RAGE and the innate immune response in Infection and Inflammation

van Zoelen, M.A.D.

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Receptor for advanced glycation end products is protective during murine tuberculosis

Marieke A.D. van Zoelen¹,², Catharina W. Wieland¹,², Gerritje J. W. van der Windt¹,², Jennie M. Pater¹,², Sandrine Florquin³, Peter Nawroth⁴, Angelika Bierhaus⁴ and Tom van der Poll¹,²

Submitted

¹Center for Infection and Immunity Amsterdam (CINIMA), ²Center for Experimental and Molecular Medicine (CEMM), ³Department for Pathology; Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
⁴Department of Internal Medicine, University of Heidelberg, Heidelberg, Germany
ABSTRACT

The development of active tuberculosis after infection with *Mycobacterium (M.) tuberculosis* is almost invariably caused by a persistent or transient state of relative immunodeficiency. The receptor for advanced glycation end products (RAGE) is a promiscuous receptor that is involved in pulmonary inflammation and infection. To investigate the role of RAGE in tuberculosis, we intranasally infected wild-type (Wt) and RAGE deficient (RAGE−/−) mice with live virulent *M. tuberculosis*. While lungs of infected Wt mice expressed RAGE, in particular on endothelium, *M. tuberculosis* pneumonia was associated with an enhanced expression of pulmonary RAGE. Lung inflammation was increased in RAGE−/− mice, as indicated by histopathology, percentage of inflamed area, lung weight and cytokine and chemokine levels. In addition, lung lymphocyte and neutrophil numbers were increased in the RAGE−/− mice. RAGE−/− mice displayed higher mycobacterial loads in the lungs after 3 weeks of infection, while they showed similar loads in the liver at 3 and 6 weeks. Finally, RAGE−/− mice displayed body weight loss and a worsened *M. tuberculosis* induced mortality. These data suggest that RAGE plays a beneficial role in the host response to pulmonary tuberculosis.
INTRODUCTION

Tuberculosis remains a major health burden world-wide, responsible for eight million new cases and two million deaths each year (1, 2). The causing microorganism *Mycobacterium (M.) tuberculosis* is one of the most effective pathogens in man, with approximately one-third of the world’s population being infected. Multidrug-resistant strains are on the rise and the frequent occurrence of co-infection with the human immunodeficiency virus make the treatment and outcome of tuberculosis even more worrisome.

By far the most common site of infection in tuberculosis is the lung. The receptor for advanced glycation end products (RAGE) is a promiscuous receptor that is ubiquitously expressed in the pulmonary compartment (3). RAGE was first extracted and sequenced from bovine lungs (4, 5). Subsequent studies have confirmed the expression of RAGE in normal healthy lungs (6-9) and in addition demonstrated that RAGE expression is up-regulated during pulmonary inflammation (7-9). RAGE interacts with a variety of ligands such as advanced glycation end products (10), amyloid (11), β-sheet fibrils (12), high mobility group box 1 (13, 14) and some members of the S100 family (15-17). Binding of these ligands to RAGE triggers sustained receptor-dependent signaling and activation of nuclear factor-κB and mitogen-activated protein kinase pathways. The notion that RAGE is an important pro-inflammatory receptor is supported by experimental studies in which inhibition of RAGE signaling attenuated inflammatory responses; these studies included models of hepatic injury (18-20), diabetic atherosclerosis (21, 22), delayed type hypersensitivity (23, 24), type II collagen-induced arthritis (24), experimental autoimmune encephalomyelitis (25) and sepsis (23).

Recently, RAGE expression was visualized in the lungs of two patients with tuberculosis in particular in epitheloid and giant cells, as well as in reactive pneumocytes (7). This prompted us to study the role of RAGE in the chronic lung inflammation that accompanies pulmonary tuberculosis.

MATERIALS AND METHODS

Mice
Pathogen-free 8 to 10 weeks old Wt C57Bl/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). RAGE−/− mice, backcrossed ten times to a C57Bl/6 background, were generated as described previously (23). The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.
**Experimental infection**

A virulent laboratory strain of *M. tuberculosis* H37Rv (American Type Culture Collection, Rockville, MA) was grown for 4 days in liquid Dubos medium containing 0.01% Tween-80. A replicate culture was incubated at 37 °C, harvested at mid-log phase, and stored in aliquots at −70 °C. For each experiment, a vial was thawed and washed with sterile 0.9% NaCl. Tuberculosis was induced as described previously (26-28). Briefly, mice were anesthetized by inhalation with isoflurane (Abbott Laboratories LTD., Kent, United Kingdom) and infected intranasally (i.n.) with \(10^5\) live *M. tuberculosis* H37Rv bacilli in 50 µl saline, as determined by viable counts on Middlebrook 7H11 plates. Mice were sacrificed 3 or 6 weeks after infection (\(N = 7-8\) per group at both time points) or 28 weeks after infection (experiment started with 12-14 mice per group). In addition, in order to check infection efficacy, 3 mice per group were sacrificed one day post-infection. Lungs and liver were removed aseptically, and homogenized in 5 volumes of sterile 0.9% NaCl. Ten-fold dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days incubation at 37 °C. Numbers of CFU are provided per g of lungs.

**Histology**

Lungs were removed 3, 6 or 28 weeks after inoculation with *M. tuberculosis*, fixed in 10% buffered formaldehyde for 24 h and embedded in paraffin. Hematoxilin and eosin stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: Interstitial inflammation, vasculitis, bronchitis, edema, granuloma formation and pleuritis by a pathologist blinded for groups. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 24 (29). Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung surface.

**Characterization of inflammatory infiltrates in the lungs**

Pulmonary cell suspensions were obtained by crushing lungs through a 40-µm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) as described previously (26, 27). Erythrocytes were lysed with ice-cold isotonic NH4Cl solution (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium), and counted by using a hemocytometer. The percentages of macrophages, polymorphonuclear cells and lymphocytes were determined using cytopsin preparations stained with H&E. In addition, cells were brought to a concentration of \(1 \times 10^7\) cells per mL of FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN₃ and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 minutes at 4 °C using directly labeled antibodies against CD3 (CD3-phycoerythin), CD4 (CD4-allophycocyanin), CD8 (CD8-peridinin...
chlorophyl protein) or GR-1 (GR-1-FITC). All antibodies were used in concentrations recommended by the manufacturer (BD Pharmingen, San Diego, CA). After staining, cells were fixed in 2 % paraformaldehyde, and determined using flow cytometric analysis using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Percentages of polymorphonuclear cells (PMNs), macrophages and lymphocytes were determined using GR-1 expression (GR-1 high, intermediate and low, respectively) and T cell surface proteins were analyzed on CD3+ cells within the lymphocyte gate.

Assays
For cytokine measurements, lung homogenates were diluted two times in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 2 % Triton X-100, and AEBSF (4-(2-aminoethyl)benzeneisothiocyanate), EDTA-NA₂, Pepstatin and Leupeptin (all 8 µg/ml; pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at 1500 x g at 4 °C for 15 min., and supernatants were sterilized using a 0.22 µm filter (Corning Incorporated, Corning, NY) and stored at –20 °C until assays were performed. Interferon (IFN)-γ, interleukin (IL)-4, TNF-α, IL-10, IL-1β, IL-6, keratonicyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) were measured by ELISA using matched antibody pairs according to the manufacturer’s instructions (R&D Systems Inc., Minneapolis, Minnesota, USA).

Statistical analysis
All values are expressed as mean ± SE. Differences between groups were analyzed by Mann-Whitney U test. Survival curves were compared by log rank test. When comparing two groups at multiple time points two way ANOVA was used. Statistical analyses of bacterial counts were performed after log transformation. Values of P < .05 were considered statistically significant.

Results
M. tuberculosis pneumonia results in enhanced RAGE expression in the lungs
Earlier studies indicated that normal, healthy lungs express RAGE (6-9) and that expression of RAGE in the lungs may be increased in patients with tuberculosis (7). To study whether RAGE expression changes during experimentally induced pulmonary tuberculosis, we performed immunohistochemical stainings of RAGE of lung tissue from Wt mice 3 and 6 weeks after inoculation with M. tuberculosis. In line with previous studies (6-9), normal healthy mice displayed broad RAGE staining in their lungs (Figure 1A). RAGE was predominantly present in the interalveolar septae, showing an endothelial pattern, while bronchial epithelial cells were negative for RAGE staining (Figure 1A, arrow). Immunohistochemical analysis of lungs
obtained from RAGE−/− mice, used as negative controls, confirmed the specificity of the RAGE staining (Figure 1B). Lungs from *M. tuberculosis* infected mice showed a similar pattern of RAGE positivity as the lungs from normal healthy mice, i.e. the interalveolar septae were positive for RAGE staining displaying an endothelial pattern 3 and 6 weeks after inoculation (Figure 1C and D, respectively).

**RAGE−/− mice demonstrate enhanced pulmonary inflammation**

We next evaluated whether RAGE influences the inflammatory response in the lungs during tuberculosis. Histopathological examination of lung tissue revealed profound differences between Wt and RAGE−/− mice at 3 and to a lesser extent at 6 weeks after inoculation, revealing an enhanced inflammatory response in RAGE−/− mice (Figure 2A-D). In line, the mean total histology score of the lungs (determined using the scoring system described in “Materials and Methods”) was higher in the RAGE−/− mice compared to that of the Wt mice (Figure 2E; *P* < .05 at both time points). Furthermore, the lungs of RAGE−/− mice displayed more confluent areas of inflammation (Figure 2F; *P* < .005 at both time points). At 3 weeks, lungs of Wt mice displayed granulomatous inflammatory infiltrates primarily located around small bronchi and vessels composed of lymphocytes and macrophages with few granulocytes, confirming earlier observations (26, 27, 30). Moreover, edema and pleuritis were more pronounced in RAGE−/− than in Wt mice. Furthermore, the lung weights of RAGE−/− mice at 3 and 6 weeks post inoculation with *M. tuberculosis* were much higher than those of Wt mice (Figure 2F; *P* < .005 at both time points), indicative for enhanced inflammation and edema. To evaluate the cellular composition of the pulmonary infiltrates in Wt and RAGE−/− mice, we prepared whole lung suspensions at 3 weeks after infection (Table 1). The lungs of RAGE−/− mice contained...
more leukocytes ($P < .001$ versus Wt mice), which was due to a profound rise in the number of lymphocytes and neutrophils ($P < .0005$ and $P < .005$ versus Wt mice, respectively). To determine whether the enhanced influx of lymphocytes in RAGE$^{-/-}$ mice was restricted to a certain subset, we analyzed whole lung cell suspensions by flow cytometry (Table 2). This revealed that the percentages of CD4$^+$ and CD8$^+$ T-cells within the CD3$^+$ population did not differ between RAGE$^{-/-}$ and Wt mice. Moreover, the CD8$^+$ T-cells in the CD3$^+$ populations of both mouse strains equally expressed the “early” activation marker CD69, while CD69 expression on CD4$^+$ T-cells was decreased after weeks in the RAGE$^{-/-}$ mice compared to the Wt mice ($P < .0006$, Table 2).

**Figure 2.** RAGE$^{-/-}$ mice display enhanced lung inflammation. Wt and RAGE$^{-/-}$ mice were inoculated intranasally with $10^5$ CFUs *M. tuberculosis*. Representative HE stainings of lung tissue at 3 (A and B) and 6 (C and D) weeks post inoculation (PI) in Wt (A and C) and RAGE$^{-/-}$ (B and D) mice. Original magnification 4x, inserts 20x. Graphical representation of the degree of lung inflammation at 3 and 6 weeks (E) and percentage of inflamed area (F), determined according to the scoring system described in the Materials and Methods section. Lung weight in grams (F). Data are means ± SE of 8 mice per genotype at each time point. *, $P < .05$ vs Wt mice; ***, $P < .005$ vs Wt mice.
Table 1. Leukocyte counts in lung homogenates

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>RAGE⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
<td>180 ± 16</td>
<td>344 ± 40*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>19 ± 3</td>
<td>39 ± 6†</td>
</tr>
<tr>
<td>Macrophages</td>
<td>91 ± 8</td>
<td>109 ± 16</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>71 ± 7</td>
<td>197 ± 21‡</td>
</tr>
</tbody>
</table>

Data are mean numbers ± SE of cells x 10⁵/ml at 3 weeks after i.n. inoculation of 10⁵ CFUs *M. tuberculosis*. N = 7-8 mice per genotype.

* P < .001 versus Wt mice.
† P < .005 versus Wt mice.
‡ P < .0005 versus Wt mice.

Table 2. Cellular composition in the lungs at 3 weeks after inoculation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Wt</th>
<th>RAGE⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺</td>
<td>64.0 ± 0.6</td>
<td>64.2 ± 1.4</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>36.1 ± 0.6</td>
<td>35.8 ± 1.4</td>
</tr>
<tr>
<td>CD4⁺/CD69⁺</td>
<td>66.7 ± 2</td>
<td>58.4 ± 3 ‡</td>
</tr>
<tr>
<td>CD8⁺/CD69⁺</td>
<td>71.4 ± 2</td>
<td>68.3 ± 1</td>
</tr>
</tbody>
</table>

Data are mean numbers ± SE of cells x 10⁵/ml at 3 weeks after i.n. inoculation of 10⁵ CFUs *M. tuberculosis*. N = 7-8 mice per genotype.

‡ P < .0005 versus Wt mice.

**RAGE⁻/⁻ mice have elevated lung levels of pro-inflammatory cytokines and chemokines**

Cytokines and chemokines play a pivotal role in the regulation of the immune response to tuberculosis (2, 31-33). Therefore, we measured the concentrations of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-1β, the anti-inflammatory cytokines IL-4 and IL-10 and the chemokines KC and MIP-2 in lung homogenates obtained 3 and 6 weeks after infection (Table 3). At 3 weeks post infection, the pulmonary concentrations of TNF-α, IL-6 and IL-1β were higher in RAGE⁻/⁻ mice (P < .01 vs. Wt mice); after 6 weeks IL-6 and IL-1β levels were still elevated in the RAGE⁻/⁻ mice (P < .05 vs. Wt mice).
Of the anti-inflammatory cytokines, IL-4 levels were increased after 3 weeks in RAGE-/- mice, while IL-10 levels were similar in both mouse strains at both time points. With regard to chemokines, both KC and MIP-2 concentrations were higher in lungs from RAGE-/- than in lungs from Wt mice at 3 weeks after infections (P < .05).

**Table 3. RAGE-/- mice display reduced cytokine and chemokine levels in their lungs**

<table>
<thead>
<tr>
<th></th>
<th>3 wks</th>
<th>6 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt</td>
<td>Wt</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>199 ± 13</td>
<td>204 ± 12</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1156 ± 82</td>
<td>1580 ± 58 †</td>
</tr>
<tr>
<td>IL-6</td>
<td>232 ± 46</td>
<td>674 ± 190 †</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5119 ± 657</td>
<td>12358 ± 668 †</td>
</tr>
<tr>
<td>IL-4</td>
<td>67 ± 4 *</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>IL-10</td>
<td>156 ± 9</td>
<td>168 ± 7</td>
</tr>
<tr>
<td>KC</td>
<td>2358 ± 140</td>
<td>3209 ± 251 *</td>
</tr>
<tr>
<td>MIP-2</td>
<td>720 ± 12</td>
<td>865 ± 36 †</td>
</tr>
</tbody>
</table>

Data are mean numbers ± SE of cells x 10^5/ml at 3 weeks after i.n. inoculation of 10^5 CFUs *M. tuberculosis*. N = 7-8 mice per genotype.

* P < .001 versus Wt mice.
† P < .005 versus Wt mice.
‡ P < .0005 versus Wt mice.

**RAGE-/- mice display a transiently enhanced outgrowth of *M. tuberculosis***

Having established that RAGE is involved in the regulation of pulmonary inflammation in our model of lung tuberculosis, we determined the impact of RAGE deficiency on the growth and dissemination of *M. tuberculosis*. RAGE deficiency had a modest effect on the growth of mycobacteria in the lung: at 3 weeks after infection the number of CFUs recovered from the lungs of RAGE-/- mice was 2.4-fold higher than that harvested from Wt lungs (Figure 3A, P < 0.01). At 6 weeks post infection, the mycobacterial burdens in the lungs of RAGE-/- and Wt mice were similar (Figure 3A). To study the dissemination of *M. tuberculosis* to distant organs, we determined the bacterial loads in the livers. No differences in bacterial outgrowth were observed between the livers from RAGE-/- and Wt mice at either 3 or 6 weeks after infection (Figure 3B).
Chapter 5

RAGE-/- mice demonstrate an enhanced lethality after \( M. \) \textit{tuberculosis} infection

To investigate the role of RAGE in the outcome of tuberculosis, mice were inoculated with \( M. \) \textit{tuberculosis}, their body weight was measured and the mice were followed for 28 weeks. RAGE-/- but not Wt mice demonstrated a loss of body weight after \( M. \) \textit{tuberculosis} infection (Figure 4A). In addition, RAGE-/- mice showed an increased mortality. The first deaths among RAGE-/- mice occurred 18 weeks after infection and overall mortality was 58\% (Figure 4B). In contrast, mortality among Wt mice did not occur until 22 weeks post infection and overall mortality was only 12\% (\( P < .05 \) for the difference between RAGE-/- and Wt mice).

\textbf{Discussion}

The primary aim of this study was to examine the role of RAGE in the regulation of the pulmonary response to tuberculosis. We hypothesized that RAGE would have a marked influence on the chronic lung inflammation that accompanies tuberculosis, considering its strong expression within the pulmonary compartment (3-9) and its established role as a pro-inflammatory receptor in multiple disease models (11, 21-25, 34). Much to our surprise, RAGE deficiency resulted in an enhanced inflammatory response in the lungs of mice infected with \( M. \) \textit{tuberculosis} via the airways, which was associated with an adverse

\textbf{Figure 3.} Decreased early pulmonary bacterial outgrowth in lungs of RAGE-/- mice after \( M. \) \textit{tuberculosis} infection. Mycobacterial loads in lungs: Wt (open symbols) and RAGE-/- (closed symbols) mice were infected with i.n. with \( 10^5 \) CFUs of \( M. \) \textit{tuberculosis}. After 1 day, 3 and 6 weeks of infection, mice were sacrificed and bacterial loads were determined in lung (A) and liver (B) homogenates. After 1 day, \( M. \) \textit{tuberculosis}, could not be recovered from livers. Data are means ± SE of 8 mice per genotype for 3 and 6 weeks and of 3 mice for day 1. **, \( P < .01 \) vs Wt mice.
long-term outcome as reflected by accelerated weight loss and an increased mortality. These data suggest that RAGE is important for a balanced inflammatory reaction in the lungs during tuberculosis.

In accordance with previous studies (3-9), we showed that normal mouse lungs abundantly express RAGE. Our finding that experimentally induced pulmonary tuberculosis results in increased RAGE expression in lung tissue extends a previous investigation that reported enhanced RAGE expression in two patients with tuberculosis (7). Additional investigations have indicated that RAGE expression becomes upregulated during bacterial pneumonia as well (7). In contrast, patients with the acute respiratory distress syndrome did not have increased pulmonary expression of RAGE (9) and rats with acute lung injury induced by intratracheally administered lipopolysaccharide displayed no change in the distribution of RAGE-expressing cells (8). These data suggest that infectious lung inflammation, resulting from a gradually growing (myco)bacterial load providing a sustained proinflammatory stimulus, has a stronger impact on RAGE expression than sterile lung inflammatory disorders.

RAGE−/− mice demonstrated more lung inflammation, especially 3 weeks after infection, as reflected by histopathology and cytokine/chemokine levels. This is remarkable since inhibition of the interaction of RAGE with its ligands led to a reduced inflammation in models of hepatic injury, diabetic atherosclerosis, delayed type hypersensitivity, type II

Figure 4. Body weight and survival of Wt and RAGE−/− mice during M. tuberculosis infection. Wt (open circles) and RAGE−/− mice (closed circles) were inoculated with 10^5 CFUs of M. tuberculosis. Body weight relative to day 0 (A). Survival of Wt and RAGE−/− mice (B). Data are means ± SE, starting with 12-16 mice per genotype at the beginning of the survival experiment. *, P < .05 vs Wt mice.
collagen-induced arthritis, experimental auto-immune encephalomyelitis and sepsis (18-25). Moreover, RAGE has been implicated as a pro-inflammatory receptor in a model of lung fibrosis induced by intratracheal administration of bleomycin (35). These earlier reports all support the concept that activation of RAGE elicits inflammation and are in accordance with investigations showing that RAGE triggers multiple pro-inflammatory intracellular signaling pathways (36, 37). Of note, 3 weeks after infection the mycobacterial loads were higher in RAGE−/− mice than in Wt mice, which may have contributed to the increased inflammatory response in these animals. However, the difference in mycobacterial burden was modest at best, and we therefore consider this not a major factor. Considering that there is no evidence for a direct role for RAGE in host defense against M. tuberculosis at the cellular level, it is conceivable that the transiently increased growth of mycobacteria in RAGE−/− mice was the consequence of the less controlled inflammatory reaction in the lung resulting in an impaired containment of the infection. Together these data suggest that the role of RAGE in the regulation of lung inflammation during pulmonary tuberculosis is rather specific for this particular infectious disease.

RAGE has been implicated in the regulation of cell migration. RAGE is a counter-receptor for leukocyte integrins; in particular, RAGE has been identified as a binding partner for the β2 integrins Mac-1 and p150,95 (38). The interaction of RAGE with β2 integrins mediated leukocyte recruitment in vivo: RAGE−/− mice displayed a diminished number of adherent inflammatory cells on the peritoneum after cecal ligation and puncture (23) and a reduction in neutrophil influx in the peritoneal cavity after thioglycollate peritonitis (38). Hence, in theory RAGE can enhance leukocyte influx by virtue of its capacity to act as an endothelial cell adhesive receptor, as well as – in a more indirect way – by acting as a pro-inflammatory receptor enhancing the release of pro-inflammatory mediators. However, in our model of pulmonary tuberculosis RAGE−/− mice demonstrated enhanced rather than diminished leukocyte recruitment to infected lungs, which involved both neutrophils and lymphocytes. Our current data do not provide direct insight into how the attraction of leukocytes is increased in RAGE−/− mice. The mechanisms underlying cell recruitment and granuloma formation during lung tuberculosis are complex, involving chemokines, chemokine receptors and adhesion molecules (32). Of considerable interest, however, similar results were reported in the model of bleomycin-induced chronic lung inflammation, wherein RAGE−/− mice displayed an enhanced pulmonary influx of lymphocytes and neutrophils (35), suggesting that RAGE exerts an inhibitory effect on leukocyte migration to the lungs during chronic inflammatory reactions in general.

We here tested the hypothesis that RAGE, which is strongly expressed in healthy lungs and becomes upregulated after infection with M. tuberculosis, plays an important role in the chronic inflammation that accompanies lung tuberculosis. In contrast to our expectation,
RAGE deficiency resulted in enhanced rather than reduced lung inflammation. This finding not only suggests that RAGE is not important for induction of sustained pulmonary inflammation after infection with *M. tuberculosis* via the airways, but also that the delicate balance between benefit and harm resulting from the inflammatory response during tuberculosis can be disturbed by intact RAGE signaling.

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REFERENCES


