Streptococcal M1 protein triggers chemokine formation, neutrophil infiltration, and lung injury in an NFAT-dependent manner

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RECEIVED FEBRUARY 27, 2014; REVISED NOVEMBER 17, 2014; ACCEPTED NOVEMBER 26, 2014. DOI: 10.1189/jlb.3HI0214-123RR

ABSTRACT

*Streptococcus pyogenes* of the M1 serotype can cause STSS, which is associated with significant morbidity and mortality. The purpose of the present study was to examine the role of NFAT signaling in M1 protein-induced lung injury. NFAT-luc mice were treated with the NFAT inhibitor A-285222 before administration of the M1 protein. Neutrophil infiltration, edema, and CXC chemokines were quantified in the lung, 4 h after challenge with the M1 protein. Flow cytometry was used to determine Mac-1 expression. Challenge with the M1 protein increased NFAT-dependent transcriptional activity in the lung, spleen, and liver in NFAT-luc mice. Administration of the NFAT inhibitor A-285222 abolished M1 protein-evoked NFAT activation in the lung, spleen, and liver. M1 protein challenge induced neutrophil recruitment, edema, and CXC chemokine production in the lung, as well as up-regulation of Mac-1 on circulating neutrophils. Inhibition of NFAT activity attenuated M1 protein-induced neutrophil infiltration by 77% and edema formation by 50% in the lung. Moreover, administration of A-285222 reduced M1 protein-evoked pulmonary formation of CXC chemokine >80%. In addition, NFAT inhibition decreased M1 protein-triggered Mac-1 up-regulation on neutrophils. These findings indicate that NFAT signaling controls pulmonary infiltration of neutrophils in response to streptococcal M1 protein via formation of CXC chemokines and neutrophil expression of Mac-1. Thus, the targeting of NFAT activity might be a useful way to ameliorate lung injury in streptococcal infections. *J. Leukoc. Biol.* 97: 1003-1010; 2015.

Introduction

The microbial causes of septic shock have been dominated by Gram-negative bacteria, but a recent resurgence of Gram-positive bacterial infections has changed the microbial etiology in septic patients [1, 2]. *S. pyogenes* is a common cause of Gram-positive infections, presenting with a wide severity spectrum ranging from uncomplicated cases of pharyngitis to severe and fatal conditions, such as STSS, which is a feared condition associated with a high mortality rate [3, 4]. *S. pyogenes* contain a versatile spectrum of virulence factors, including M proteins. At present, >80 different M serotypes have been documented in *S. pyogenes*. Notably, accumulating evidences have demonstrated that the M1 serotype is most frequently associated with STSS [3, 5]. M1 protein is a potent activator of innate immune cells, such as neutrophils [6] and monocytes [5]. Neutrophils protect against invading microorganisms, but excessive activation and infiltration of neutrophils are also considered to be a rate-limiting step in acute lung damage [7, 8]. The lung is one of the most critical organs being compromised in STSS patients [9]. Extravascular infiltration of neutrophils at sites of tissue damage is controlled by specific adhesion molecules, including CD11 [10], CD44 [11], CD62 [12], and CD162 [13], as well as CXC chemokines, such as KC (CXCL1) and MIP-2 (CXCL2) [14]. It has been shown that M1 protein-induced lung recruitment of neutrophils is dependent on the function of CXC chemokines [15]. Thus, the role of adhesion molecules and chemokines in mediating neutrophil accumulation in the lung is relatively well known, but the signaling pathways regulating M1 protein-provoked infiltration of neutrophils and lung damage remain elusive.

Extracellular stress signals trigger intracellular signaling cascades converging on specific transcription factors, controlling gene expression and generation of inflammatory compounds. Cytosolic calcium is a key determinant in the activation of immune cells [16]. One critical target of calcium in leukocytes is calcineurin, a unique calcium/calmodulin-activated serine/threonine protein phosphatase, exerting important roles in cellular processes and calcium-dependent signal transduction pathways [17, 18]. One key target of calcineurin is the transcription factor NFAT, consisting of four

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Abbreviations: BALF = bronchoalveolar lavage fluid, FTS = farnesylthiosalicylic acid, KC = keratinocyte-derived cytokine, Mac-1 = membrane-activated complex-1, MNL = monomorphonuclear leukocytes, MPO = myeloperoxidase, NFAT-luc = NFAT-luciferase reporter, PMNL = polymorphonuclear leukocytes, RLU = relative luciferase unit(s), STSS = streptococcal toxic shock syndrome

Volume 97, June 2015    Journal of Leukocyte Biology    1003

0741-5400/15/0097-1003 © Society for Leukocyte Biology

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isoforms (NFATc1–c4). NFATs are heavily phosphorylated and located in the cytosol under resting conditions. Upon stimulation, NFATs are dephosphorylated by calcineurin, allowing nuclear translocation [19]. NFAT activation initiates a cascade of transcriptional processes regulating several physiologic and pathologic events [20–22]. NFAT was first discovered as a transcriptional regulator of T cells [23, 24], but NFAT is now considered to exert functions in numerous different cell types outside of the immune system [20]. However, the role of NFAT signaling in regulating pathologic inflammation and tissue damage in the lung in response to M1 protein challenge is not known.

Based on these considerations, the purpose of this study was to define the functional significance of NFAT signaling in regulating CXC chemokine production, neutrophil activation, and infiltration, as well as edema formation in acute lung injury provoked by streptococcal M1 protein.

MATERIALS AND METHODS

Animals

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 (Lund, Sweden). Phenotypically normal adult FVB/N 9X-NFAT-luc mice were used. NFAT-luc mice expressing nine copies of an NFAT-binding site from the IL-4 promoter (5'-TGAGAAAAT-3') were positioned 5' to a minimal promoter from the α-myosin heavy-chain gene (−164 to +16) and inserted upstream of a luciferase reporter gene [25]. Originally, on an FVB/N background, mice were backcrossed for 4 generations to C57Bl/6 mice for various studies requiring this background, hence, having a mix background (C57/Bl6/FVB/N; 94%/6%). Female mice, weighing 21–25 g, were used for experiments and maintained on a 12–12 h light–dark cycle and fed standard laboratory diet and water ad libitum. Animals were anesthetized with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight i.p.

Experimental protocol

M1 protein was purified from the isogenic mutant MC25 strain (derived from the API 3. f lag genes strain 40/38 from the WHO Collaborating Centre for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic), as described previously [26]. Mice were challenged i.v. with 15 μg M1 protein-dissolved protein in PBS. Sham mice were treated with i.v. PBS alone. Vehicle or the NFAT inhibitor A285222 (0.29 mg/kg body weight, administered i.p., once daily for 7 consecutive days and in the morning of operation) was administered before M1 protein challenge. Treatment with A285222 was well tolerated and performed according to a previously established protocol [27, 28]. A285222 was kindly provided by Abbott Laboratories (Abbott Park, IL, USA). Experiments were terminated 4 h after M1 protein challenge or sham procedure, at which point, mice were re-anesthetized, 10 μl blood was obtained from the tail vein for measurements of leukocyte numbers, and 0.5 ml blood was obtained from the vena for flow cytometry. One part of the left lung was ligated and excised for edema measurement. The other part of the left lung, together with spleen and liver, was dissected out for luciferase measurements. The right lung was used for sampling and quantifying BALF 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 MPO assays and ELISA, as described subsequently.

Luciferase reporter assay

Luciferase activity was measured as described previously in tissue homogenates from lung, spleen, and liver in NFAT-luc mice from each group, as specified in the text [28]. OD was measured by use of an Infinite M200 instrument (Tecan Nordic AB, M¨olndal, Sweden) and data expressed as RLU/μg protein. Protein quantification was done by use of Biochrominonic Acid Protein Assay Reagent (Pierce, Thermo Scientific, Rockford, IL, USA).

Systemic leukocyte counts

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% v/v) in a 1:20 dilution. Leukocytes were identified as MNL and PMNL cells in a Burker chamber [10].

Lung edema

The left lung was excised, washed in PBS, gently dried by use of 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 an indicator of lung edema formation [10].

MPO activity

Lung tissue was thawed and homogenized in 0.02 M phosphate buffer (pH 7.4). Supernatant was discarded after centrifugation for 10 min at 14,000 rpm at room temperature, and then, the pellet was dissolved by adding 1 ml 0.5% hexadecyltrimethylammonium bromide. Samples were stored at −20°C overnight and then thawed and kept at in a 60°C water bath for 2 h, followed by 90 s sonication. Supernatant was collected after 5 min centrifugation at 14,000 rpm, and the MPO activity in the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (150 nm, with a reference filter 540 nm, 25°C), as described previously [10]. Values were expressed as MPO units/g tissue.

ELISA

Levels of CXCL1/KC and CXCL2/MIP2 in lung homogenates were analyzed by use of Quantikine ELISA kits (R&D Systems Europe, Abingdon, Oxon, United Kingdom) by use of murine rCXCL1/MIP2 and rCXCL2/KC as standards and according to the manufacturer’s instructions. The lower limit of the assay was 0.5 pg/ml.

Flow cytometry

For analysis of the expression of surface molecules on circulating 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/CD52 (BD Biosciences Pharmingen, San Jose, CA, USA) antibody for blocking FcγRIII/II to reduce nonspecific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, San Diego, CA, USA) and FITC-conjugated anti-Mac-1 (clone M1/70, integrin αM chain, rat IgG2b, BD Biosciences Pharmingen). The mean fluorescence intensity was determined by comparisons with an isotype control antibody (FITC-conjugated rat IgG2b). Cells were fixed, and erythrocytes were lysed by lysis buffer (BD Biosciences Pharmingen), 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 these gates on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells.

Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Sections (6 μm) were stained with H&E. Lung injury was quantified in a blinded manner by adoption of a pre-existing scoring system, as described [29, 30], including alveolar spaces, thickness of alveolar septas, alveolar fibrin deposition, and neutrophil infiltration, graded on a 0 (absent)–4 (extensive) scale.
 Statistics
Data are presented as mean values ± SEM. The percentage reduction caused by A-285222 was compared with vehicle treatment in animals exposed to M1 protein. Statistical evaluations were performed by use of Mann-Whitney rank sum test for comparing 2 groups. *P < 0.05 was considered significant. The experiments were repeated in 5 animals (n = 5) in each group.

RESULTS
M1 protein challenge increases NFAT-dependent transcriptional activity
Challenge with the M1 protein dramatically increased luciferase activity in the lung, spleen, and liver of transgenic NFAT-luc reporter mice (Fig. 1; *P < 0.05 vs. sham, n = 5). Administration of the NFAT inhibitor A-285222 markedly decreased M1 protein-evoked NFAT activation in studied organs, indicating that A-285222 is a powerful inhibitor of NFAT transcriptional activity (Fig. 1; #P < 0.05 vs. vehicle + M1 protein, n = 5). A-285222 prevents NFAT nuclear accumulation, without affecting AP-1 or NF-kB activation or calcineurin phosphatase activity [31].

NFAT regulates M1 protein-induced lung injury
M1 protein challenge caused clear-cut lung damage (Fig. 2). Histologic examination revealed intact lung morphology in sham mice (Fig. 2A), whereas M1 protein-treated mice exhibited disrupted and abnormal lung-tissue microarchitecture typified by interstitial edema, capillary congestion, and neutrophil accumulation (Fig. 2B). Inhibition of NFAT activity significantly limited all M1 protein-induced changes in the lung (Fig. 2C). Quantification of the morphologic changes revealed that M1 protein increased the lung injury score (Fig. 2D; *P < 0.05 vs. sham, n = 5) and that administration of the A-285222 effectively attenuated lung injury in mice challenged with the M1 protein (Fig. 2D; #P < 0.05 vs. vehicle + M1 protein, n = 5). Moreover, M1 protein increased the lung wet/dry ratio from 4.4 ± 0.1 to 5.1 ± 0.0 in M1 protein-treated mice (Fig. 3; P < 0.05 vs. sham, n = 5). This M1-induced edema was attenuated in animals that had been treated with the NFAT inhibitor, as shown by a decreased lung wet/dry ratio to 4.7 ± 0.0 (Fig. 3; P < 0.05 vs. vehicle + M1 protein, n = 5).

NFAT regulates M1 protein-induced neutrophil recruitment
Challenge with the M1 protein enhanced lung activity of MPO by >20-fold (Fig. 4A; *P < 0.05 vs. sham, n = 5). Inhibition of NFAT activity decreased the M1 protein-triggered increase in MPO activity by 77% (Fig. 4A; #P < 0.05 vs. vehicle + M1 protein, n = 5). Quantification of BALF neutrophils showed a substantial increase in the number of alveolar neutrophils after injection of the M1 protein (Fig. 4B; *P < 0.05 vs. sham, n = 5), which was attenuated in animals treated with A-285222 from 89.6 ± 8.0 × 10^5 to 56.0 ± 2.1 × 10^5, corresponding to a 60% decrease in the number of pulmonary neutrophils (Fig. 4B; #P < 0.05 vs. vehicle + M1 protein, n = 5). In addition, we observed that challenge with the M1 protein attenuated the circulating numbers of PMNLs and MNLs (Table 1). This is in line with what others have reported previously [15, 32–35]. Inhibition of NFAT activity reversed this M1 protein-induced reduction in PMNLs and MNLs in the blood (Table 1).

Figure 1. Luciferase activity (RLU/µg protein) in (A) lung, (B) spleen, and (C) liver of NFAT-luc mice. Sham animals were treated with PBS (i.v.) only. Separate mice were pretreated with vehicle (PBS) or A-285222 (0.29 mg/kg) i.p. once daily for 7 consecutive days and in the morning before challenge with the M1 protein (15 µg i.v.). Samples were obtained 4 h after injection of the M1 protein. Data represent means ± SEM. *P < 0.05 versus sham; #P < 0.05 versus vehicle + M1 protein (n = 5 mice/group).

NFAT regulates M1 protein-induced Mac-1 expression on neutrophils
Mac-1 is an important adhesion molecule mediating neutrophil recruitment [10, 35]. Injection of the M1 protein caused a significant up-regulation of Mac-1 on circulating neutrophils (Fig. 5; *P < 0.05 vs. sham, n = 5). NFAT inhibition markedly decreased neutrophil surface expression of Mac-1 in mice exposed to M1 protein (Fig. 5; #P < 0.05 vs. vehicle + M1 protein, n = 5).

NFAT regulates M1 protein-induced CXC chemokine formation in the lung
Recent data have shown that the CXCL2–CXCR2 axis controls neutrophil up-regulation of Mac-1 in M1 protein-induced inflammation [34]. Therefore, we next analyzed the role of
NFAT in regulating production of CXC chemokines in the lung. Pulmonary levels of CXCL1 and CXCL2 were low in sham mice. Challenge with the M1 protein increased lung levels of CXCL-1 by 110-fold and CXCL-2 by 897-fold (Fig. 6; *P < 0.05 vs. sham, n = 5). Administration of A-285222 decreased M1 protein-induced formation of CXCL-1 and CXCL-2 by 81% and 88%, respectively (Fig. 6; #P < 0.05 vs. vehicle + M1 protein, n = 5).

DISCUSSION

Our data show that NFAT activity constitutes an important component in streptococcal M1 protein-evoked acute lung damage. These results indicate NFAT inhibition reduces M1 protein-induced formation of CXC chemokines, neutrophil activation, and infiltration in the lung. Indeed, we found that inhibition of NFAT not only attenuated M1 protein-evoked neutrophil recruitment but also abolished edema formation and tissue injury in the lung. Considered together, our novel findings suggest that the targeting of the NFAT signaling pathways might be a useful way to protect against acute lung injury in streptococcal infections.

*S. pyogenes* of the M1 serotype can cause potentially fatal infections, such as STSS [4]. During bacterial invasion, the M1 protein is shed from the surface of *S. pyogenes* into the circulation, triggering widespread activation of the host innate-immune cells. It is well appreciated that activation of the host
innate immune system is a critical step in sepsis, leading to lung dysfunction and impaired gaseous exchange [9]. M1 protein is a potent activator of neutrophils and monocytes, causing massive secretion of proinflammatory cytokines and chemokines [5, 6]. Alveolar accumulation of neutrophils is known to be a rate-limiting step in M1 protein-induced lung injury [6, 15], which is considered to be regulated by secreted chemokines and adhesion molecules expressed on activated leukocytes and endothelial cells [35]. However, the signaling pathways regulating neutrophil infiltration and lung injury in response to M1 protein remain elusive. NFAT activity is known to regulate key components in tissue development, such as formation of blood vessels and the musculoskeletal system, as well as neuronal growth and development of the immune system [36–39]. Nonetheless, accumulating data suggest that NFAT activity also plays an important role in pathologic inflammation, including atherosclerosis [40], autoimmune diseases [41], and acute pancreatitis [27]. In the present study, we could demonstrate that challenge with the M1 protein triggered NFAT-dependent transcriptional activity, not only in the lung but also in the spleen and liver, suggesting that NFAT is widely activated in M1 protein-induced inflammation. Moreover, it was found that administration of the NFAT inhibitor A-285222 attenuated M1 protein-evoked NFAT activity in all studied tissues, indicating that A-285222 is an effective inhibitor of NFAT signaling. Herein, we show for the first time that inhibition of NFAT decreases lung edema and tissue damage (66% reduction) in the lungs of mice exposed to M1 protein, suggesting that NFAT activity plays an important role in the regulation of acute lung damage in response to systemic exposure of the M1 protein. With the knowledge that neutrophil recruitment is a key component in M1 protein-induced lung damage [15], we were interested in studying the effect of A-285222 on neutrophil recruitment in the lung.

Notably, we found that treatment with A-285222 reduced lung levels of MPO by 77% in response to M1 protein challenge, suggesting that NFAT signaling exerts a potent role in regulating pulmonary infiltration of neutrophils in streptococcal infections. This A-285222-mediated reduction in MPO activity correlated very well with the reduction in the numbers of neutrophils in the alveolar compartment (60% reduction) in M1 protein-treated mice. In this context, we recently observed that inhibition of the small GTPase Ras, by use of the Ras signaling inhibitor FTS, also protected against M1 protein-induced lung injury [42]. FTS was slightly less potent than the NFAT inhibitor used herein, reducing lung damage by 45% and

**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>4.0 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>Vehicle + M1 protein</td>
<td>0.8 ± 0.1*</td>
<td>0.3 ± 0.1*</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>A-285222 + M1 protein</td>
<td>2.6 ± 0.4†</td>
<td>1.3 ± 0.3†</td>
<td>3.9 ± 0.7†</td>
</tr>
</tbody>
</table>

Sham animals were treated with PBS (i.v.) only. Separate mice were pretreated with vehicle (PBS) or A-285222 (0.29 mg/kg) i.p. once daily for 7 consecutive days and in the morning before challenge with the M1 protein (15 µg i.v.). Blood samples were obtained 4 h after injection of the M1 protein. Cells were identified as MNLs and PMNLs. Data represent means ± sem. *P < 0.05 versus sham; †P < 0.05 versus vehicle + M1 protein; n = 5 mice/group.
alveolar accumulation of neutrophils by 48% (compared with 66% and 60%, respectively, for A-285222) [42]. It is interesting to speculate whether Ras and NFAT signaling might interact at some level, a possibility worth exploring in future studies. Nonetheless, with the consideration of the tight relationship between neutrophil recruitment on one hand and lung injury on the other hand [8], it might be assumed that at least a part of the protective effect of A-285222 is related to the reduction in pulmonary neutrophilia.

We next wanted to examine the role of NFAT signaling in the regulation of neutrophil recruitment in the lung in response to systemic challenge with the M1 protein. A recent study reported that activation and up-regulation of Mac-1 on neutrophils are key components at the initiation of acute lung damage caused by streptococcal M1 protein [34]. Indeed, Mac-1 is not only a marker of neutrophil activation but also a fundamental adhesion molecule that mediates neutrophil-endothelium interactions in the pulmonary microvasculature, both in endotoxemia and abdominal sepsis [10, 43]. As a result of the potent reduction in pulmonary neutrophils in mice treated with the NFAT inhibitor, we next asked whether NFAT might regulate Mac-1 expression on the surface of neutrophils in M1 protein-induced lung damage. In the present study, we found that the targeting of NFAT activity greatly attenuated M1 protein-induced expression of Mac-1 on neutrophils, indicating that inhibition of neutrophil up-regulation Mac-1 might help explain the inhibitory impact of the NFAT inhibitor on neutrophil accumulation and tissue injury in streptococcal M1 protein lung damage. Extravascular navigation of neutrophils is coordinated by secreted chemokines. Neutrophil activation and trafficking are controlled by CXC chemokines, such as CXCL1 (KC) and CXCL2 (MIP-2) [14]. In the present study, we found that NFAT inhibition markedly suppressed M1 protein-evoked pulmonary formation of CXCL1 and CXCL2. With the consideration that CXC chemokines and their receptor CXCR2 are critical in mediating M1 protein-induced neutrophil up-regulation of Mac-1 [34], our findings suggest that inhibition of CXC chemokine formation could be a mechanism that helps to explain the inhibitory effect of the NFAT inhibitor on neutrophil activation, Mac-1 expression, and recruitment into the lung in response to streptococcal M1 protein. Taken together, this is the first study to demonstrate that NFAT signaling exerts a significant function in streptococcal M1 protein-provoked neutrophil recruitment and tissue injury in the lung. In this context, it is interesting to note that it has been reported that cyclosporine and FK506, 2 calcineurin inhibitors, can reduce neutrophil responses and protect against endotoxemia and acute lung injury [44, 45]. With the knowledge that NFAT activity is regulated by calcineurin [46], these present findings suggest that inhibition of CXC chemokine formation could be a mechanism that helps to explain the inhibitory effect of the NFAT inhibitor on neutrophil activation, Mac-1 expression, and recruitment into the lung in response to streptococcal M1 protein.
findings might help explain the protective effects of calcineurin inhibitors on endotoxemia and pulmonary injury. Considered together, our findings indicate that a calcium/calcineurin-NFAT signaling axis is an important component in lung damage associated with streptococcal infections, as has been demonstrated in a list of other conditions, including atherosclerosis [40], acute pancreatitis [27], cardiac hypertrophy [25], and diabetes-induced vascular inflammation [47].

In conclusion, these novel results demonstrate that NFAT inhibition attenuates CXC chemokine generation, neutrophil activation, and recruitment in M1 protein-induced lung edema formation and tissue injury. Thus, our findings suggest that the targeting of the NFAT signaling pathway might be a useful way to protect against pulmonary damage in severe infections caused by *S. pyogenes.*

**AUTHORSHIP**

Songen Zhang performed experiments, analyzed the data, and wrote the manuscript. Su Zhang performed experiments and analyzed the data. E.G.-V. performed experiments. H.H. supervised the project and provided the M1 protein. M.F.G. supervised the project and provided NFAT-luc mice. H.T. supervised the project, designed the experiments, and wrote the manuscript. All authors provided feedback on the manuscript.

**ACKNOWLEDGMENTS**

This work was supported by grants from the Swedish Medical Research Council (2011-3900 and 2012-3685), Swedish Heart and Lung Foundation, Crafoord Foundation, Einar and Inga Nilsson Foundation, Harald and Greta Jaensson Foundation, Greta and Johan Kock Foundation, Fröken Agnes Nilsson Foundation, Franke and Margareta Bergqvisit Cancer Foundation, Lundgren Foundation, Magnus Bergvall Foundation, Mössfelt Foundation, Nanna Svertz Foundation, Ruth and Richard Julin Foundation, Albert Pahlsson Foundation, Knut and Alice Wallenberg Foundation, Lund University Diabetes Centre, Skåne University Hospital, and Lund University.

**DISCLOSURES**

The authors report no conflict of interest.

**REFERENCES**


KEY WORDS: leukocytes · infection · sepsis · migration
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J Leukoc Biol 2015 97: 1003-1010 originally published online January 12, 2015
Access the most recent version at doi:10.1189/jlb.3HI0214-123RR

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