγ (32). In addition, IFNγ production in post-septic animals did not appear affected in this study, suggesting that the increased susceptibility to RSV infection observed in this model is not strictly due to a decrease in IFNγ.

Lymphocyte-derived cytokines appear skewed towards T_H2-type responses following sepsis (24). This skewing towards T_H2-type cytokine responses is also supported by the depressed IL-12 production by accessory myeloid cells (such as dendritic cells) following severe injury and/or sepsis (33). Previous studies have indicated a propensity for IL-17 production by post-septic CD4+ T cells both *in vivo* and *in vitro* (5, 6). In agreement with these studies, we show that CD4+ T cells from post-septic animals expressed increased IL-17 following *in vitro* skewing with exogenous cytokines, suggesting that the sepsis response may have conditioned the surviving CD4+ T cells to produce IL-17 upon secondary stimulation. Interestingly, this increase in IL-17 production appears dependent on STAT3 and not RORγt. As IL-17a is not thought to signal via IL-17 receptor-dependent STAT3 phosphorylation(34), the observed increased in STAT3 binding to the IL-17a promoter appears to be further evidence of the increased propensity of post-septic CD4+ T cells to produce IL-17. STAT3 plays an important role in initiating IL-17 gene transcription, and STAT3-dependent signals (such as IL-6 receptor signaling) play an important role in the development of IL-17 producing CD4+ T cells (35). Aberrant STAT3 signaling in post-septic T cells may participate in the increased IL-17 production observed by these cells in response to T_H17-opposing cytokines (such as IFNγ and IL-4).

In the present study, increased IL-17 production by post-septic CD4+ T cells resulted in increased viral titers and increased mucus production following airway RSV infection. This increase in IL-17 has a deleterious effect on viral immunity despite being a pro-inflammatory response, as it mediates immunopathology (i.e. mucus) without providing protection against the infectious agent (i.e. RSV viral titers). The results of this study provide further evidence for the susceptibility of survivors of severe sepsis to secondary infections, and respiratory viral infections in particular. In addition, these studies provide evidence that severe sepsis can shift immune responses towards IL-17 production, in addition to T_H2 responses. Importantly, these studies implicate increased IL-17 production by CD4+ T cells as an indicator of post-septic immune dysfunction, restricting the ability of the post-septic immune system to respond effectively to pulmonary viral infection.

Acknowledgments

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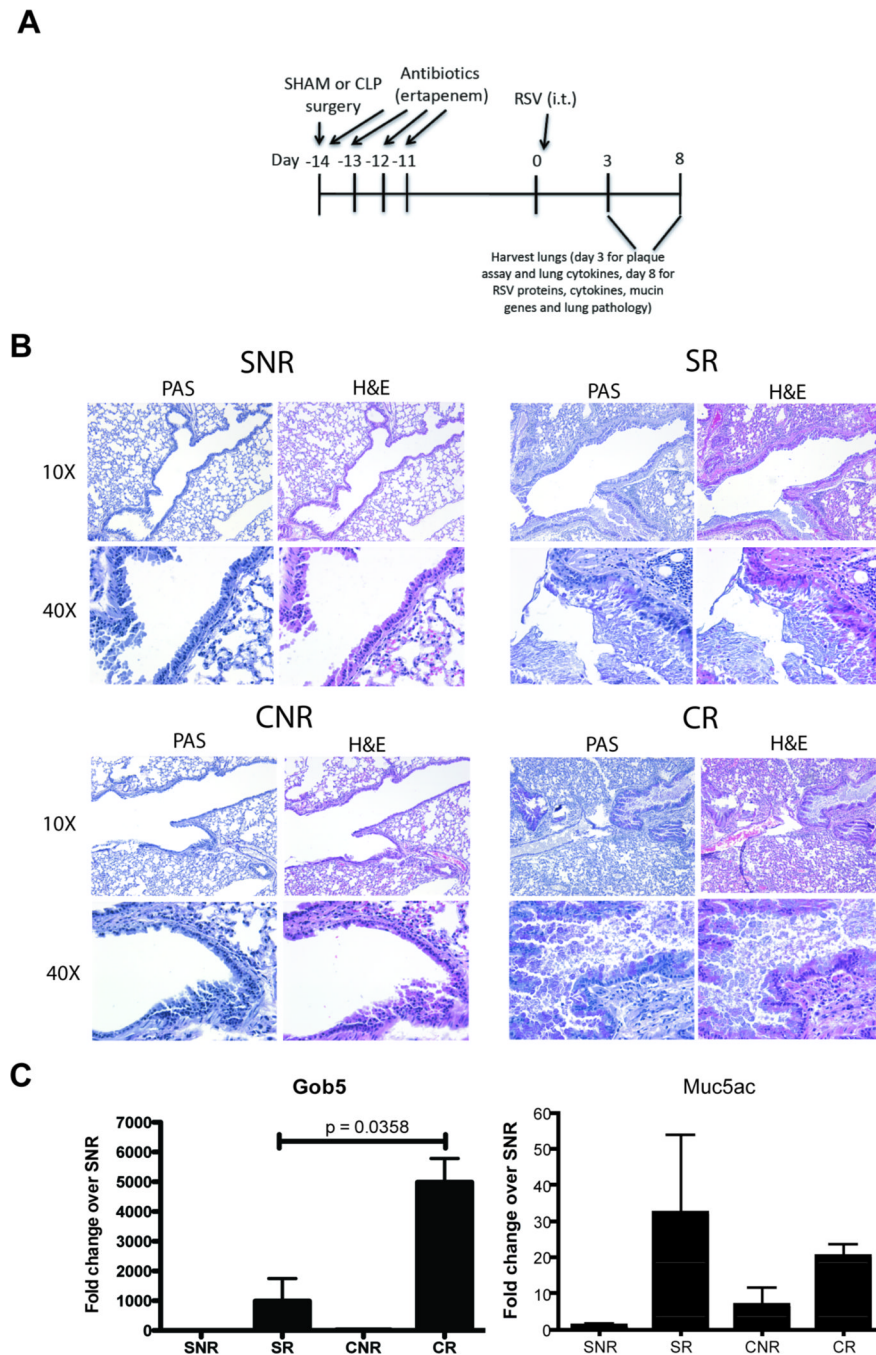
A

B

C

Fig. 1. Secondary RSV infection in post septic mice induces hyper mucus production
A) Schematic of RSV infection in Sham or CLP. B) Sham or CLP mice were infected with RSV or left uninfected and 8 days post infection mice were euthanized and their lungs were harvested and stained with H&E or PAS. B) RNA was extracted from the lungs and mucin associated genes were analyzed by RT-PCR. Data is representative of three independent experiments, n=3–5 mice/group. The data is expressed as mean ± S.E.

A
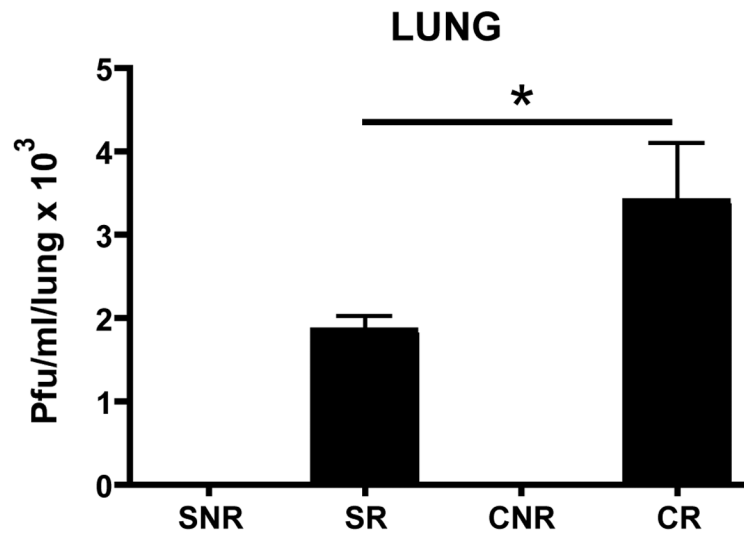
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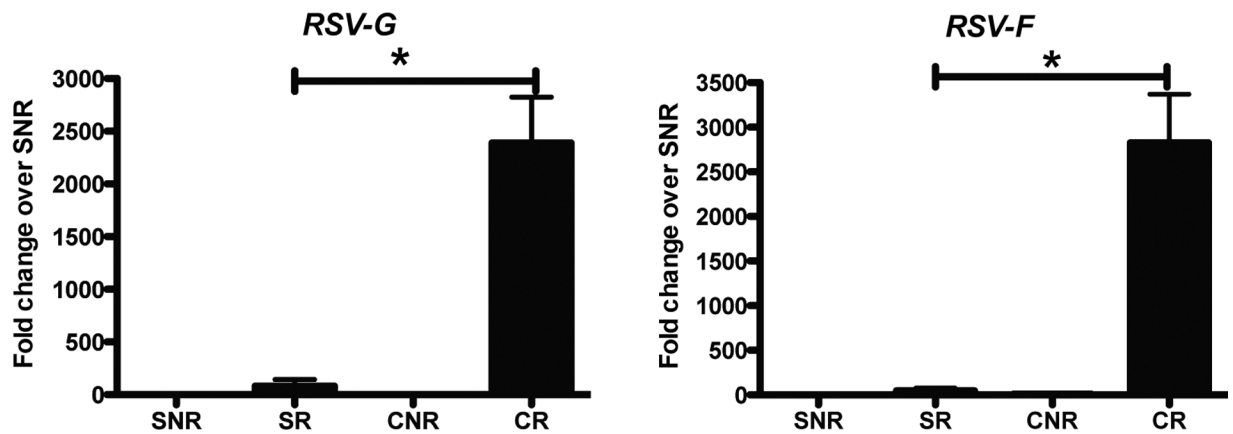
Fig. 2. Increased viral burden in the lungs of RSV infected post-septic mice
Sham or CLP mice infected with or without RSV were euthanized 3 days post infection. A)
The lungs were disrupted to assess the viral burden using a plaque assay. B) RNA from 8
day post infected mice was analyzed for RSV proteins (G and F). The data is expressed as
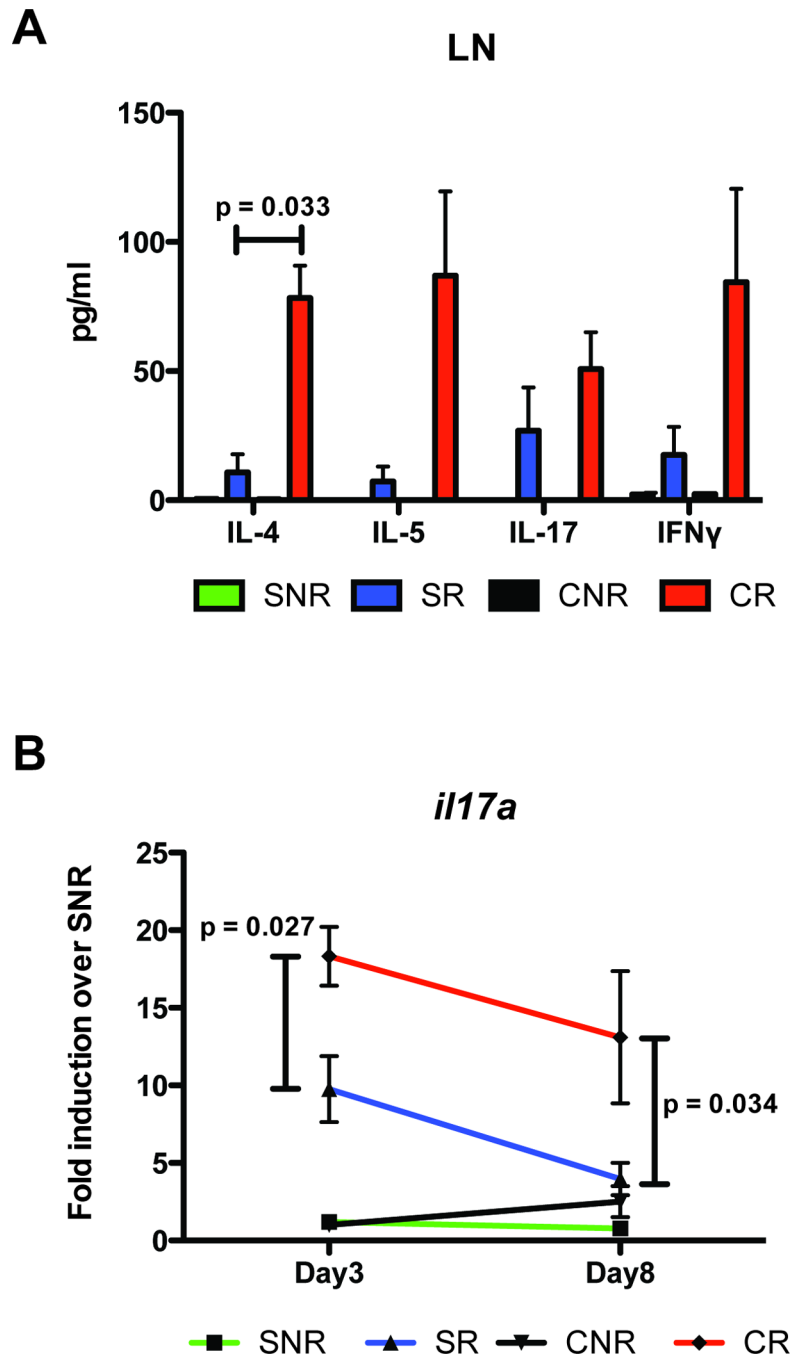mean ± S.E and * represents $p < 0.04$.

A LN

B *il17a*

Fig. 3. Post septic mice exhibit increased pulmonary Th2 cytokines to secondary viral infection
A) Mediastinal lymph nodes from Sham and CLP mice infected with RSV were collected on day 8 post infection, individually plated in 96-well plates, and restimulated *ex vivo* with RSV for an additional 48 hours. Supernatants were then collected and cytokines were analyzed by bead-based multiple cytokine analysis (Luminex). B) Sham or CLP mice were infected with RSV or left uninfected. On day 3 day and day 8 post RSV infection, mice were euthanized and lungs harvested for RNA expression of IL-17 by RT-PCR. The data is expressed as mean ± S.E. Each experiment was repeated twice, n=3–5 mice/group.
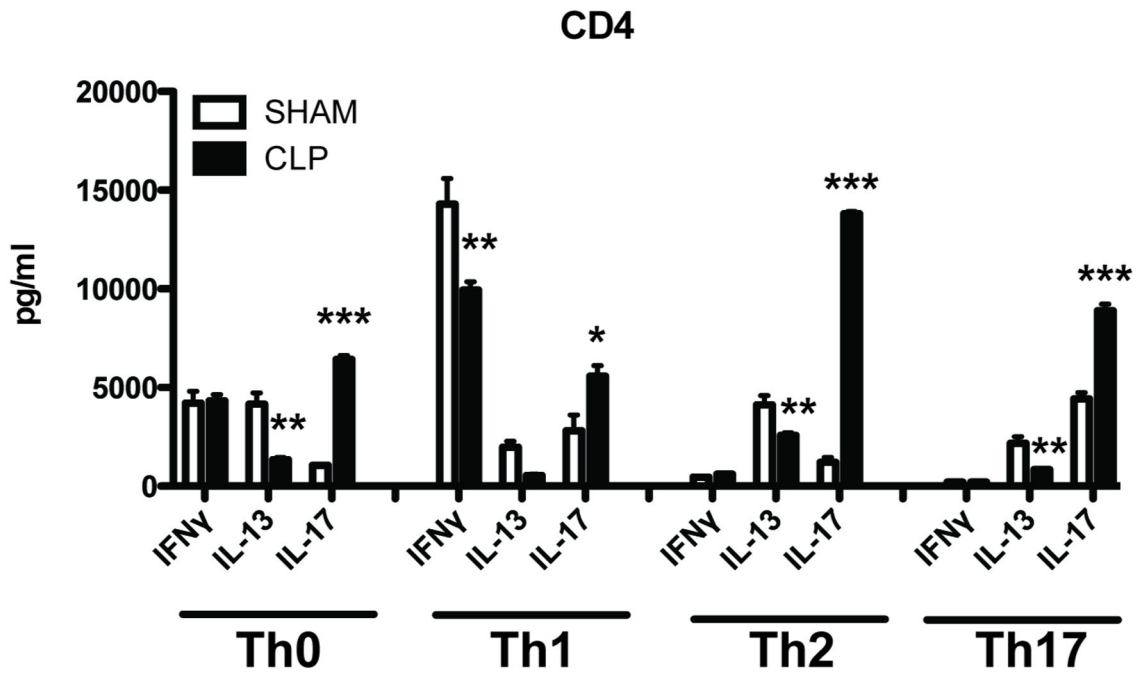
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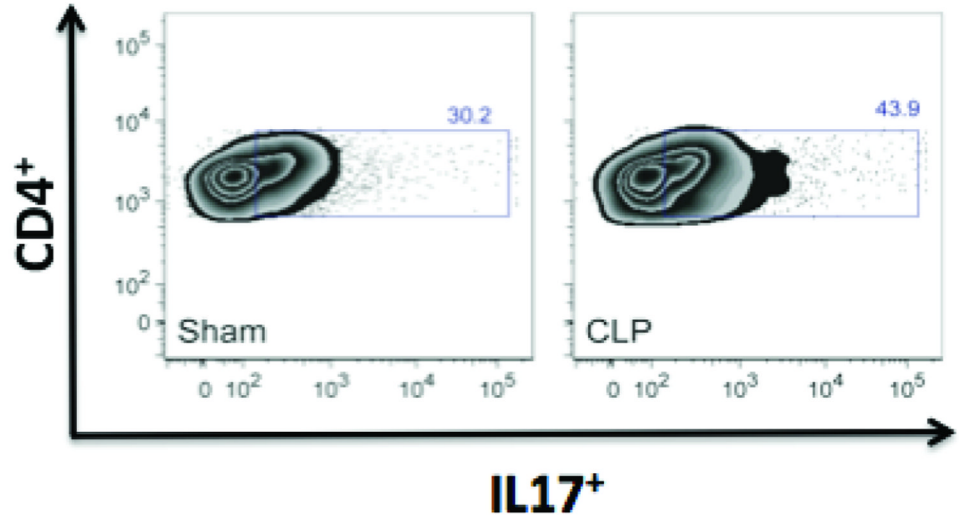
B

Fig. 4. Skewed CD4 T cells from post septic mice produce increased levels of IL-17
CD4 T cells were MACS enriched from the spleens of sham treated or post septic (CLP) mice on day 14 and assayed for cytokine production *in vitro.* (A) Cells were re-stimulated with anti CD3 and anti CD28 in the presence of golgi-plug overnight *in vitro* and stained intracellularly for IL-17 levels and analyzed by flow cytometry. (B) Cells were seeded into 96 well plates at a density of 1×10^6 cells per well. The cells were either un-skewed (Th0) or skewed towards Th1, Th2 and Th17 for 48 hours. The supernatants were then collected and analyzed for Th1, Th2 and Th17 cytokines by bead-based multiple cytokine analysis (Luminex).
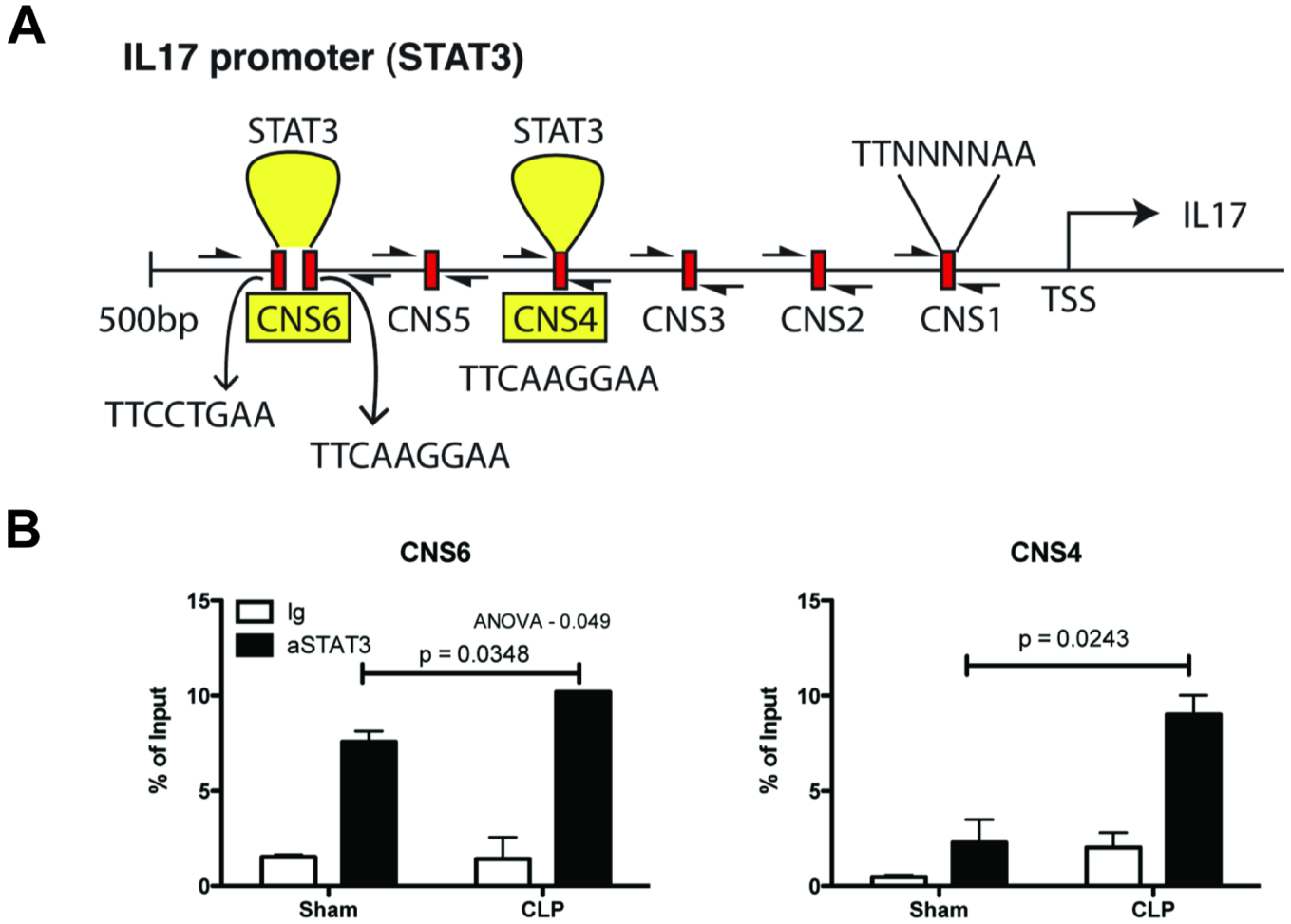
A

B

Fig. 5. CD4+ T cells from post septic mice exhibit increased STAT3 binding to the IL-17 promoter

A) Putative STAT3 binding sequences on Il17 promoter (CNS 1–6) with 0.5kb upstream of the transcription start site was analyzed by ChIP assay. (B) Chromatin was immunoprecipitated-using control Ig or STAT3 antibody and DNA was quantified using CNS 1–6 specific primers. The data is expressed as mean ± S.E. * p = 0.05, ** p <0.04, *** p <0.001. All experiments were repeated twice.
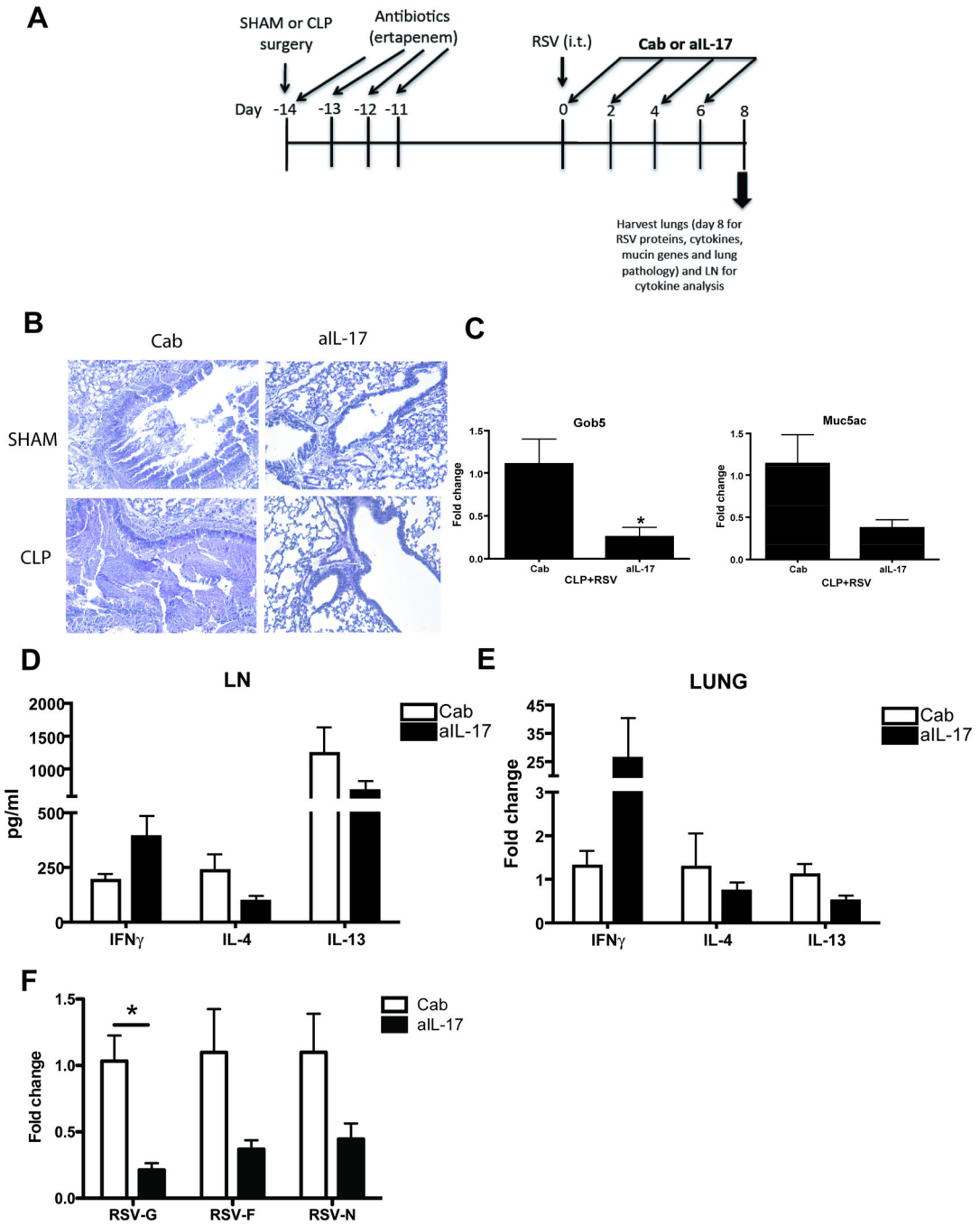
Fig. 6. Neutralizing IL-17 in post septic mice reduces viral load and reverses RSV induced pulmonary pathology

A) Schematic of treatment with neutralizing antibody to IL-17 in RSV infected post septic mice. B) Sham or post septic (CLP) mice were treated with control antibody (Cab) or neutralizing antibody to IL-17, 2.5 hours prior to RSV infection on day 0. The mice were treated with 3 more doses of Cab or anti-IL-17 on days 2, 4 and 6 before they were euthanized and their lungs were harvested for PAS staining for mucus, and C) RNA analysis of mucus associated genes – Gob5 and Muc5ac. D) The LN from CLP mice infected with RSV and treated with Cab or anti-IL-17 were further re-stimulated with RSV for 48 hours. The supernatants were collected and cytokine analysis was performed using the bioplex-200

system. Lungs from CLP mice infected with RSV and treated with Cab or anti-IL-17 were analyzed for RNA expression of E) cytokines IFNγ, IL-4 and IL-13 and F) RSV-G, RSV-F and RSV-N proteins. Data is representative of two independent experiments, n=3–7 mice/group. The data is expressed as mean ± S.E. and * $p < 0.05$ was considered significant.

Table 1

Lymphocyte distribution in the lungs of Sham and CLP mice following secondary RSV infection

Days Following RSV infection	Cell type and Number (Mean ± SEM)	Cell type and Number (Mean ± SEM)	Cell type and Number (Mean ± SEM)	Cell type and Number (Mean ± SEM)
	CD4+ T cells	CD8+ T cells	ϒ/δ T cells	Pan NK cells
	× 10^5	× 10^5	× 10^4	× 10^4
Day 2				
Sham No RSV	2.18 ± 1.3	8.84 ± 5.1	1.81 ± 1.0	6.00 ± 3.5
Sham RSV	3.01 ± 1.7	12.90 ± 7.4	1.83 ± 1.0	5.07 ± 2.92
CLP No RSV	2.86 ± 1.6	12.68 ± 7.3	2.00 ± 1.2	3.54 ± 2.0
CLP RSV	2.61 ± 1.1	12.57 ± 5.6	2.49 ± 1.1	4.59 ± 2.1
Day 4				
Sham No RSV	8.19 ± 4.7	3.47 ± 2.0	0.16 ± 0.09	14.51 ± 8.4
Sham RSV	4.99 ± 2.5	2.56 ± 1.3	0.26 ± 0.13	16.41 ± 8.2
CLP No RSV	11.99 ± 6.9	5.33 ± 3.1	0.12 ± 0.07	19.25 ± 11.1
CLP RSV	6.96 ± 3.11	3.88 ± 1.7	0.24 ± 0.11	15.98 ± 7.1