STAT3-dependent CXC chemokine formation and neutrophil migration in streptococcal M1 protein-induced acute lung inflammation

Songen Zhang,1 Rundk Hwaiz,1 Lingtao Luo,1 Heiko Herwald,2 and Henrik Thorlacius1

1Department of Clinical Sciences, Section for Surgery, Lund University, Malmö, Sweden and 2Division of Infection Medicine, Lund University, Lund, Sweden

Submitted 3 November 2014; accepted in final form 1 April 2015

Zhang S, Hwaiz R, Luo L, Herwald H, Thorlacius H. STAT3-dependent CXC chemokine formation and neutrophil migration in streptococcal M1 protein-induced acute lung inflammation. Am J Physiol Lung Cell Mol Physiol 308: L1159–L1167, 2015. First published April 3, 2015; doi:10.1152/ajplung.00324.2014.—Streptococcus pyogenes cause infections ranging from mild pharyngitis to severe streptococcal toxic shock syndrome (STSS). The M1 serotype of Streptococcus pyogenes is most frequently associated with STSS. Herein, it was hypothesized that STAT3 signaling might be involved in M1 protein-evoked lung inflammation. The STAT3 inhibitor, S3I-201, was administered to male C57Bl/6 mice before iv challenge with M1 protein. Bronchoalveolar fluid and lung tissue were harvested for quantification of STAT3 activity, neutrophil recruitment, edema, and CXC chemokine formation. Neutrophil expression of Mac-1 was quantified by use of flow cytometry. Levels of IL-6 and HMGB1 were determined in plasma. CXCL2-induced neutrophil chemotaxis was studied in vitro. Administration of S3I-201 markedly reduced M1 protein-provoked STAT3 activity, neutrophil recruitment, edema formation, and inflammatory changes in the lung. In addition, M1 protein significantly increased Mac-1 expression on neutrophils and CXC chemokine levels in the lung. Treatment with S3I-201 had no effect on M1 protein-induced expression of Mac-1 on neutrophils. In contrast, inhibition of STAT3 activity greatly reduced M1 protein-induced formation of CXC chemokines in the lung. Interestingly, STAT3 inhibition markedly decreased plasma levels of IL-6 and HMGB1 in animals exposed to M1 protein. Moreover, we found that S3I-201 abolished CXCL2-induced neutrophil migration in vitro. In conclusion, these novel findings indicate that STAT3 signaling plays a key role in mediating CXC chemokine production and neutrophil infiltration in M1 protein-induced acute lung inflammation.

Address for reprint requests and other correspondence: Henrik Thorlacius, Dept. of Surgery, Malmö Univ. Hospital, Lund Univ., 205 02 Malmö, Sweden (e-mail: henrik.thorlacius@med.lu.se).

SEVERE INFECTIONS WITH Streptococcus pyogenes can cause streptococcal toxic shock syndrome (STSS), which is associated with a high mortality rate (5, 33). One of the most insidious aspects of STSS is acute lung damage with impaired gaseous exchange (31, 32). Current management of patients suffering from STSS is largely limited to antibiotics and supportive care, which is related to an incomplete understanding of the underlying mechanisms in sepsis-induced lung injury. Streptococcus pyogenes contains numerous virulence factors, such as M proteins. More than 80 different M serotypes of Streptococcus pyogenes have been described in the literature (15, 26). In general, the M1 serotype is most commonly associated with STSS and mortality (26). Convincing data have shown that M1 protein is a potent activator of innate immune cells, including neutrophils and monocytes (31, 46). Neutrophils are part of the initial defense against invading microbes but overwhelming neutrophil responses are also known to be a rate-limiting step in septic lung injury (12, 20). Several studies have reported that M1 protein increase Mac-1 expression on neutrophils needed for neutrophil recruitment (45, 48, 50). CXC chemokines, including CXCL1 and CXCL2, orchestrate extravascular localization of neutrophils at sites of inflammation (51). Notably, one investigation showed that M1 protein-induced neutrophil accumulation in the lung is dependent on CXC chemokines (50). Thus the adhesive and chemokine-dependent mechanisms regulating pulmonary accumulation of neutrophils are relatively well known, but the signaling pathways controlling M1 protein-evoked neutrophil recruitment and lung inflammation remain elusive.

Signal transducer and activator of transcription 3 (STAT3) was first identified as an acute-phase response gene in the liver (40). Accumulating data have shown that STAT3 regulates formation and signaling of numerous cytokines and chemokines (9, 43). Several studies have shown that STAT3 activity is increased in the lung challenged with endotoxin (21). IgG immune complexes (36), and allergens (30) as well as in models of single bacterial infection (7) and abdominal sepsis (38). The functional role of STAT3 in lung inflammation has been difficult to study because genetic deletion of STAT3 causes early embryonic lethality (35). However, development of tissue or cell-specific deletion of the STAT3 gene and new specific STAT inhibitors, such as S3I-201 and Stattic, suggest that STAT3 might play an important role in pulmonary inflammation. For example, it has been demonstrated that STAT3 maintains surfactant homeostasis (16) and surfactant lipid synthesis (17) in acute lung injury. Moreover, by regulating formation of cytokines and chemokines, several studies have reported that STAT3 controls neutrophil recruitment into the inflamed lung (18, 27). On the other hand, STAT3 has also been shown to mediate anti-inflammatory responses induced by IL-10 (42). Therefore, it is difficult to predict the potential effect of STAT3 blockade on inflammatory reactions. Notably, the potential role of STAT3 signaling in regulating lung inflammation in response to M1 protein challenge has never been examined.

Based on the considerations above, the aim of the present investigation was to define the functional significance of STAT3 signaling in controlling cytokine and chemokine production, neutrophil Mac-1 expression, and recruitment as well as tissue damage in a model of acute lung injury based on intravenous (iv) administration of streptococcal M1 protein.

MATERIALS AND METHODS

Animals. Male C57Bl/6 mice (20–25 g) were kept under standard laboratory conditions, fed a laboratory diet and water ad libitum, and
maintained on a 12:12-h light-dark cycle. Animals were anesthetized with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram body weight. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the regional ethical committee for animal experimentation at Lund University, Sweden.

**Experimental model.** M1 protein was purified from the isogenic mutant MC25 strain derived from the API Streptococcus pyogenes strain 40/58 from the WHO Collaborating Centre for references and research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic as described previously (48). Thus M1 protein was purified from a mutated Streptococcus pyogenes strain, making the possibility of endotoxin contamination close to zero. Mice were injected with 15 µg iv of M1 protein in phosphate-buffered saline (PBS). Sham mice received iv PBS alone. The STAT3 inhibitor S3I-201 (7.5 mg/kg, Sigma-Aldrich, Stockholm, Sweden) or vehicle (0.05% DMSO) was given intraperitoneally (ip) 10 min before challenge with M1 protein. In separate experiments, S3I-201 (7.5 mg/kg) was given ip to sham animals. S3I-201 was dissolved in 0.05% DMSO and diluted in PBS just before injection. Animals were reanesthetized 4 h after challenge with M1 protein. Blood was collected for systemic leukocyte counts, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) to quantify the number of alveolar neutrophils. Then the lung was excised and one lobe was fixed in formaldehyde for histology and the remaining lung tissue was snap-frozen in liquid nitrogen and stored at −80°C for later STAT1, STAT3, and myeloperoxidase (MPO) assays and ELISA as described subsequently.

**Western blot.** Lung tissues were homogenized in ice-cold lysis buffer (25 mM Tris·HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, and 5% glycerol) containing protease inhibitors (Halt Protease Inhibitor Cocktail-EDTA Free) and centrifuged (16,000 g, 15 min at 4°C). Protein concentration of the supernatant was determined by the Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). An aliquot of 25 µg of protein was mixed with 3× protein loading buffer and boiled for 5 min before loading onto a 10–12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in Tris-buffered saline/Tween 20 buffer containing 5% bovine serum albumin powder for 1 h at room temperature. Protein immunoblots were performed with specific antibodies to phospho-STAT1 (Tyr701) (Cell Signaling Technology, Beverly, MA) and phosphothesynine (Tyr705) STAT3 (Cell Signaling Technology) overnight in 4°C. The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized by using a commercial chemiluminescence detection kit (ECL Plus; Amersham Biosciences, Piscataway, NJ) as described by the manufacturer. To measure total STAT1 or STAT3, the membrane incubated with Restore Western Blot Stripping Buffer (Pierce Biotechnology) for 5 min and then reblocked in Tris-buffered saline/Tween 20 buffer containing 5% bovine serum albumin powder for 1 h at room temperature and then incubated with STAT1 or STAT3 (Cell Signaling Technology) overnight at 4°C. The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized by using a commercial chemiluminescence detection kit as described by the manufacturer.

**Systemic leukocyte count.** Blood was collected from the tail vein and mixed with Turk’s solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) in a 1:20 dilution. Leukocytes were defined as monomorphonuclear (MLNs) and polymorphonuclear (PMNs) cells in a Burker chamber.

**Lung edema.** The left lung was excised, washed in PBS, gently dried with a blotting paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet weight to dry weight was used as indicator of lung edema formation.

**MPO activity.** Lung tissue was thawed and homogenized in 1 ml of 0.5% hexadeyltrimethylammonium bromide. Samples were freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (450 nm, with a reference filter 540 nm, 25°C) as previously described (2). Values were expressed as MPO units per gram tissue.

**BALF.** Mice were placed in a supine position and the trachea was surgically exposed. A catheter was inserted into the trachea. BALF was collected by five washes of 1 ml of PBS (5 mM EDTA). The numbers of MLN and PMN cells were counted in a Burker chamber.

**ELISA.** Levels of CXCL1 and CXCL2 in lung homogenates and levels of IL-6 and HMGB1 in plasma were quantified by use of double-antibody Quantikine ELISA kits (R & D Systems, Europe, Abingdon, Oxon, UK) with recombinant murine CXCL1, CXCL2, IL-6, and HMGB1 as standards. The lower limit of the assay was 0.5 pg/ml.

**Histology.** Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded, and 6-µm sections were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a preexisting scoring system as described (13), including size of alveolar collapse, thickness of alveolar septum, alveolar fibrin deposition, and neutrophil infiltration graded on a 0 (absent) to 4 (extensive) scale. In each tissue sample, five random areas were scored and mean value was calculated. The histology score is the sum of all four parameters.

**Flow cytometry.** Mac-1 was quantified on blood neutrophils. Blood was collected (1:10 acid citrate dextrose) 4 h after M1 protein challenge and incubated (10 min, room temperature) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce nonspecific labeling and then incubated with phycocerythrin-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, San Diego, CA), and FITC-conjugated anti-Mac-1 (clone M1/70, integrin αmβ2, rat IgG2b). The mean fluorescence intensity was determined by comparisons to an isotype control antibody (FITC-conjugated rat IgG2b). All antibodies were purchased from BD Biosciences Pharmingen, San Jose, CA, except when indicated. Cells were fixed and erythrocytes were lysed by BD lysis buffer (BD Biosciences) and then neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics and then Mac-1 expression was determined on Gr-1+ cells in this gate on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). A viable gate was used to exclude dead and fragmented cells.

**In vitro activation of neutrophils.** Blood was collected from healthy animals in syringes containing 1:10 acid citrate dextrose. Whole blood was incubated with M1 protein (1 µg/ml) with or without S3I-201 (100 µM, Sigma-Aldrich) and vehicle at 37°C for 20 min. Cells were stained for flow cytometric analysis of Mac-1 expression on neutrophils (Gr-1+) as described above.

**Chemotaxis assay.** Neutrophils isolated from bone marrow by use of Ficol-Paque were preincubated with S3I-201 (100 µM, Sigma-Aldrich) for 30 min and 1.5 × 10⁶ neutrophils were placed in the upper chamber of the Transwell inserts (5 µm pore size; Corning Costar, Corning, NY). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml; R&D Systems). After 120 min, inserts were removed, and migrated neutrophils were 1:1 transferred into 0.5% hexadeyltrimethylammonium bromide. Chemotaxis was determined by MPO levels in the wells as described above.

**Statistics.** Data are presented as mean values ± SE. Mann-Whitney rank-sum test was used for comparing two groups. P < 0.05 was considered significant and n represents the number of animals.
RESULTS

**STAT3 activity in the lung.** To investigate STAT3 activation in the lung after challenge with M1 protein and the potential effect of S3I-201, lungs from sham and M1 protein-treated mice were harvested for analysis of STAT3 phosphorylation. It was found that M1 protein challenge increased STAT3 phosphorylation in the lung (Fig. 1). Administration of S3I-201 decreased M1 protein-induced STAT3 activation in the lung (Fig. 1A). Quantification of STAT3 phosphorylation revealed that S3I-201 attenuated M1 protein-induced STAT3 activation by 63% in the lung (Fig. 1B). M1 protein challenge also increased phosphorylation of STAT1 in the lung (Fig. 2, A and B). However, administration of S3I-201 had no effect on pulmonary STAT1 activity in animals exposed to M1 protein (Fig. 2, A and B).

**Lung inflammation.** Challenge with M1 protein caused a clear-cut lung injury typified by enhanced lung edema formation (Fig. 3). Thus lung wet-to-dry ratio increased from 4.2 ± 0.2 in sham mice to 5.0 ± 0.0 in animals exposed to M1 protein (Fig. 3). Administration of the STAT3 inhibitor S3I-201 (7.5 mg/kg) reduced lung wet-to-dry ratio down to 4.1 ± 0.1 in mice challenged with M1 protein (Fig. 3). Moreover, morphological analysis showed normal lung microarchitecture in sham animals (Fig. 4A), whereas M1 protein increased interstitial edema, capillary congestion, and neutrophil infiltration (Fig. 4B). It was found that inhibition of STAT3 signaling reduced M1 protein-provoked changes of the tissue architecture and neutrophil recruitment in the lung (Fig. 4C). Quantification of the tissue changes showed that M1 protein increased pulmonary injury score and that administration of S3I-201 significantly attenuated the M1 protein-induced lung inflammation (Fig. 4D). Administration of S3I-201 had no effect on lung morphology in sham animals (Fig. 3 and 4).

---

**Fig. 1.** M1 protein-evoked STAT3 activity in the lung. Mice were treated with the STAT3 inhibitor S3I-201 (7.5 mg/kg) or vehicle (0.05% DMSO) 10 min prior to M1 protein injection. Mice treated with PBS served as sham animals. A: STAT3 phosphorylation (pY705-STAT3) was determined by use of Western blot as described in MATERIALS AND METHODS, and data are shown as percentage change from sham. B: aggregate data on STAT3 phosphorylation. Samples were harvested 4 h after M1 protein challenge. Data represent means ± SE; *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + M1 protein; n = 5.

**Fig. 2.** M1 protein-evoked STAT1 activity in the lung. Mice were treated with the STAT3 inhibitor S3I-201 (7.5 mg/kg) or vehicle (0.05% DMSO) 10 min prior to M1 protein injection. Mice treated with PBS served as sham animals. A: STAT1 phosphorylation (Tyr701) rabbit monoclonal antibody was determined by use of Western blot as described in MATERIALS AND METHODS, and data are shown as percentage change from sham. B: aggregate data on STAT1 phosphorylation. Samples were harvested 4 h after M1 protein challenge. Data represent means ± SE; *P < 0.05 Vehicle vs. Sham; n = 5.

**Fig. 3.** STAT3 controls M1 protein-evoked edema in the lung. Mice were treated with the STAT3 inhibitor S3I-201 (7.5 mg/kg) or vehicle (0.05% DMSO) 10 min prior to M1 protein injection. Mice treated with PBS served as sham animals. A: STAT3 phosphorylation (pY705-STAT3) was determined by use of Western blot as described in MATERIALS AND METHODS, and data are shown as percentage change from sham. B: aggregate data on STAT3 phosphorylation. Samples were harvested 4 h after M1 protein challenge. Data represent means ± SE; *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + M1 protein; n = 5.
Neutrophil accumulation in the lung. M1 protein challenge increased pulmonary levels of MPO by more than 14-fold (Fig. 5A). Inhibition STAT3 activity reduced the M1 protein-evoked enhancement in the lung activity of MPO by 48% (Fig. 5A). The number of BALF neutrophils was greatly enhanced 4 h after injection of M1 protein (Fig. 5B). It was found that treatment with S3I-201 attenuated the number of neutrophils from 100.0 ± 6.1 × 10³ down to 63.2 ± 4.6 × 10³ in the lung, which corresponds to a 52% decrease, 4 h after M1 protein challenge (Fig. 5B). Moreover, it was observed that challenge with M1 protein reduced the number of circulating PMNLs and MNLs (Table 1). Inhibition of STAT3 activity reduced this M1 protein-evoked systemic leukopenia (Table 1). Injection of S3I-201 had no effect on pulmonary accumulation of neutrophils in sham mice (Fig. 5, A and B).

Mac-1 expression and CXC chemokine generation. M1 protein challenge greatly increased Mac-1 expression on circulating neutrophils compared with sham animals (Fig. 5C). We observed that STAT3 inhibition had no effect on M1 protein-evoked increases of Mac-1 expression on circulating neutrophils (Fig. 5C). Several studies have demonstrated that CXC chemokines are potent regulators of pulmonary neutrophilia. Thus we next examined the role of STAT3 activity in regulating CXCL1 and CXCL2 formation in the lung. Lung levels of CXC chemokines were low but detectable in sham mice whereas challenge with M1 protein induced a 13-fold and
86-fold increase in the pulmonary levels of CXCL1 and CXCL2, respectively (Fig. 6). Administration of S3I-201 decreased lung levels of CXCL1 by 71% (Fig. 6A) and CXCL2 by 80% (Fig. 6B) in animals exposed to M1 protein. Administration of S3I-201 had no effect on pulmonary levels of CXC chemokines in sham mice (Fig. 6). It was found that M1 protein induced generation of IL-6 to 80% (Fig. 6A) in animals exposed to M1 protein. Administration of S3I-201 reduced M1 protein-induced generation of IL-6 to 10.2 ± 0.3 pg/ml down to 2.1 ± 0.4 ng/ml in animals exposed to M1 protein (Fig. 7B). Thus STAT3 inhibition reduced plasma levels of IL-6 and HMGB1 by more than 88% in mice exposed to M1 protein. Injection of S3I-201 had no effect on plasma levels of IL-6 and HMGB1 in sham mice (Fig. 7A). In addition, CXCL2 increased surface expression of Mac-1 on isolated neutrophils (Fig. 8B). However, it was found that coinubation of neutrophils with S3I-201 had no impact on CXCL2-induced increases of Mac-1 expression on isolated neutrophils (Fig. 8B).

**DISCUSSION**

This study documents an important role of STAT3 in streptococcal-induced acute lung inflammation. Our findings show that STAT3 signaling regulates CXC chemokine formation and neutrophil migration in the inflamed lung. Moreover, inhibition of STAT3 activity reduced plasma levels of IL-6 and HMGB1 and reversed leukocytopenia in mice exposed to M1 protein, indicating that STAT3 also regulates the systemic inflammatory response in streptococcal infections.

**Streptococcus pyogenes** of the M1 serotype is most frequently linked to the development of STSS and high mortality (15, 26). During systemic invasion, *Streptococcus pyogenes* shed M1 protein from their surface, which subsequently triggers systemic activation of innate immune cells, such as neutrophils and monocytes (15, 31). Herein, we demonstrate for the first time that M1 protein increases STAT3 activation is increased in the lung and that administration of the STAT3

---

**Table 1. Systemic leukocyte differential counts**

<table>
<thead>
<tr>
<th></th>
<th>MNL</th>
<th>PMNL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>3.9 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Vehicle + M1 protein</td>
<td>1.4 ± 0.2*</td>
<td>0.5 ± 0.1*</td>
<td>2.0 ± 0.4*</td>
</tr>
<tr>
<td>S3I-201 + M1 protein</td>
<td>3.2 ± 0.1†</td>
<td>1.7 ± 0.2†</td>
<td>4.9 ± 0.3†</td>
</tr>
</tbody>
</table>

Sham animals were treated with PBS (iv) only. Separate mice were treated with vehicle (0.05% DMSO) or S3I-201 (7.5 mg/kg IP) before iv challenge with M1 protein (15 µg). Blood samples were obtained 4 h after injection of M1 protein. Cells were identified as monomorphonuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL). Data are mean ± SE and 10⁶ cells/ml, *P < 0.05 vs. sham and †P < 0.05 vs. vehicle + M1 protein; n = 5 mice per group.
inhibitor effectively inhibits STAT3 phosphorylation, but not STAT1 phosphorylation, in lungs of animals exposed to M1 protein. It is interesting to note that the STAT3 inhibitor almost abolished lung edema but only reduced STAT3 activity by 63%. The reason for this discrepancy is not known but might be due to edema formation being more dependent on full STAT3 activity in M1 protein-induced lung inflammation. In this context, it should be mentioned that administration of the STAT3 inhibitor after challenge with M1 protein had no significant effect on edema formation and inflammation in the lung (not shown). Nonetheless, our findings are in line with previous studies showing that STAT3 is activated in different models of acute lung damage (11, 30, 36). For example, several studies have shown that endotoxin causes an early and clear-cut increase in STAT3 phosphorylation in the lung (18, 21), suggesting a pathological role of STAT3 in acute lung injury. However, functional studies of STAT3 in acute lung injury have been hampered by the fact that deletion of the STAT3 gene causes embryonic lethality in mice (35). As a consequence, functional studies have relied on the targeted disruption of STAT3 in one or a couple of cell types (epithelial and myeloid cells) in mice and have reported enhanced pulmonary inflammation and tissue injury in response to endotoxin (16, 21, 34). In this context, it is important to note that the net effect of STAT3 deletion is dependent on timing and type of cell types being deleted. This notion is supported by data showing that STAT3 deletion in T cells is protective in an experimental enterocolitis model (3) and that targeting STAT3 in respiratory cells reduces lung inflammation in allergen- and IgG immune complex-induced lung damage (30, 36). The development of specific drugs against STAT3, such as S3I-201, as used herein opens new possibility to study the global effect of targeting STAT3 function in different diseases. Nonetheless, our findings suggest that global inhibition STAT3 exerts a protective effect in streptococcal-induced acute lung injury. Our observation is also indirectly supported by studies showing that blocking JAK/JAK2 signaling, which is upstream of STAT3, ameliorates lung inflammation and tissue injury in models of acute lung damage (29).

Considering the critical role of neutrophil infiltration M1 protein-provoked lung injury (31, 46), it was of interest to examine the effect of S3I-201 on neutrophil accumulation in the lung. Herein, we found that S3I-201 decreased the M1 protein-evoked increase in lung levels of MPO by 48%, indicating that STAT3 signaling constitutes a significant feature in

Fig. 6. STAT3 controls M1 protein-induced CXC chemokine formation in the lung. Animals were treated with the STAT3 inhibitor S3I-201 (7.5 mg/kg) or vehicle (0.05% DMSO) 10 min prior to M1 protein injection. Mice treated with PBS served as sham animals. ELISA was used to quantify the levels of CXCL1 and CXCL2 in the lung of mice 4 h after M1 protein challenge. Data represent means ± SE; *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + M1 protein; n = 5.

Fig. 7. STAT3 controls plasma levels of IL-6 and HMGB1 in mice exposed to M1 protein. Animals were treated with the STAT3 inhibitor S3I-201 (7.5 mg/kg) or vehicle (0.05% DMSO) 10 min prior to M1 protein injection. Mice treated with PBS served as sham animals. ELISA was used to quantify the plasma levels of IL-6 and HMGB1 4 h after M1 protein challenge. Data represent means ± SE; *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + M1 protein; n = 5.
neutrophil infiltration in streptococcal infections. This STAT3-dependent decrease in MPO levels correlated very well with the attenuation in the number of alveolar neutrophils (52% decrease) in animals exposed to M1 protein. These findings are in line with previous studies on bacterial infections showing a prominent role of STAT3 activity in mediating neutrophil recruitment into the lung (30, 36). Taking into consideration the tight relationship between neutrophil accumulation and lung injury (1), it can be forwarded that the lung protective effect of S3I-201 is related to the decrease in pulmonary neutrophilia. Extravascular localization of neutrophils at sites of tissue inflammation is orchestrated by secreted CXC chemokines. Previous studies have reported a functional role of CXC chemokines in streptococcal infections (47, 48), and we have shown that M1 protein is a potent inducer of CXCL1 and CXCL2 formation in the lung (48). Herein, it was observed that treatment with S3I-201 markedly reduced CXCL1 and CXCL2 production in the lung, indicating that STAT3 activity is a key regulator of M1 protein-induced CXC chemokine generation in the lung. CXC chemokines are potent stimuli for neutrophil migration (44, 49); it was therefore also of interest to study the role of S3I-201 in regulating chemokine-dependent chemotaxis herein. We found that STAT3 inhibition abolished CXCL2-induced neutrophil migration in vitro. This finding is in line with a previous study showing that CXCL2-induced migration of neutrophil from the bone marrow to the blood is dependent on STAT3 signaling (24). Neutrophil trafficking is also mediated by specific adhesion molecules on neutrophils, such as P-selectin glycoprotein ligand-1 and Mac-1 (2, 10), and therefore we asked whether STAT3 regulates neutrophil expression of Mac-1. However, it was found that STAT3 inhibition had no impact of Mac-1 upregulation on neutrophils in vivo or in vitro, suggesting that the lung-protective effect of S3I-201 is independent of neutrophil expression of Mac-1 in streptococcal infections. Taken together, our findings suggest that STAT3 regulates M1 protein-induced neutrophil recruitment in the lung at two distinct levels, i.e., both formation and function (i.e., migration) of CXC chemokines.

Systemic inflammation in patients with bacterial infections is typified by enhanced plasma levels of IL-6 and HMGB1 (6, 8, 39). Studies have shown a correlation between high IL-6 levels and mortality of septic patients (28, 37). A previous study has shown that M1 protein is a potent stimulator of monocytes causing massive release of cytokines (i.e., IL-6) via binding of TLR2 on monocytes (26). IL-6 is a complex cytokine exerting both pro- and anti-inflammatory effects (19), which might be related to different signaling pathways of IL-6 (4, 14). In the present study, we observed that inhibition of STAT3 signaling markedly reduced plasma levels of IL-6 in animals exposed to M1 protein. The impact of this reduction of IL-6 on M1 protein-induced acute lung damage is not known at present but this observation supports the concept that STAT3 signaling also controls the systemic inflammatory response in streptococcal infections. Another indicator of systemic inflammation is HMGB1, which is a potent inducer of vascular leakage and a late mediator in endotoxemia and septicemia (23, 25, 41) as well as a predictor of clinical outcome in patients with severe sepsis (22). Herein, it was found that M1 protein challenge triggered a massive increase in the plasma levels of HMGB1. Interestingly, STAT3 inhibition reduced HMGB1 levels in the plasma by 90% in mice exposed to M1 protein, indicating a key role of STAT3 in M1 protein-induced sys-

---

**Fig. 8. STAT3 controls neutrophil migration in vitro.**

A: neutrophil migration was determined in response to medium alone (Control), medium plus CXCL2 (100 ng/ml), with or without preincubation of neutrophils with the STAT3 inhibitor S3I-201 (100 μM). B: Mac-1 expression on neutrophils in vitro. Whole blood was incubated with PBS only, or CXCL2 (100 ng/ml) and vehicle (0.05% DMSO) or S3I-201 (100 μM). Data represent means ± SE; *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + CXCL2; n = 5.
temic inflammation. This is the first study showing that STAT3 regulates IL-6 and HMGB1 formation in M1 protein-induced inflammation and future studies should address the functional role IL-6 and HMGB1 in streptococcal infections.

In conclusion, our novel results indicate that STAT3 signal...

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (2012-3685), Crafoordstiftelsen, Einar och Inga Nilssonstiftelse, Harald och Greta Jaenssontstiftelse, Greta och Johan Kockstiftelse, Fröken Agnes Nilssonstiftelse, Franke och Margareta Bergqviststiftelse för främjande av cancerforskning, Magnus Bergvallsstiftelse, Mossfelsstiftelse, Nanna Svatrstiftelse, Ruth och Richard Julinsstiftelse, Svenska läkaresällskapet, Allmänna sjukhusets i Malmö stiftelse för bekämpande av cancer, MAS fonder, Malmö University Hospital, and Lund University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


