Staphylococcal enterotoxin B (SEB) - induced microRNA-155 targets suppressor of cytokine signaling-1 (SOCS1) to promote acute inflammatory lung injury.

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Running Title: SEB-induced miR-155 promotes inflammatory ALI
Staphylococcal enterotoxin B (SEB) causes food poisoning in humans. It is considered a biological weapon, and inhalation can trigger lung injury and sometimes respiratory failure. Being a superantigen, SEB initiates an exaggerated inflammatory response. While the role of microRNAs (miR) in immune cell activation is getting increasing recognition, their role in the regulation of inflammatory disease induced by SEB has not been studied. In this investigation, we demonstrate that exposure to SEB by inhalation results in acute inflammatory lung injury accompanied by altered miR expression profile in lung infiltrating cells. Amongst the miRs that were significantly elevated, miR-155 was the most over-expressed. Interestingly, miR-155−/− mice were protected from SEB-mediated inflammation and lung injury. Further studies revealed a functional link between SEB-induced miR-155 and pro-inflammatory cytokine IFN-γ. Through the use of bioinformatics tools, suppressor of cytokine signaling -1 (SOCS1), a negative regulator of IFN-γ, was identified as a potential target of miR-155. While miR-155−/− mice displayed increased Socs1, the overexpression of miR-155 led to its suppression, thereby enhancing IFN-γ levels. Additionally, the inhibition of miR-155 resulted in restored Socs1 expression. Together, our data demonstrate an important role for miR-155 in promoting SEB-mediated inflammation in the lungs through Socs1 activation, and suggest that miR-155 may be an important target in preventing SEB-mediated inflammation and tissue injury.
INTRODUCTION

Staphylococcal Enterotoxin B (SEB), a superantigen produced by *Staphylococcus aureus* has deleterious effects in humans such as food poisoning (1) and toxic shock (2). Because it can be easily aerosolized, SEB is classified as a Category B agent by the Centers for Disease Control and Prevention (3). Upon inhalation exposure, SEB can trigger acute inflammatory lung injury characterized by immune cell infiltration, excessive cytokine production, tissue damage and pulmonary edema (4, 5).

Due to the distinct manner in which SEB binds to the non-polymorphic regions of MHC II on antigen presenting cells and the specific Vβ regions of the T-cell receptor (TCR) such as murine Vβ8 (6), SEB exposure leads to the activation and proliferation of a large population (5-30%) of T-lymphocytes (7). Activation of such a substantial number of T-lymphocytes results in the robust production of inflammatory cytokines such as IL-2, TNF-α and IFN-γ (8, 9). In most cases, IFN-γ is the main culprit in mediating the damaging and often lethal effects seen upon SEB exposure. For example, transgenic mice deficient in IFN-γ, were protected from SEB-mediated Toxic Shock Syndrome (TSS) and subsequent mortality(10). Additionally, the neutralization of IFN-γ, after SEB exposure was shown to prevent lethal systemic inflammation (11) further suggesting the importance of SEB-mediated IFN-γ production. While the interaction between SEB and TCR, along with the subsequent T-cell proliferation and cytokine secretion have been extensively studied (12-14), the role of miRNA in mediating SEB-induced inflammation has not yet been elucidated.

MicroRNA (miR) are ~21-23 nt long, single stranded non-coding RNA molecules that can translationally repress or target mRNA for degradation, thereby acting as primary modulators of gene expression (15). Several studies have demonstrated a role for miR in modulating immune responses under various inflammatory conditions (16). For example, while miR-125b is highly expressed in naïve
CD4+ T-cells, it becomes significantly downregulated upon T-cell activation (17). Similarly, studies have demonstrated that overexpression of miR-17-92 cluster in T-cells leads to lymphoproliferative disorder due to the repression of the pro-apoptotic molecule, BIM (18). Furthermore, mice deficient in miR-155 are resistant to developing experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (19), while the overexpression of miR-155 exacerbates the symptoms associated with the disease. Taken together, these studies strongly suggest that miRs play a major role in modulating immune cell activation, particularly T-cells, as well as promoting pro-inflammatory responses.

In the current study of SEB-induced acute inflammatory lung injury, we applied microarray analysis and quantitative real time PCR (q-RT PCR) to establish important miRs that are dysregulated in response to SEB. Further, our data identified miR-155 as a major contributor to SEB-mediated lung inflammation. While it is known that SEB exposure leads to inflammation and the production of copious amounts of IFN-γ, we provide mechanistic insight through gain and loss of function experiments, into the role of miR-155 in this process. Our results may present an opportunity to further therapeutically target miR-155 in the treatment of SEB-mediated acute inflammatory lung injury.

**MATERIALS AND METHODS**

**Mice**

Female C57BL/6 mice (6-8 weeks) were purchased from the National Cancer Institute (NCI). miR-155−/− (B6.Cg-Mir155tm1.1Rsky/J) were purchased from The Jackson laboratory. All mice were housed under pathogen free conditions at the Animal Resource Facility (ARF), University of South Carolina (USC) School of Medicine. The use of vertebrate animals in the experiments performed was pre-approved by the Institutional Animal Care and Use Committee (IACUC) at USC. This study was carried out in strict...
accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (20).

**Induction of SEB-induced acute lung injury (ALI)**

SEB was obtained from Toxin Technologies (Sarasota, Florida). SEB dissolved in sterile PBS (2 mg/mL) was administered by the intranasal (i.n) route in a volume of 25 μL for a dose of 50 μg per mouse, as described (4, 21, 22). Mice were euthanized 48 hours after SEB exposure.

**Lung histopathological analysis**

At the time of euthanasia, lungs were obtained and fixed in 10% formalin. The tissue was then paraffin embedded and serial sections (5 μm) were made. The sections were subsequently deparaffinized by dissolving with xylene, followed by rehydration in several changes of alcohol (100%, 95%, and 90%). The slides were then stained with hematoxylin and eosin (H&E) and evaluated with a Nikon E600 light microscopy system.

**Antibodies**

Fluorescein isothiocyanate-conjugated anti-CD8 (clone: 53.6.7) and phycoerythrin-conjugated anti-CD4 (clone: GK1.5) Abs were purchased from Biolegend (San Diego, CA).

**Preparation of lung-infiltrating cells and flow cytometry**

Mice were exposed to SEB as described above. Forty eight hours after SEB exposure, lungs were harvested and homogenized using Stomacher® 80 Biomaster blender from Seward (Davie, FL) in 10 ml of sterile PBS. After washing with sterile PBS, the cells were carefully layered on Ficoll - Histopaque®-1077 from Sigma-Aldrich (St Louis, MO) and separated by density gradient centrifugation at 500 x g for 30 minutes at 24°C with brake off. The mononuclear cell layer isolated was then enumerated using...
the Trypan blue exclusion method. To determine the subsets of immune cells infiltrating the lung, cells were stained with fluorescent conjugated antibodies (anti-CD4, anti-CD8) and analyzed using the Beckman Coulter 500 Flow cytometer (Indianapolis, IN).

**Recovery of bronchoalveolar lavage fluid (BALF) and cytokine detection**

Forty eight hours after SEB exposure, mice were euthanized and tracheae from vehicle or THC-treated mice were tied with a suture and the lung was excised as an intact unit. With 1 ml sterile ice-cold PBS, the trachea was lavaged to collect the BALF fluid. Cytokine analysis for interferon-γ (IFN-γ) was carried out using BALF. All cytokines were measured using Biolegend (San Diego, CA) ELISA MAX™ Standard kits.

**Total RNA isolation**

Total RNA (including small RNAs) was isolated from lung-infiltrating mononuclear cells or in vitro from lymph nodes or splenocytes using miRNeasy kit from Qiagen (Valencia, CA) following manufacturer’s instructions. The purity and concentration of the RNA was confirmed spectrophotometrically, while the integrity of miRNA was further assessed using Agilent 2100 BioAnalyzer (Agilent Tech, Palo Alto, CA).

**miRNA expression profiling and analysis**

To profile miRNA expression in the lung, the Affymetrix GeneChip® miRNA 1.0 array platform was used. The array which comprises of 609 mouse miRNA probes makes use of FlashTag™ Biotin HSR hybridization technique and was carried out according to manufacturer’s instructions (Affymetrix, Santa Clara, CA). Fluorescent intensities obtained from hybridization were log-transformed and visualized in the form of a heatmap. Hierarchical clustering was carried out using Ward’s method and Similarity
measurement was calculated using half square Euclidean distance. miRNA expression fold change obtained from the microarray were then further analyzed using the commercially available analysis tool Ingenuity Systems®-Ingenuity Pathway analysis –(IPA), (Mountain View, CA, USA.) In brief, the dataset of 609 miRNA were uploaded into IPA and only miRNA that were 3 fold or higher were considered for analysis. Core analysis was carried out and a ‘Top Network’ of miRNA and its associated molecules was generated. All microRNA microarray data were deposited in ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2379.

Analysis of miRNA target genes

IPA was also used to determine and collate the highly predicted, moderately predicted and experimentally observed mRNA target genes of those miRNA that were highly (≥3 fold) upregulated using IPA’s miRNA target filter tool. These targets were further sorted based on their role in cytokine signaling, cellular growth and proliferation and cellular immune response. Additionally, to assign Immunological functions to our list of miRNA targets, Gene Ontology (GO) mapping of miRNA target genes was carried out using Cytoscape 3.0.1 equipped with ClueGO and CluePedia applications.

Quantitative real-time PCR (qRT-PCR)

Total RNA (miRNA and mRNA) were converted to cDNA using the miScript cDNA synthesis kit (Qiagen) according to manufacturer’s instructions. For miRNA validation, the miScript SYBR Green PCR kit (Qiagen) was used and fold change of miRNA was determined using $2^{-\Delta\Delta Ct}$ method. Snord 96a was used as small RNA endogenous control. For mRNA validation, SSO advanced™ SYBR Green PCR kit from Biorad (Hercules, CA) was used according to manufacturer’s instructions and β-actin was used as the endogenous control. The following primers were used: β-actin (F) 5'-

GGCTGTATTCCCCTCAT G-3' and (R) 5'-CCAGTT GGTAACAATGCCATGT-3'; SOCS-1 (F) 5'-
GGTTGTAGCAGCTTGTGTC-3' and (R) 5'-AATGAAGCCAGAGACCCTC-3'; IFN-γ (F) 5'-
GCGTCATTGAATCACACCTG-3' and (R) 5'-GAGCTCATTGAATGCTTGGC-3'

**Transfection with miR-155 mimic and inhibitors**

Lymph nodes (axillary and inguinal) from naïve C57Bl/6 mice were harvested and cultured in 10 ml of complete media at 37°C and 5% CO₂. Complete media comprised of RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS, 10mM L-glutamine, 10mM Hepes, 50 μM β-Mercaptoethanol, and 100 μg/mL penicillin. Cells were seeded at 2x10⁵ cells in 24 well plates and transfected with either 40 nM synthetic mmu-miR-155-3p miScript miRNA mimic (CUCCUACCUGUUAGCAUAAC) or AllStar negative control siRNA. For inhibition of miR-155, cells were activated with SEB (1 μg/ml) and treated with 100 nM Anti-mmu-miR-155-3p miScript miRNA inhibitor (CUCCUACCUGUUAGCAUAAC) or miScript Inhibitor negative control for 24 hours using HiperFect transfection reagent (Qiagen) according to manufacturer’s instructions.

**Luciferase assay**

The following plasmids were purchased from GeneCopoeia (Rockville, MD) – 3'UTR- Socs1 (MmiT028883) and control plasmid (CmiT000001-MT01). Chinese Hamster Ovary (CHO) cells were co-transfected with 100 ng of plasmid and 100nM of miRIDIAN microRNA mmu-miR-155-5p mimic or miRIDIAN microRNA mimic negative control using DharmaFECT DUO transfection reagent following manufacturer’s instructions (Thermo Scientific, Pittsburgh, PA). 24 hr following transfection, Luciferase activity was measured using LucPair™ miR-Duo Luciferase Assay kit from GeneCopoeia.
Statistics

All statistical analyses were carried out using GraphPad Prism Software (San Diego, CA). In all experiments, the number of mice used was 4-5 per group, unless otherwise specified. Results are expressed as means ± SEM. Student’s t-test was used to compare WT and miR-155⁻/⁻ data, whereas multiple comparisons were made using one-way analysis of variance (ANOVA), followed by post hoc analysis using Tukey’s method. A p-value of <0.05 was considered statistically significant. Individual experiments were performed in triplicate and each experiment was performed independently at least three times to test reproducibility of results.
RESULTS

SEB exposure triggers inflammation in the lung.

Previously, a single dose of SEB (50 μg) by intranasal delivery was found to induce cellular infiltration, increase cytokine production, cause histopathological lesions and edema in C57BL/6 mice (4, 21, 22) mimicking the symptoms of acute inflammatory lung injury in humans (23). In this study, we first sought to investigate the inflammatory effect of SEB-exposure in the lungs. Forty eight hours after SEB exposure, H&E stained sections of the lungs from SEB-exposed mice showed massive infiltration of cells and signs of edema as evidenced by fluid filled bronchioles (Figure 1A). Because SEB is a potent activator of T-cells, we examined the effect of SEB on T-cell subsets within the lung. Immediately after euthanasia, the lungs were harvested and mononuclear cells were isolated from the lungs by density gradient centrifugation to determine the phenotypic characteristics of the cells. SEB exposure not only led to an overall increase in mononuclear cells but specifically, an increase in CD4+ and CD8+ T cells (Figure 1B) was seen. Because SEB exposure triggers an increase in IFN-γ, a major pro-inflammatory cytokine previously reported to orchestrate the inflammatory cascade and cause tissue damage(10, 24, 25), we analyzed the concentration of IFN-γ in the bronchoalveolar lavage fluid (BALF) and found a high concentration (upto 3000 pg/ml) of IFN-γ in the lungs of SEB exposed mice (Figure 1C). These data suggested that SEB administration via the intranasal route triggers acute inflammation in the lungs.

SEB exposure modulates miRNA expression in the lungs.

The dysregulation of specific miRs in response to SEB exposure has not been elucidated. Because miRs play a critical role in mediating inflammation, we examined the miR profile after SEB exposure. Total miR was isolated from lung-infiltrating mononuclear cells and the relative abundance of miR in SEB exposed and vehicle treated mice was determined using microarray miRNA analysis. A
heatmap was generated based on hierarchical clustering of miRNA highlighting a stark difference between vehicle- and SEB-exposed mice (Figure 2A). Further examination of miR expression revealed that of the 609 miR assessed, most remained unchanged, but a few showed significant up- or downregulation as seen in the fold change distribution plot (Figure 2B). Ingenuity Pathway Analysis (IPA) generated a ‘Top Network’ of miR that comprised of five upregulated miRs, including miR-155, miR-31, miR-182, miR-20b, and miR-222 and their associated molecules (Figure 2C). This network was characterized by IPA as responses involving inflammation, cellular development, cellular growth and proliferation. To assign significant Immunological functions to the genes in the aforementioned ‘Top Network’, Cytoscape (ClueGO+CluePedia application) was employed. Gene Ontology (GO) mapping revealed that the genes associated with the miR in the ‘Top Network’ were functionally relevant to T-cell activation (GO: 0042110) and proliferation (GO: 0042098), Interferon-γ signaling (GO: 0060333) and Toll-like receptor signaling (GO: 0002224) (Figure 2D). Next, we validated the expression levels of these miRs in lung infiltrating mononuclear cells by q-RT PCR, which corroborated the expression patterns seen using the microarray (Figure 2E). Amongst the miRs we validated, miR-155 was the most highly expressed (~ 8 fold) in the lungs upon SEB exposure. Based on this, the role of miR-155 in the development of SEB-induced ALI was further investigated.

miR-155 is important for SEB-mediated inflammation

Because miR-155 was highly upregulated in response to SEB, we hypothesized that it might play a crucial role in facilitating the inflammation observed during the disease. To that end, WT and miR-155 Δ/Δ mice were exposed to SEB to determine the effects on disease parameters. H&E stained sections of the lung revealed that WT mice exposed to SEB exhibited numerous layers of infiltration interspersed with edema. Interestingly, miR-155 Δ/Δ mice exposed to SEB presented with almost normal lung architecture (Figure 3A). Additionally, the miR-155 deficient mice expressed significantly decreased
total numbers of mononuclear cells within the lung upon SEB exposure when compared to their WT counterparts. Upon closer examination of the mononuclear cell phenotype within the lungs, absolute numbers of CD4+ and CD8+ in the miR-155−/− mice were decreased compared to WT mice (Figure 3B). Additionally, cytokine analysis of the bronchoalveolar lavage fluid (BALF) in the lungs of WT mice demonstrated high concentrations of pro-inflammatory cytokine IFN-γ. In contrast, IFN-γ levels were significantly diminished in miR-155−/− mice (Figure 3C). Taken together, these results provided clear evidence that miR-155−/− mice were protected from SEB mediated ALI, suggesting that miR-155 plays a critical role in SEB-induced inflammation.

miR-155 expression is critically linked to IFN-γ production

Because SEB exposure leads to the release of copious amounts of IFN-γ and also results in increased miR-155 expression, we considered if there was a positive correlation between IFN-γ secretion and the expression of miR-155. To explore this possibility, we first assessed the expression of IFN-γ after transfection of LN T-cells with a synthetic miR-155 mimic. Interestingly, we found a substantial increase in IFN-γ levels not only in WT mice (Figure 4A) but also in miR-155 deficient mice that were transfected with the mimic (Figure 4B). Additionally, inhibition of miR-155 with a synthetic inhibitor conversely resulted in the diminished expression of IFN-γ (Figure 4C), suggesting a crucial link between miR-155 expression and that of IFN-γ after SEB exposure.

miR-155 targets Socs1, a negative regulator of IFN-γ

miRs regulate the expression of genes by binding the 3’UTR of their respective target mRNA. To examine the link between miR-155 and its potential target genes, we undertook a bioinformatics-based approach. First, IPA miRNA target filter tools were used, selecting only those miR-155 target genes that were relevant to cytokine signaling, cellular immune response, and cellular growth and...
proliferation. Thirty six targets common to the filtering criteria applied were selected (Figure 5A). Next, an IPA generated network was used to sort the targets based on those that were highly predicted and experimentally observed (Figure 5B). Conclusively, a gene known as suppressor of cytokine signaling-1 (Socs1) was found to be a prominent miR-155 target due to miR-155’s ability to bind to the 3’ UTR of the Socs1 mRNA (Figure 5C). Because Socs1 is induced by IFN-γ and acts a negative regulator of IFN-γ, the relationship between miR-155 and Socs1 in the context of IFN-γ production was examined. miR-155−/− mice that were exposed to SEB had significantly increased expression of Socs1 mRNA in lung infiltrating mononuclear cells when compared to WT (Figure 5D). Accordingly, we hypothesized that miR-155 may target Socs1 to promote IFN-γ-mediated inflammation during SEB-induced inflammatory ALI. To confirm Socs1 as a miR-155 target, we first measured relative luciferase activity after co-transfection of miR-155 mimic and plasmid containing the 3’ UTR of Socs1. Compared to mimic control, miR-155 mimic led to a significant decrease in luciferase activity (Figure 6A) validating Socs1 as a target for miR-155. Next, the impact of miR-155 mimic on Socs1 levels was explored. We found that both, in WT (Figure 6B) and miR-155 deficient cells (Figure 6C) that were transfected with miR-155 mimic, Socs1 levels remained suppressed. On the other hand, whereas SEB activation continued to lead to a repression in Socs1, miR-155 inhibition of SEB-activated cells, resulted in its derepression (Figure 6D), confirming the role of miR-155 in suppressing Socs1 during SEB-mediated activation of immune cells.
With the discovery of miR, a novel and exciting mechanism of gene regulation has arisen. miRs are small non-coding endogenous RNA molecules that bind 3’ UTR of genes carrying complimentary sites. A single miR usually targets several mRNA, acting as a fine-tuner of gene regulation rather than an on-off system (26). In the context of inflammation, miRs have been found within a variety of immune cells often targeting genes involved in the regulation of inflammatory response (27). In the current study, we closely examined the miR profile after inhalation exposure to SEB. We observed that amongst the miRs that were dysregulated in response to SEB, miR-155 was one of the most significantly altered. It has been reported that naïve CD4+T cells initially display low levels of miR-155, which is increased after the engagement of TCR by an antigen (28, 29). This is consistent with our observation that miR-155 was upregulated following SEB activation.

Recent studies have reported that miR-155−/− mice are resistant to EAE, demonstrating its importance in mediating disease development (30). In other studies, miR-155−/− mice failed to control *H. pylori* infection due to defective Th1 signaling (31). Moreover, in a mouse model of collagen-induced arthritis (CIA), the deficiency of miR-155 led to a decrease in pathogenic T-cells. In humans, it has also been reported that soldiers undergoing a battle-field like stress program demonstrated an increase in hsa-miR-155 levels in leucocytes exposed to SEB *ex vivo* (32) indicating that stress-related inflammation could also potentially lead to the increase in miR-155. The current study further suggests that the acute inflammatory response in the lungs to a bacterial superantigen is also regulated by miR-155, inasmuch as, SEB-exposed miR-155−/− mice having fewer infiltrating T-cells and almost normal lung histopathology.
Usually an appropriate regulation of IFN-γ is necessary for mediating Th1 responses and blunting infection (33). SEB exposure, however, causes an excessive release of IFN-γ. T-cells exposed to IFN-γ, proliferate further, thus perpetuating a cycle of inflammation (34, 35). Studies carried out with SEB activation of splenocytes have demonstrated that early cytokines released include IL-2 and TNF-α, followed by the massive production of IFN-γ by T-helper cells (36). Additionally, in vitro SEB activation of rat splenocytes results in the release of IFN-γ for up to 48 hours post-activation and promotes the proliferation of CD4+ T-cells, similar to that seen in mice and humans (37). We have noted in the current model (unpublished) that the peak of acute inflammatory lung injury occurs at 48 hours after SEB exposure. In line with the typical kinetics of cytokine release seen with SEB activation, we did not detect early cytokines, TNF-α and IL-2, in the BALF at this time point (data not shown). However, our data indicated substantial release of SEB-induced IFN-γ in WT mice suggesting that this particular cytokine may significantly contribute to SEB-induced inflammation. Moreover, the correlation between decreased IFN-γ in the BALF of miR-155−/− mice exposed to SEB and lack of significant inflammation in the lungs is suggestive of a major role for IFN-γ in our model.

The relationship between miR-155 and IFN-γ has been briefly explored in previous studies. For example, when miR-155 is overexpressed in human NK cells, the subsequent downregulation of a target, SHIP-1, promotes IFN-γ expression (38). During collagen-induced arthritis, miR-155−/− mice display significantly lower number of IFN-γ producing cells than WT (39). Likewise, our data demonstrated that while transfection with a synthetic miR-155 mimic, leads to the induction of IFN-γ, the blockade of miR-155, diminishes IFN-γ production. Our results clearly indicate that the SEB-mediated induction of IFN-γ can be explained, at least in part, by the induction of miR-155.

To uncover the relationship between miR-155 and IFN-γ in response to SEB exposure, we employed bioinformatics tools and conducted extensive literature search. These efforts suggested...
suppressor of cytokine signaling 1 (SOCS1) as a possible link between miR-155 and IFN-γ. SOCS1 belongs to a family of eight proteins (SOCS1–SOCS7) that regulate the production of several cytokines (40). In particular, SOCS1 is induced by IFN-γ for auto regulation of the IFN-γ pro-inflammatory response by inhibiting the JAK/STAT1 signaling pathway (41). Recent experiments in numerous cell types have revealed that miR-155 targets SOCS1 (42-44). For example, macrophages infected with an RNA virus demonstrated enhanced Type I interferon production due to miR-155 targeting of Socs1 (45). In the current study we noted that the miR-155−/− mice that are exposed to SEB, showed increased expression in Socs1 mRNA levels compared to SEB exposed WT mice. The expression of Socs1 correlated inversely with IFN-γ production in the BALF. In addition, our gain and loss of function studies with miR-155 mimic and inhibitor, clearly demonstrated that miR-155-mediated targeting of Socs1 regulates IFN-γ production.

The results of the present study highlight the role of miR-155 in SEB induced acute inflammatory lung injury. Specifically, we demonstrate that the high levels of IFN-γ production associated with SEB exposure can be attributed to the miR-155 mediated repression of Socs1, a critical regulator of IFN-γ (Figure 7). Furthermore, the importance of miR-155 is made particularly evident as miR-155 deficient mice were found to be protected from SEB-mediated inflammation and acute lung injury, thereby suggesting that therapeutic targeting of miR-155 may be useful in the treatment of SEB-triggered acute inflammatory lung injury.

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Figure 1. SEB induces lung inflammation  
(A) Representative H&E images (20x) of cross sections of the lung from mice exposed to either Vehicle or SEB.  
(B) Lung infiltrating mononuclear cells obtained by density gradient centrifugation and total number of viable cells were counted using a hemocytometer. Cells were further stained with mAb to identify CD4+ and CD8+ cells and analyzed on a flow cytometer. The percentage of the immune cell subsets was multiplied by the total number of cells found in the lung and divided by 100 to yield the absolute cell numbers shown.  
(C) The concentration of IFN-γ protein present in the BALF was determined using a standard ELISA kit. Data are mean ± SEM (n=5) and are representative of three independent experiments. Statistical significance as compared to SEB + Vehicle is indicated as *p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001.
Figure 2. SEB exposure leads to dysregulation of miRNA. Forty eight hours after vehicle or SEB administration, miRNA was isolated from lung infiltrating mononuclear cells. (A) Heatmap depicting differential expression of miRNA in the lungs of mice exposed to SEB + vehicle as compared to vehicle. (B) Fold change distribution of the 609 miRNA indicating several upregulated and downregulated miRNA. (C) Ingenuity Pathway analysis generated ‘Top network’ with network function denoted as ‘inflammatory response, cellular development, cellular growth and proliferation’. (D) Cytoscape generated Gene Ontology (GO) network based on Immunological processes for the molecules associated in ‘Top Network’ using ClueGo 2.0.7 application. Analysis criteria consisted of two-sided hypergeometric test with Benjamin Hochberg correction. Only results with kappa score = 0.3 are displayed. (E) qRT PCR validation of the IPA generated ‘Top upregulated miRNA’. Total RNA was isolated from lung infiltrating mononuclear cells. Snord96a was used as the small RNA endogenous control and the expression level of SEB-induced miRNA shown here was normalized to vehicle. Data is represented as mean ± SEM from replicate samples (*p<0.05, **p <0.01 as compared to vehicle).
Figure 3. miR-155 plays a critical role in SEB-induced ALI. WT (C57BL/6) and miR-155-/- (B6.Cg-Mir155 tm1.1 Rsky/J) mice were exposed to SEB and euthanized 48 hours later. (A) Representative H&E images (20x) of sections of lung indicating immune cell infiltration. (B) Phenotypic characterization of cells infiltrating the lung was determined by staining of mononuclear cells with fluorescent conjugated mAb against CD4 and CD8. (C) Levels of IFN-γ cytokine in the BALF was determined by ELISA. Data are mean ± SEM (n=5) and are representative of two independent experiments. Statistical significance as compared to WT is indicated as *p<0.05, ** p<0.01.
Figure 4. IFN-γ forms a critical link between SEB and subsequent miR-155 induction. (A) Lymph node (LN) T cells obtained from naïve wildtype (WT) mice were transfected either with miR-155 mimic (Mimic) or mimic control (Control) for 24 hours. IFN-γ levels were determined by RT-PCR. (B) LN T cells obtained from miR-155-/- were also transfected with 10nM miR-155 mimic or mimic control as indicated for 24 hours and IFN-γ levels were assessed. (C) LN cells were activated with SEB (1μg/ml) for 24 hours. Cells were then transfected with 50nM miR-155 inhibitor (Inh) or Inhibitor Control (Inh Control) for another 24 hours. IFN-γ levels were determined via RT-PCR. Data is represented as mean ± SEM from replicate samples. Statistical significance is indicated as *p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001.
Figure 5. Identification of SEB-induced miR-155 targets. (A) miR-155 targets were filtered based on their role in cytokine signaling, cell growth and proliferation and cellular immune response using IPA. A proportional Venn diagram indicating the miR-155 targets common to all three filtering criteria was generated. The list of targets is indicated within brackets and *Socs1*, a highly predicted target is highlighted (red). (B) IPA network was generated highlighting the highly predicted (yellow) and experimentally observed (brown) miR-155 targets, in addition to *Socs1* (red), the target of interest. (C) Schematic illustration of the predicted target site for miR-155 within the 3’ UTR of *Socs1* mRNA. (D) Total mRNA was isolated from lung-infiltrating mononuclear cells of WT and miR-155 /-/- mice exposed to SEB. Relative expression of *Socs1* mRNA was determined by qRT PCR using β-actin as endogenous control. Data is represented as mean ± SEM from replicate samples. Statistical significance is indicated as *p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001.
Figure 6. miR-155 targets Socs1. (A) Chinese Hamster Ovary (CHO) cells were co-transfected with miR-155 mimic or mimic control along with plasmid containing either the 3’UTR of Socs1 or control plasmid for 24 hours. Relative luciferase activity (firefly normalized to renilla) was determined following transfection. (B) Lymph nodes (LN) cells obtained from naïve wildtype (WT) mice were transfected either with 40nM miR-155 mimic (Mimic) or mimic control (Control) for 24 hours. Socs1 levels were determined by RT-PCR. (C) LN cells obtained from miR-155-/- were also transfected with 40nM miR-155 mimic or mimic control as indicated for 24 hours and Socs1 levels were assessed. (D) LN cells were activated with SEB (1μg/ml) for 24 hours. Cells were then transfected with 100nM miR-155 inhibitor (Inh) or Inhibitor Control (Inh Control) for another 24 hours. Socs1 levels were determined via qRT-PCR. Data is represented as mean ± SEM from replicate samples statistical significance is indicated as *p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001.
Figure 7. Schematic of SEB-mediated downregulation of Socs1 via miR-155. SEB exposure leads to the release of IFN-γ and subsequent expression of miR-155. miR-155 mediated suppression of Socs1 prevents appropriate control of IFN-γ leading to cell proliferation and sustained cytokine signaling and damage to the lung.
AUTHOR CORRECTION

Correction for Rao et al., Staphylococcal Enterotoxin B-Induced MicroRNA-155 Targets SOCS1 To Promote Acute Inflammatory Lung Injury

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