Regulator of Calcineurin 1 Suppresses Inflammation during Respiratory Tract Infections

Robert D. Junkins, Adam J. MacNeil, Zhengli Wu, Craig McCormick and Tong-Jun Lin

*J Immunol* 2013; 190:5178-5186; Prepublished online 15 April 2013; doi: 10.4049/jimmunol.1203196

http://www.jimmunol.org/content/190/10/5178

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/04/15/jimmunol.1203196.DC1

**References**

This article cites 78 articles, 42 of which you can access for free at: http://www.jimmunol.org/content/190/10/5178.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Regulator of Calcineurin 1 Suppresses Inflammation during Respiratory Tract Infections

Robert D. Junkins,*†‡ Adam J. MacNeil,*†‡ Zhengli Wu,*†‡ Craig McCormick,*‡ and Tong-Jun Lin*†‡

Respiratory tract infection with Pseudomonas aeruginosa is a common cause of hospitalization in immune-compromised individuals. However, the molecular mechanisms involved in the immune response to P. aeruginosa lung infection remain incompletely defined. In this study, we demonstrate that the regulator of calcineurin 1 (RCAN1) is a central negative regulator of inflammation in a mouse model of acute bacterial pneumonia using the opportunistic bacterial pathogen P. aeruginosa. RCAN1-deficient mice display greatly increased mortality following P. aeruginosa lung infection despite enhanced neutrophil recruitment and bacterial clearance. This mortality is associated with higher systemic levels of proinflammatory cytokines in RCAN1-deficient animals. These aberrant inflammatory responses coincide with increased transcriptional activity of proinflammatory RCAN1-target proteins NFAT and NF-κB. In addition, we reveal a novel regulatory role for RCAN1 in the ERK/STAT3 pathway both in vitro and in vivo, suggesting that aberrant STAT3 activity may significantly contribute to delayed resolution of inflammatory responses in our model. Together, these findings demonstrate that RCAN1 is a potent negative regulator of inflammation during respiratory tract infections. The Journal of Immunology, 2013, 190: 5178–5186.

D own syndrome (DS) is caused by trismisy of chromosome 21 and represents the single most common chromosomal anomaly in live born infants, occurring with an incidence of 1 in every 600–900 live births (1–3). Along with severe mental and anatomical abnormalities, DS is associated with a wide range of immunodeficiencies (4). Included are deficiencies in adaptive immunity such as lymphopenia (5), impaired Ab response to immunization (6, 7), as well as decreased levels of IgA (8). Deficits in innate immunity have also been identified primarily involving impaired neutrophil chemotaxis (9, 10).

Despite severe anatomical and mental defects associated with the condition, the most common health complication associated with DS is recurrent and persistent respiratory tract infections (11, 12), among which the greatest cause of hospital admission are bacterial and viral pneumonias (13, 14). Although there have been multiple efforts to characterize immunodeficiencies in DS patients, the molecular and genetic mechanisms that underlie many of these defects remain poorly defined. Regulator of calcineurin 1 (RCAN1) is a DS-associated gene that has recently emerged as an attractive candidate for mediating some of the immunodeficiencies associated with this syndrome.

The Journal of Immunology, 2013, 190: 5178–5186.
Previous work has focused on the role of RCAN1 in the production of various inflammatory cytokines in response to immunological stimuli (45). However, the biological implications of RCAN1 during infection remain poorly understood. To examine the role of the RCAN1 during the innate immune response, we use a lung infection model of acute bacterial pneumonia that employs the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, which is associated with high levels of morbidity and mortality among cystic fibrosis patients and immunocompromised individuals (46). We find that RCAN1 deficiency results in greatly increased mortality following *P. aeruginosa* infection despite enhancement of neutrophil recruitment and bacterial clearance from the lungs. The increased mortality was associated with an inappropriate early cytokine response following infection with *P. aeruginosa* mediated through increased activation of the proinflammatory transcription factors NF-κB and NFAT. Unexpectedly, we further identified a novel regulatory role for RCAN1 in a third inflammatory pathway leading to the activation of STAT3. These data confirm that RCAN1 is a central negative regulator of inflammatory cytokine production, and trisomy of the gene warrants further investigation as a contributing factor to immune dysfunction in DS patients.

**Materials and Methods**

**Animals**

The RCAN1 gene was targeted for deletion by standard homologous recombination in embryonic stem cells (5v129 strain), followed by generation of chimeric mice, which were subsequently bred to pass the targeted allele into the germline in the C57BL/6 genetic background, as described elsewhere (23). These mice were originally provided by J. Molkentin (Cincinnati Children’s Hospital Medical Center, University of Cincinnati, Cincinnati, OH). C57BL/6 mice purchased from Charles River Laboratories and bred in the same facility as RCAN1-deficient mice were used as wild-type controls. All animal protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care.

**Animal survival and antibiotic therapy studies**

For survival experiments, the laboratory strain of *P. aeruginosa* PAK (a gift of J. Boyd, Institute of Marine Bioscience, National Research Council, Halifax, NS, Canada) was used. Mice were intranasally infected with 1 LD50 of *P. aeruginosa* (2.23 × 10^7 CFU/mouse). For antibiotic therapy studies, mice were treated as previously described (47). Briefly, animals were administered 200 mg/kg ceftazidime (Sigma-Aldrich, St. Louis, MO) s.c. every 10 d, and the weight, body temperatures, and disease scores were measured. Similar to those observed clinically (47, 48). Animals were monitored daily for signs of infection and were treated as described previously (52). Briefly, samples in duplicate (75 µL) were mixed with equal volumes of the substrate (3.3’,5’-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 µM; and H2O2, 2.2 mM) for 2 min. The reaction was stopped by adding 150 µL 2 M H2SO4. The OD was measured at 450 nm.

**Nuclear extract preparation and EMSA**

Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer’s protocol. EMSA was performed, as previously described (53). Briefly, probe labeling was accomplished by treatment with T4 kinase (Life Technologies, Burlington, ON, Canada) in the presence of32P adenosine triphosphate (Perkin Elmer, Waltham, MA). Labeled oligonucleotides were purified on a Sephadex G-25M column (GE Healthcare, Pittsburgh, PA). Ten micrograms of nuclear protein was added to a 10 µL vol of binding buffer supplemented with 1 µg poly-(dI-dC) (GE Healthcare) for 15 min. Labeled double-stranded oligonucleotide was added to each reaction mixture, which was incubated at room temperature for 30 min and separated by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris–boric acid–EDTA buffer. Gels were vacuum dried and subjected to autoradiography. The following synthesized double-stranded oligonucleotides were used: NFAT-binding consensus sequence on the IL-6 promoter, 5’-AGTTGAGGGACCTTCCCAGGC-3’ (Promega, Madison, WI).

**Statistics**

Data are presented as mean ± SE of the indicated number of experiments. Statistical significance was determined by assessing means with an unpaired t test. Differences were considered significant at *p < 0.05, **p < 0.01, and ***p < 0.005.

**Western blot and scanning densitometry**

Cell lysates (15–20 µg) were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane, blotted with primary and secondary Abs, as indicated, and detected by an ECL detection system (Western Lightning Plus-ECL, Perkin Elmer). Scanning densitometry was performed using Scion Image (Scion, Frederick, MD).

**Results**

**RCAN1-deficient mice display increased mortality despite enhanced bacterial clearance and neutrophil infiltration in vivo**

Previous reports have implicated the DS critical region gene RCAN1 as a negative regulator of various inflammatory pathways.
However, the biological impact of RCAN1 during bacterial pneumonia remains unclear. To address this question, we used a P. aeruginosa model of acute bacterial pneumonia in wild-type (+/+), and RCAN1-deficient mice (−/−) to assess the impact of the protein on mortality and bacterial clearance in vivo. Wild-type and RCAN1-deficient mice were infected with 1 LD50 of the laboratory strain of P. aeruginosa PAK. Animal mortality was then monitored for 10 days postinfection (Fig. 1A). Unexpectedly, whereas 50% of wild-type animals succumbed to the infection, primarily between 24 and 48 hpi, 100% of the RCAN1-deficient mice died within the first 18 hpi, suggesting that RCAN1 plays a critical role in host defense against P. aeruginosa lung infection. To examine whether antibiotic therapy affects RCAN1-regulated response in P. aeruginosa infection, mice were treated with a concentration of the antibiotic ceftazidime, which yields serum concentrations similar to those achieved clinically (47, 48). Whereas antibiotic therapy was able to increase survival in wild-type animals, it was unable to rescue RCAN1-deficient mice, which all succumbed to the infection in the first 18 hpi (Fig. 1A).

To further characterize the role of RCAN1 in host defense against P. aeruginosa, the impact of RCAN1 on bacterial burden was assessed in wild-type and RCAN1-deficient animals. To address this question, mice were infected intranasally with P. aeruginosa strain 8821 [isolated from the lungs of a cystic fibrosis patient (51)], as this strain is associated with lower mortality in vivo. Bacterial burden was assessed in the lung and BALF of wild-type and RCAN1-deficient mice at 4 and 24 hpi. No differences were observed at 4 hpi, most likely due to the high bacterial burden, and time required for the recruitment and phagocytosis of the bacteria by neutrophils (data not shown). Contrary to expectation, there was significantly more bacteria detected in the lungs of wild-type mice compared with RCAN1-deficient animals 24 hpi (Fig. 1B). A similar trend was observed in the BALF of RCAN1-deficient animals, but it did not reach statistical significance (Fig. 1C). To determine whether RCAN1 deficiency affects bacterial dissemination, the bacterial burden in the spleen and blood was assessed. The levels of bacteria in both the serum and spleen were decreased in RCAN1-deficient mice (Fig. 1D, 1E). These results suggest that the decreased bacterial load in the lungs was not a result of impaired containment of the infection. The bacterial burden in the RCAN1-deficient mice is lower than that in wild-type mice.

As the clearance of P. aeruginosa from the lungs is largely dependent upon the recruitment of neutrophils to the site of infection (54), we next set out to measure the infiltration of these cells into the respiratory tract. Assessment of neutrophil infiltration was carried out using an assay to determine the activity of the neutrophil granule-specific enzyme MPO. MPO activity was greatly enhanced at 4 hpi in both the lung (Fig. 1F) and BALF (Fig. 1G) of RCAN1-deficient mice. These levels of MPO activity remained elevated in the lung tissue 24 hpi. However, no differences in activity were observed in the BALF at this time point. These data stand in stark contrast to previous results in other gene knockout mice we and others have generated using this P. aeruginosa lung infection model, in which increased bacterial burden is usually associated with increased mortality (49, 55, 56). Altogether, these data suggest that the increased mortality in RCAN1-deficient animals may not be directly caused by bacterial pneumonia.

**FIGURE 1.** RCAN1-deficient mice display increased mortality, but decreased bacterial burden following P. aeruginosa lung infection. RCAN1 wild-type (+/+) and knockout (−/−) mice were infected intranasally with 1 LD50 (2.25 × 107 CFU) of P. aeruginosa strain PAK. Mice were then left untreated, or were treated with 200 mg/kg ceftazidime (Ceft) s.c. every 8 h. Animal survival was monitored daily (A) (n = 10, *p < 0.05, ****p < 0.0005). Wild-type and RCAN1-deficient mice were also infected intranasally with 107 (4–8 h) or 105 (24 h) CFU of P. aeruginosa strain 8821, or an equivalent volume of saline (NT). Lungs and BALF were collected 4 or 24 hpi. Serial dilution of homogenized lung tissue (B), BALF (C), spleens (D), and blood (E) was streaked on LB agar plates and incubated 24 h at 37°C. The resultant colonies were counted to determine bacterial load (n = 8–10 ± SEM, *p < 0.05). Samples were then lysed, and MPO activity was assayed in the lungs (F) and BALF (G) (n = 6–11 ± SEM, *p < 0.05).

RCAN1-deficient mice have greatly enhanced serum levels of inflammatory cytokines following P. aeruginosa lung infection

Due to the reported roles for RCAN1 as a regulator of inflammation, we next set out to determine whether the discrepancies between the enhanced bacterial clearance and increased mortality in RCAN1-deficient mice could be explained by susceptibility to systemic inflammation. To explore this possibility, we examined the levels of four cytokines whose increased expression has been linked to mortality associated with systemic inflammation. These cytokines are TNF (57), IL-1β (58), IL-6 (59), and a murine homolog of human IL-8, MIP-2 (60). Wild-type and RCAN1-deficient mice were infected intranasally with 1 LD50 of P. aeruginosa strain PAK. Mice were then left untreated, or were treated with the antibiotic ceftazidime every 8 h, starting 2 hpi. Serum was then collected at various time points and analyzed for levels of inflammatory cytokines. We observed that the levels of IL-6 (Fig. 2A), MIP-2 (Fig. 2B), and IL-1β (Fig. 2C) started to increase in the serum of RCAN1-deficient mice as early as 3 hpi. Levels of IL-1β and MIP-2 were then determined in the serum of RCAN1-deficient mice when compared with wild-type controls. Antibiotic treatment with ceftazidime modestly decreased IL-6 and IL-1β level in the serum in wild-type mice. Similarly, MIP-2 and IL-1β level in the serum of RCAN1-
deficient mice was modestly reduced after ceftazidime treatment. However, the levels of these inflammatory mediators in RCAN1-deficient mice remained well above those seen in wild-type animals, suggesting that the antibiotic therapy was unable to prevent aberrant systemic inflammation that occurs in the absence of RCAN1. Together these data demonstrate that, despite enhanced neutrophil infiltration and bacterial clearance from the lungs following P. aeruginosa infection, RCAN1-deficient mice display enhanced mortality most likely due to increased systemic inflammation.

We next further determined the inflammatory response in the lungs of RCAN1-deficient mice. Wild-type and RCAN1-deficient mice were infected intranasally with 10^7 or 10^6 CFU P. aeruginosa strain 8821 for 4- and 24-h infections, respectively. A mock infection with saline was used as a control. At the indicated time points, mice were sacrificed, and lung tissue and BALF were collected and analyzed for the levels of proinflammatory and immunoregulatory cytokines (Table I). The levels of all the proinflammatory cytokines examined were greatly enhanced in both the lung and BALF of RCAN1-deficient mice infected with P. aeruginosa at the early 4-hpi time point compared with wild-type mice. At the later 24-hpi time point, few differences in inflammatory cytokine levels were observed, and the differences that persisted were smaller than those observed at 4 hpi. Interestingly, the enhanced proinflammatory cytokine response observed at 4 h was accompanied by decreased levels of the immunomodulatory cytokine TGF-β in both the lungs and the BALF of RCAN1-deficient mice. These results indicate that RCAN1 opposes the generation of an inflammatory environment through inhibiting proinflammatory cytokine production and promoting immunomodulatory TGF-β, particularly during the early stages post-infection.

Cultured RCAN1-deficient macrophages display enhanced early production of inflammatory cytokines following infection with P. aeruginosa

Macrophages are important during host defense against P. aeruginosa infection. We set out to further characterize the inflammatory response in wild-type and RCAN1-deficient bone marrow–derived macrophages in vitro. To examine RCAN1 expression in macrophages during P. aeruginosa infection, bone marrow–derived macrophages were cultured from wild-type and RCAN1-deficient mice. These macrophages were then infected with P. aeruginosa strain 8821 at a multiplicity of infection (MOI) of 1:10. Lysates were prepared at various time points and subjected to Western blot analysis. The long form of RCAN1 (37 kDa) was found to be constitutively expressed in wild-type cells; however, the short (25 kDa) form of the protein was rapidly induced in response to P. aeruginosa infection, supporting a role for the protein early in P. aeruginosa infection (Supplemental Fig. 1A, 1B). To assess the impact of this RCAN1 induction on inflammatory cytokine production, wild-type and RCAN1-deficient cultured macrophages were exposed to P. aeruginosa at various MOIs to determine the dose resulting in the optimal production of various inflammatory cytokines (Supplemental Fig. 2A–D). The optimal MOI was determined to be 1:1, as higher doses resulted in substantial cell death. Thus, this dose was used for subsequent experiments.

Wild-type and RCAN1-deficient macrophages were then infected for various time points up to 48 h. The levels of secreted inflammatory cytokines, including IL-6, IL-1β, TNF, and MIP2, were tested. The levels of all the inflammatory cytokines were significantly enhanced in the supernatants of RCAN1-deficient macrophages treated with P. aeruginosa at early time points (Fig. 3A–D). Interestingly, no significant differences were observed at the later 24-hpi time point. Similar results were obtained using P. aeruginosa strain 8821 at a MOI of 1:10 (Supplemental Fig. 3A–D). These results suggest that RCAN1 plays an important negative regulatory role in inflammatory cytokine production early in infection.

Reports have implicated a role for RCAN1 in regulating two distinct proinflammatory transcription factors. First, RCAN1 inhibits the activity of calcineurin, a phosphatase required for the activation of NFAT (17, 19, 24). Second, calcineurin-independent and -dependent roles for RCAN1 have been characterized for the inhibition of NF-κB through decreased phosphorylation of the inhibitory protein IκBα, leading to increased protein stability and inhibition of the NF-κB pathway (43, 44). To assess the contribution of this second pathway to enhanced cytokine production in the absence of RCAN1, lysates were collected from wild-type and RCAN1-deficient bone marrow–derived macrophages treated with P. aeruginosa strain PAK at a 1:1 MOI for various times. These lysates were subjected to Western blot analysis for total IκBα as well as the phosphorylated form of the protein and an actin-loading control (Fig. 3E). Blots from three separate experiments were quantified using scanning densitometry, and the average fold increase relative to wild-type untreated samples was determined (Fig. 3F). IκBα was found to be significantly hyperphosphorylated at the early 3- and 6-hpi time points in the RCAN1-deficient cells, whereas no significant differences were observed with longer treatments. Similar results were obtained when cells were treated with P. aeruginosa strain 8821 at a 1:10 MOI (Supplemental Fig. 3E, 3F), showing that RCAN1 impairs NF-κB activation through decreased phosphorylation of IκBα during P. aeruginosa infection.
Enhanced inflammatory cytokine production in RCAN1-deficient mice is accompanied by increased proinflammatory transcription factor activity following P. aeruginosa lung infection

NFAT and NF-κB are master regulators of cytokine and chemokine production. We next assessed the activity of these transcription factors by performing EMSAs on nuclear extracts prepared from the lungs of wild-type and RCAN1-deficient mice infected with P. aeruginosa strain 8821 for 4 or 24 h. The activity of NF-κB was greatly enhanced in the lungs of RCAN1-deficient mice 4 hpi, whereas only a marginal increase was observed at 24 h that did not reach statistical significance (Fig. 4A, 4C). However, the activity of the proinflammatory transcription factor NFAT was greatly enhanced in RCAN1-deficient mice at both the early 4-hpi and late 24-hpi time points (Fig. 4B, 4D). Similar results were obtained for 4-h infection with 1 LD₅₀ of P. aeruginosa strain PAK for both NFAT and NF-κB (Supplemental Fig. 4A, 4B). These results confirm that the RCAN1 greatly impacts the inflammatory response in vivo through temporally distinct negative regulation of two distinct proinflammatory transcription factors.

The ERK/STAT3 axis is regulated by RCAN1 following P. aeruginosa infection in vitro and in vivo

Recently, increased STAT3 activity has been reported in DS patients, supporting a clinically relevant role for dysregulating of the pathway (61). However, a role for RCAN1 in the regulation of the STAT3 pathway has not been explored. To assess the activation of the STAT3 pathway in vitro, bone marrow–derived macrophages from wild-type and RCAN1-deficient mice were treated with P. aeruginosa strain 8821 at a MOI of 1:10. Lysates were collected at various times, and Western blot analysis was performed. The MAPK ERK was found to be hyperphosphorylated in both untreated and RCAN1-deficient cells, as well as at early time points postinfection with P. aeruginosa (Fig. 5A, 5D). Two other MAPKs, JNK and p38, showed no differences in activation state (data not shown).

As STAT3 is a target of ERK kinase activity at serine 727, a phosphorylation event required for transcriptional activity of the transcription factor (62), we next set out to determine the effects of increased ERK activation in RCAN1-deficient cells on the STAT3 pathway. Western blot analysis indicated that STAT3 was hyperphosphorylated at serine 727 in untreated RCAN1-deficient macrophages, as well as at early time points postinfection with P. aeruginosa (Fig. 5A, 5B).

In addition to phosphorylation at serine 727, STAT3 activity also requires phosphorylation at tyrosine residue 705 to drive dimerization and nuclear translocation of the protein, which is induced

Table I. RCAN1-deficient mice display enhanced early inflammatory cytokine production

<table>
<thead>
<tr>
<th></th>
<th>Uninfectedᵃ</th>
<th>4 hᵇ</th>
<th>24 hᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>93.7 ± 44.4</td>
<td>63 ± 25.6</td>
<td>117.8 ± 17.4***</td>
</tr>
<tr>
<td>IL-6β</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1793.9 ± 201.6***</td>
</tr>
<tr>
<td>TNF</td>
<td>17.1 ± 2.4</td>
<td>19.5 ± 3.1</td>
<td>89.6 ± 4.4**</td>
</tr>
<tr>
<td>MIP-2</td>
<td>24.7 ± 7.4</td>
<td>66.9 ± 2.4</td>
<td>1912.1 ± 207.9***</td>
</tr>
<tr>
<td>LIX</td>
<td>197.8 ± 86.2</td>
<td>498.1 ± 196.6</td>
<td>1135.3 ± 53.0**</td>
</tr>
<tr>
<td>KC</td>
<td>318.2 ± 116.3</td>
<td>819.5 ± 207.3</td>
<td>6811.5 ± 329.8***</td>
</tr>
<tr>
<td>TGF-β</td>
<td>241.2 ± 99.9</td>
<td>379.2 ± 57.6</td>
<td>350.3 ± 35.2*</td>
</tr>
<tr>
<td>BALF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>57.4 ± 4.5</td>
<td>64.1 ± 8.6</td>
<td>330.8 ± 81.0**</td>
</tr>
<tr>
<td>IL-6β</td>
<td>N.D.</td>
<td>N.D.</td>
<td>134.3 ± 3.1*</td>
</tr>
<tr>
<td>TNF</td>
<td>19.9 ± 1.2*</td>
<td>32.4 ± 2.9*</td>
<td>502.3 ± 35.3***</td>
</tr>
<tr>
<td>MIP-2</td>
<td>49.4 ± 18.0</td>
<td>91.9 ± 36.4</td>
<td>2475.5 ± 467.3***</td>
</tr>
<tr>
<td>LIX</td>
<td>191.3 ± 102.4</td>
<td>77.8 ± 24.4</td>
<td>402.7 ± 136.3*</td>
</tr>
<tr>
<td>KC</td>
<td>14.4 ± 7.5*</td>
<td>87.8 ± 16.0*</td>
<td>6454.8 ± 348.7*</td>
</tr>
<tr>
<td>TGF-β</td>
<td>30.0 ± 18.1</td>
<td>139.2 ± 28.2*</td>
<td>42.6 ± 28.1*</td>
</tr>
</tbody>
</table>

ᵃData are the mean ± SE of 6 mice per group (pg/ml).
ᵇData are the mean ± SE of 3 mice per group (pg/ml).
ᶜData are the mean ± SE of 10 mice per group (pg/ml).

* p < 0.05, ** p < 0.01, *** p < 0.001.
N.D., Not detected.

FIGURE 3. RCAN1-deficient macrophages display enhanced production of proinflammatory cytokines in response to P. aeruginosa infection. Wild-type (+/+ ) and RCAN1-deficient (−/− ) bone marrow–derived macrophages were left untreated (NT) or exposed to P. aeruginosa strain PAK at a MOI of 1:1. Supernatants were collected at various time points and analyzed for the proinflammatory cytokines MIP-2 (A), TNF (B), IL-1β (C), and IL-6 (D) (n = 3 ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001). Lysates were prepared and subjected to Western blot for phospho and total-IκBα as well as actin-loading control (E). Blots are representative of three separate experiments, which were quantified by scanning densitometry (F) (n = 3 ± SEM, * p < 0.05, *** p < 0.001).
three individual experiments. Scanning densitometry was performed (k labeled NF-phosphorylation in both resting and (63). Western blot analysis showed greatly enhanced tyrosine 705 via IL-6R (CD126) through a JAK/STAT-dependent mechanism 2P. aeruginosa transcription factor activation in vivo following RCAN1-deficient mice display enhanced inflammatory tran- 5C). To assess the impact on aberrant STAT3 phosphorylation in the absence of RCAN1 on transcriptional activity, nuclear extracts collected from wild-type and RCAN1-deficient macrophages were prepared and subjected to EMSA. STAT3 transcriptional activity was found to be significantly impaired at early time points postinfection in RCAN1-deficient macrophages (Fig. 5E, 5G). However, at later time points postinfection, STAT3 activity was significantly increased in RCAN1-deficient macrophages. Probe specificity was confirmed using competition and super shift assays (data not shown). Together these results indicate that the STAT3 pathway is dysregulated in RCAN1-deficient macrophages. Probe specificity was confirmed with DS-associated gene RCAN1 as a negative regulator of inflammation in a P. aeruginosa model of bacterial pneumonia. RCAN1 deficiency was found to be associated with an inappropriate inflammatory response following P. aeruginosa infection both in vitro and in vivo. This unchecked inflammatory response was associated with dysregulation of three critical inflammatory pathways involving the transcription factors NFAT, NF-kB, and STAT3. The resulting increase in inflammatory cytokine production led to increased neutrophil infiltration and bacterial clearance from the lungs of RCAN1-deficient mice. However, the end result of RCAN1 deficiency was increased mortality associated with systemic inflammation, which could not be corrected with antibiotic therapy. To our knowledge, these results show for the first time that RCAN1 is a central negative regulator of inflammation during P. aeruginosa lung infections.

It is worthy to note that the NFAT, NF-kB, and STAT3 pathways shown in this study to be regulated by RCAN1 are universally important during respiratory tract infections and have been extensively studied during Pneumococcus infection (64–66). Thus, it is likely that the phenotype observed in RCAN1-deficient animals may not be specific to P. aeruginosa infection. RCAN1 may represent a general host defense mechanism in greater clinical settings such as Pneumococcus infection.

In addition to the novel insights into the regulation of the inflammatory response by RCAN1, this study also shed light on a previously unrecognized protective role for RCAN1 against systemic inflammation. Upon infection with 1 LD50 of Paerugi- nosa, RCAN1-deficient mice all succumbed within the first 18 h. This mortality was associated with elevated levels of serum cytokines, demonstrating widespread systemic inflammation that could not be corrected with antibiotic therapy. Collectively, our findings help to elucidate the protective biological functions of RCAN1 in the context of infection and inflammation and implicate dysregulation of NFAT, NF-kB, and STAT3 signaling in the enhanced mortality observed in RCAN1-deficient mice.

RCAN1 is widely expressed in various tissues throughout the body (17, 20, 21) and is overexpressed in DS patients (18). Intriguingly, previous studies have proposed RCAN1 as both an inhibitor (67–70) and an activator (19, 23, 71–73) of inflammation via calcineurin, suggesting that the biological activity of the protein might be context dependent. To our knowledge, our find- ings in this work provide the first evidence of a negative regulatory role for RCAN1 in inflammation during respiratory tract infec- tions using an in vivo model of bacterial pneumonia. Interestingly, we found temporally distinct effects of RCAN1 on the kinetics of two important proinflammatory transcription factors. NFAT activity was greatly enhanced in the lungs of RCAN1-deficient mice throughout the course of bacterial infection. However, NF-kB activity was only observed to be enhanced early postinfection in via IL-6R (CD126) through a JAK/STAT-dependent mechanism (63). Western blot analysis showed greatly enhanced tyrosine 705 phosphorylation in both resting and P. aeruginosa–infected RCAN1-deficient macrophages compared with wild-type controls (Fig. 5A, 5C). To assess the impact on aberrant STAT3 phosphorylation in the absence of RCAN1 on transcriptional activity, nuclear extracts collected from wild-type and RCAN1-deficient macrophages were prepared and subjected to EMSA. STAT3 transcriptional activity was found to be significantly impaired at early time points postinfection in RCAN1-deficient macrophages (Fig. 5E, 5G). However, at later time points postinfection, STAT3 activity was significantly increased in RCAN1-deficient macrophages. Probe specificity was confirmed using competition and super shift assays (data not shown). Together these results indicate that the STAT3 pathway is dysregulated in RCAN1-deficient macrophages in both untreated and P. aeruginosa–treated cells in vitro.

Having seen altered STAT3 activation in vitro, we next set out to measure STAT3 transcriptional activity in vivo. EMSA was per- formed on lungs from wild-type and RCAN1-deficient mice treated with saline (NT) or infected with P. aeruginosa strain 8821 for 4 or 24 h, using a DNA probe specific for STAT3. No STAT3 activity was observed in uninfected lungs of wild-type or RCAN1-deficient mice, and increased activity was observed in the lungs of wild-type mice 4 hpi (Fig. 5F, 5H). However, at the later 24-hpi time point, STAT3 activity was only observed in lungs of RCAN1-deficient mice. Together these data suggest that RCAN1 defi- ciency delays and prolongs STAT3 activation following P. aeruginosa infection in vivo.

Discussion

DS is the most common chromosomal anomaly among live born infants and is associated with a variety of immunological defects (1–4). These defects combined with anatomical factors associated with DS lead to greatly increased risk of severe and persistent respiratory tract infections, including viral and bacterial pneu- monias (11–14). A handful of DS-associated genes has been im- plicated in these respiratory syndromes, and understanding their relative contributions to impaired immunological function is a critical step toward improving treatment and prevention of respiratory tract infections in these high-risk patients. In the current study, we demonstrate a critical role for the DS-associated gene RCAN1 as a negative regulator of inflammation in a P. aeruginosa model of bacterial pneumonia. RCAN1 deficiency was found to be associated with an inappropriate inflammatory response following P. aeruginosa infection both in vitro and in vivo. This unchecked inflammatory response was associated with dysregulation of three critical inflammatory pathways involving the transcription factors NFAT, NF-kB, and STAT3. The resulting increase in inflammatory cytokine production led to increased neutrophil infiltration and bacterial clearance from the lungs of RCAN1-deficient mice. However, the end result of RCAN1 deficiency was increased mortality associated with systemic inflammation, which could not be corrected with antibiotic therapy. To our knowledge, together these results help to elucidate the protective biological functions of RCAN1 in the context of infection and inflammation and implicate dysregulation of NFAT, NF-kB, and STAT3 signaling in the enhanced mortality observed in RCAN1-deficient mice.
the lungs of RCAN1-deficient mice. The differential control exerted by RCAN1 over these pathways most likely reflects distinct molecular regulatory mechanisms. RCAN1 can exert sustained inhibition of NFAT via direct interactions with calcineurin (17, 19, 24). By contrast, RCAN1 controls NF-κB activation through poorly understood mechanisms leading to the stabilization of the IκB–NF-κB complex (43, 44). It is perhaps the differential regulation of these transcription factors that accounts for the cytokine profiles observed during *P. aeruginosa* lung infection in vivo. Whereas all inflammatory cytokines monitored were greatly elevated in RCAN1-deficient mice early in infection, fewer differences were observed later in infection, and those that remained were less pronounced.

Recently, increased STAT3 expression has been reported in DS patients, implying a clinically relevant role for dysregulation of the pathway (61). As STAT3 represents an important regulator of inflammation, we set out to see what effect, if any, RCAN1 had on the pathway. Surprisingly, we identified a previously unknown regulatory role for RCAN1 in STAT3 activation, both in vitro and in vivo. Hyperactivation of the ERK MAPK was observed in vitro, coinciding with increased phosphorylation of the ERK target STAT3 at serine 727 in untreated cells, as well as at early time points postinfection. By contrast, phosphorylation of STAT3 at tyrosine 705, which was also enhanced in RCAN1-deficient cells, was only observed following *P. aeruginosa* infection. These patterns of aberrant STAT3 phosphorylation led to delayed and prolonged STAT3 activation following *P. aeruginosa* infection in vitro. Similarly, the in vivo kinetics of STAT3 activation was substantially altered in RCAN1-deficient mice, which displayed impaired early STAT3 activation that persisted later in infection, after the wild-type response had disappeared. These delayed STAT3 kinetics correlated with the levels of the immunomodulatory cy-
tokine TGF-β, suggesting that RCAN1-mediated STAT3 activation may be playing primarily an anti-inflammatory role during P. aeruginosa lung infection. These results are not entirely unanticipated as, in contrast to NFAT and NF-κB, which are strictly proinflammatory transcription factors, STAT3 acts both to drive the inflammatory response and participate in its resolution. Indeed, various in vivo knockout models have found that inflammatory cytokine production is not impaired by STAT3 deficiency, but is instead enhanced, leading to a variety of inflammatory pathologies (74), including increased susceptibility to endotoxemia (75), as is instead enhanced, leading to a variety of inflammatory pathologies

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Jean Marshall for input during the writing of the manuscript.

References


B. which are strictly proinflammatory transcription factors, STAT3 acts both to drive the inflammatory response and participate in its resolution. Indeed, various in vivo knockout models have found that inflammatory cytokine production is not impaired by STAT3 deficiency, but is instead enhanced, leading to a variety of inflammatory pathologies (74), including increased susceptibility to endotoxemia (75), as is instead enhanced, leading to a variety of inflammatory pathologies

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Jean Marshall for input during the writing of the manuscript.

References


B. which are strictly proinflammatory transcription factors, STAT3 acts both to drive the inflammatory response and participate in its resolution. Indeed, various in vivo knockout models have found that inflammatory cytokine production is not impaired by STAT3 deficiency, but is instead enhanced, leading to a variety of inflammatory pathologies (74), including increased susceptibility to endotoxemia (75), as is instead enhanced, leading to a variety of inflammatory pathologies

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Jean Marshall for input during the writing of the manuscript.

References


B. which are strictly proinflammatory transcription factors, STAT3 acts both to drive the inflammatory response and participate in its resolution. Indeed, various in vivo knockout models have found that inflammatory cytokine production is not impaired by STAT3 deficiency, but is instead enhanced, leading to a variety of inflammatory pathologies (74), including increased susceptibility to endotoxemia (75), as is instead enhanced, leading to a variety of inflammatory pathologies

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Jean Marshall for input during the writing of the manuscript.

References


B. which are strictly proinflammatory transcription factors, STAT3 acts both to drive the inflammatory response and participate in its resolution. Indeed, various in vivo knockout models have found that inflammatory cytokine production is not impaired by STAT3 deficiency, but is instead enhanced, leading to a variety of inflammatory pathologies (74), including increased susceptibility to endotoxemia (75), as is instead enhanced, leading to a variety of inflammatory pathologies

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Jean Marshall for input during the writing of the manuscript.

References


Supplementary Figure Legends:

Figure S1: RCAN1 is induced in response to *P. aeruginosa* infection. Wild-type (+/+) and RCAN1-deficient (-/-) bone marrow derived macrophages were treated with *P. aeruginosa* strain 8821 at and MOI of 1:10. At the indicated time point lysates were prepared, and western blot analysis was performed (A). The blots were subjected to scanning densitometry for quantification (B). (n=2 +/- SEM).

Figure S2: RCAN1-deficient macrophages display enhanced production of pro-inflammatory cytokines in response to *P. aeruginosa* infection: Wild-type (+/+) and RCAN1-deficient (-/-) bone marrow derived macrophages were left untreated (NT) or exposed to *P. aeruginosa* strain PAK at increasing MOIs for 6 hours. Supernatants were collected and analyzed for the pro-inflammatory cytokines MIP-2 (A), TNF (B), IL-1β (C) and IL-6 (D). (n=3 +/- SEM,*p<0.05, **p<0.01)

Figure S3: RCAN1-deficient macrophages display enhanced cytokine production and IκBα phosphorylation following *P. aeruginosa* infection. Wild-type (+/+) and RCAN1 deficient (-/-) bone marrow derived macrophages were treated with *P. aeruginosa* strain 8821 at and MOI of 1:10. Supernatants were collected at various time points and analyzed for inflammatory cytokines by ELISA (A-D). (n=3 +/-SEM, *p<0.05, **p<0.01). Lysates were also collected at each indicated time point and western blot analysis was performed for phospho and total IκBα as well as actin loading control (E). The blots were subjected to scanning densitometry for quantification (F). (n=3 +/- SEM, *p<0.05, **p<0.001).
Figure S4: RCAN1-deficient mice display enhanced inflammatory transcription factor activation *in vivo* following *P. aeruginosa* infection. RCAN1 wild type (+/+ ) and knockout (-/-) mice were infected intranasally with $2.25 \times 10^7$ *P. aeruginosa* strain PAK, or an equivalent volume of saline (NT). Nuclear proteins were extracted from lung tissues obtained from individual mice and were subjected to EMSA by incubation with $^{32}$P-labeled NFAT (A) or NF-κB (B), or DNA probes.
Fig. S1

A

<table>
<thead>
<tr>
<th></th>
<th>RCAN1 +/+</th>
<th>RCAN1 +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps.a 1:10 MOI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>1h</td>
<td>2h</td>
</tr>
</tbody>
</table>

-RCAN1.1 Long

-RCAN1 short

-Actin

B

<table>
<thead>
<tr>
<th></th>
<th>RCAN1 Long</th>
<th>RCAN1 Short</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2h</td>
<td>3.0 ± 0.2</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>4h</td>
<td>4.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>
Fig. S2

A. MIP-2 (pg/mL)

B. TNF (pg/mL)

C. IL-1β (pg/mL)

D. IL-6 (pg/mL)
Fig. S3

A. MIP-2 (ng/mL) levels over time for RCAN1 +/+ and RCAN1 -/- mice.

B. TNF (ng/mL) levels over time for RCAN1 +/+ and RCAN1 -/- mice.

C. IL-1β (ng/mL) levels over time for RCAN1 +/+ and RCAN1 -/- mice.

D. IL-6 (ng/mL) levels over time for RCAN1 +/+ and RCAN1 -/- mice.

E. Western blot analysis of total and phosphorylated IkBα for RCAN1 +/+ and RCAN1 -/- mice with and without Ps.a 1:1 MOI.

F. Fold change in p-IkBα/Total IkBα over time for RCAN1 +/+ and RCAN1 -/- mice.
Fig. S4

A

<table>
<thead>
<tr>
<th>BLANK</th>
<th>NT</th>
<th>4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>-</td>
<td>+/+</td>
</tr>
<tr>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
</tr>
</tbody>
</table>

-Free Probe

-NFAT

B

<table>
<thead>
<tr>
<th>BLANK</th>
<th>NT</th>
<th>4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>-</td>
<td>+/+</td>
</tr>
<tr>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
</tr>
</tbody>
</table>

-NFkB

-Free Probe