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Citation for published version:

Digital Object Identifier (DOI):
10.1128/IAI.74.2.1425-1430.2006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

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Macrophages Isolated from Chickens Genetically Resistant or Susceptible to Systemic Salmonellosis Show Magnitudinal and Temporal Differential Expression of Cytokines and Chemokines following *Salmonella enterica* Challenge†

Paul Wigley,* Scott Hulme, Lisa Rothwell, Nat Bumstead, Pete Kaiser, and Paul Barrow‡

Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom

Received 4 August 2005/Returned for modification 12 October 2005/Accepted 30 November 2005

Macrophages from inbred chickens that are resistant to salmonellosis show greater and more rapid expression of proinflammatory chemokines and cytokines, including the key Th1-inducing cytokine interleukin-18, upon *Salmonella* challenge than those from susceptible birds. This suggests the possibility that salmonellosis resistant-line macrophages signal more effectively and rapidly and are more able to induce protective Th1 adaptive responses.

Genetic resistance to systemic salmonellosis in the chicken is dependent on a number of factors, including *slc11a1* (Nramp1), the major histocompatibility complex, Toll-like receptor 4, and a novel genetic locus termed *sAL1* (7, 11, 13–15, 21). Previous studies of inbred White Leghorn chickens have shown that, of these factors, *sAL1* plays the greatest role in experimental infection with *Salmonella enterica* serovar Gallinarum, the causative agent of fowl typhoid, and to a lesser extent, following infection with *Salmonella enterica* serovar Typhimurium (15, 21). In these studies, birds that were resistant to *Salmonella* showed decreased mortality and morbidity and on postmortem examination they showed small granuloma-like lesions in their livers relative to the large necrotic lesions shown in susceptible birds (15, 21). No difference in initial invasion or colonization of the gastrointestinal tract was found, suggesting minimal intestinal involvement, but bacterial numbers increased rapidly in the spleens and livers of susceptible birds, suggesting that differences in systemic innate immunity played a major role, and subsequent studies showed differences in in vitro biology of macrophages from *Salmonella*-resistant and -susceptible inbred chickens (21). While no difference in uptake was found between lines, resistant W1-line macrophages cleared bacteria within 24 to 48 h of infection, whereas *Salmonella* persisted in the susceptible 72-line cells (21). Macrophages from the resistant line produced a strong oxidative response to *Salmonella*, whereas little or no detectable response was found upon challenge in macrophages from the susceptible line, though macrophages from both lines responded equally well to nonspecific stimuli. These findings suggest that macrophages play a significant role in resistance to systemic salmonellosis in the chicken. The importance of the survival of *Salmonella* serovar Gallinarum within chicken macrophages is illustrated by the complete attenuation of strains with a mutation in the *Salmonella* pathogenicity island 2 type III secretion system and that survive poorly within chicken macrophages (8). The role of heterophils, avian polymorphonuclear cells, as mediators of genetic resistance has also been investigated in lines of broiler chickens, indicating a strong correlation between heterophil function and resistance to *Salmonella enterica* serovar Enteritidis infection (19).

The role of adaptive immunity in *Salmonella* resistance in chickens has only recently begun to be explored. A number of candidate genes, including T-cell markers, cytokines, and immunoglobulin genes, have shown linkage to resistance (5, 11, 12). Single-nucleotide polymorphisms have been identified in a number of genes, including CD28 and Tlr4 genes, that appear to be associated with resistance (13), but as yet, little immune function has been ascribed to *Salmonella* resistance. Signaling through cytokines and chemokines is likely to play a major role in both the activation of innate immunity and the subsequent development of the adaptive response. Differential expression of the cytokines interleukin-6 (IL-6) and IL-18 was described in inbred chicken lines that were resistant or susceptible to Marek’s disease following infection with Marek’s disease virus (10). Recently, differential expression of cytokines has been shown in *Salmonella*-resistant and -susceptible chicken line heterophils following *Salmonella* serovar Enteritidis challenge (20), with increased expression of the proinflammatory cytokines IL-6 and IL-8 and the Th1-associated cytokine IL-18 but significantly lower levels of the anti-inflammatory cytokine transforming growth factor β4 in cells from *Salmonella*-resistant birds in comparison to the susceptible-line cells. This suggested the possibility that resistant-line heterophils would be more effective in initiating both innate and Th1-mediated adaptive responses that appear to play a pivotal role in immunity to avian systemic salmonellosis (3, 23). Here we determine differences in the expression and kinetics of expression of a range of cytokines and chemokines by macrophages from *Salmonella*-resistant and -susceptible lines in vitro.

Primary macrophages were produced from monocytes isolated from heparinized blood taken from the wing vein of

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* Corresponding author. Present address: Department of Veterinary Pathology, University of Liverpool, Leahurst, Neston CH64 0SH, United Kingdom. Phone: 44 151 7946193. Fax: 44 151 794255. E-mail: Paul.Wigley@liverpool.ac.uk.
† Sadly, Nat Bumstead passed away during this study. We fondly dedicate this paper to his memory.
‡ Present address: School of Veterinary Medicine and Science, The University of Nottingham, South Bonington, Loughborough, Leicestershire LE12 5RD, United Kingdom.
Salmonella-resistant or -susceptible chickens of 8 to 12 weeks of age. Specific-pathogen-free line W1 Salmonella-resistant and line 7₂ Salmonella-susceptible inbred White Leghorn chicks were obtained from the Poultry Production Unit, Institute for Animal Health, Compton, United Kingdom, and reared as described previously (21). To isolate peripheral blood monocytes, the blood was mixed with an equal volume of phosphate-buffered saline. Monocytes were isolated by centrifugation over Histopaque 1083 as previously described (21). Monocytes from each line, four birds for each experiment, were then pooled and cultured in supplemented RPMI 1640 for 48 h to obtain monocyte-derived macrophages (21). For both lines, cells were seeded to give a final concentration of 1 × 10⁶ macrophages per ml in 24-well tissue culture plates, with each well containing 1 ml of cells. At this point, the culture medium was replaced by antibiotic-free medium and the cells were cultured for 4 h prior to challenge.

Spontaneous nalidixic acid-resistant mutants of the well-characterized strains Salmonella enterica serovar Gallinarum 9 and Salmonella enterica serovar Typhimurium F98 were used for macrophage challenge (2, 17, 18, 21, 25). Strains were maintained as glycerol stocks at −70°C for macrophage challenge (2, 17, 18, 21, 25). Strains were maintained as glycerol stocks at −70°C for macrophage challenge (2, 17, 18, 21, 25). To isolate peripheral blood monocytes, the blood was mixed with an equal volume of phosphate-buffered saline. Monocytes were isolated by centrifugation over Histopaque 1083 as previously described (21). Monocytes from each line, four birds for each experiment, were then pooled and cultured in supplemented RPMI 1640 for 48 h to obtain monocyte-derived macrophages (21). For both lines, cells were seeded to give a final concentration of 1 × 10⁶ macrophages per ml in 24-well tissue culture plates, with each well containing 1 ml of cells. At this point, the culture medium was replaced by antibiotic-free medium and the cells were cultured for 4 h prior to challenge.

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<table>
<thead>
<tr>
<th>Time postinfection</th>
<th>W1 (resistant)</th>
<th>7₂ (susceptible)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serovar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gallinarum 9</td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>2.74 (0.17)</td>
<td>3.31 (0.33)</td>
</tr>
<tr>
<td>1 h</td>
<td>4.28 (0.11)</td>
<td>3.42 (0.05)</td>
</tr>
<tr>
<td>4 h</td>
<td>3.38 (0.21)</td>
<td>3.91 (0.23)</td>
</tr>
</tbody>
</table>

*Results shown are as determined by a gentamicin protection assay (n = 6).*
increased resistance to experimental infection and more rapid killing of *Salmonella* by macrophages of the resistant W1 chicken line (21). Recently, differences in expression of cytokines in heterophils from resistant and susceptible broiler chickens following *Salmonella* challenge have been described (19). These studies indicated increased expression of the proinflammatory cytokines IL-6 and IL-8 and the Th1 cytokine IL-18 in *Salmonella*-resistant lines. In this study, we show increased expression of proinflammatory cytokines and chemokines in response to *Salmonella* challenge. This study also demonstrates that expression of proinflammatory signals is more rapid in macrophages from *Salmonella*-resistant chickens, with rapid expression of IL-1β, IL-6, and CXC found in the challenged line W1 cells. These findings suggest that, upon stimulation by *Salmonella*, macrophages from the resistant line are able to express proinflammatory cytokines more rapidly and at a greater level. In chickens, as in mammals, expression of these cytokines would lead to increased proinflammatory activity, including an increased influx of polymorphonuclear cells, increased macrophage activation, and in the case of IL-6, activation of lymphocytes. Such a response would be consistent with the pathology and cellular changes found following experimental infection of resistant-line chickens.
Limited expression of IL-18 was found in both lines at 1 h postchallenge, with higher levels of expression following *Salmonella*-resistant and -susceptible inbred chicken lines challenged with *Salmonella* serovar Gallinarum (A and C) or *Salmonella* serovar Typhimurium (B and D) at an MOI of 10. Expression was determined by qRT-PCR from RNA isolated in triplicate challenges from three repeats of macrophages pooled from four different birds for each repeat. Significant differences in expression between chicken lines at a particular time point ($P < 0.05$) are indicated by an asterisk; highly significant differences ($P < 0.01$) are indicated by a double asterisk ($\alpha = 9$). Error bars indicate standard errors of the means.

**FIG. 2.** Expression of the CXC chemokine CXCL11 (K60) (A and B) and the MIP-family CC chemokine CCL2 (C and D) by monocyte-derived macrophages from *Salmonella*-resistant and -susceptible inbred chicken lines challenged with *Salmonella* serovar Gallinarum (A and C) or *Salmonella* serovar Typhimurium (B and D) at an MOI of 10. Expression was determined by qRT-PCR from RNA isolated in triplicate challenges from three repeats of macrophages pooled from four different birds for each repeat. Significant differences in expression between chicken lines at a particular time point ($P < 0.05$) are indicated by an asterisk; highly significant differences ($P < 0.01$) are indicated by a double asterisk ($\alpha = 9$). Error bars indicate standard errors of the means.
IL-18 than do susceptible-line cells. As well as increased antimicrobial activity to Salmonella, macrophages from the resistant W1 line may be more efficient in initiating an adaptive response that leads to the eventual clearance of Salmonella from the spleen and liver. In general, the expression of cytokines and chemokines was more rapid in Salmonella serovar Typhimurium-challenged cells than in Salmonella serovar Gallinarum-challenged cells. Salmonella serovar Typhimurium was taken up by or invaded macrophages more rapidly than the nonmotile, nonflagellated Salmonella serovar Gallinarum (Table 1). Salmonella serovar Gallinarum is generally regarded as poorly invasive in host cells (1), primarily as a consequence of its poor motility. It appears that Salmonella serovar Typhimurium will invade cells more rapidly and efficiently in vitro than will Salmonella serovar Gallinarum. This may go some way to explaining the generally slower response to Salmonella serovar Gallinarum in vitro, though as Salmonella serovar Gallinarum is highly invasive to the spleen and liver in vivo, such differences may not occur during infection.

In this study, we have shown that macrophages from chickens that are genetically resistant or susceptible to systemic salmonellosis display differential expression of cytokines to Salmonella serovar Gallinarum and Salmonella serovar Typhimurium challenge in vitro. The findings are consistent with the infection biology of Salmonella in the lines used and with previous studies indicating that macrophages from resistant-line chickens are more efficient in killing Salmonella both in vitro and in vivo. The data presented here suggest that macrophages from resistant-line chickens are capable of rapid expression of proinflammatory cytokines and chemokines following challenge and that the macrophages become activated more quickly. In addition, the differences in expression of IL-18 are intriguing, suggesting that macrophages from resistant-line chickens are more efficient in the initiation of IFN-γ-dependent adaptive immune responses. This would suggest that resistant-line chickens not only have increased innate immunity to Salmonella infection but also are more capable of stimulating a protective adaptive immune response.

We thank the Biotechnology and Biological Sciences Research Council and the European Union for funding programs relating to this work.

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FIG. 3. Expression of interleukin-18 by monocyte-derived macrophages from Salmonella-resistant and -susceptible inbred chicken lines challenged with Salmonella serovar Gallinarum (A) or Salmonella serovar Typhimurium (B) at an MOI of 10. Expression was determined by qRT-PCR from RNA isolated in triplicate challenges from three repeats of macrophages pooled from four different birds for each repeat. Significant differences in expression between chicken lines at a particular time point (P ≤ 0.05) are indicated by an asterisk; highly significant differences (P ≤ 0.01) are indicated by a double asterisk (n = 9). Error bars indicate standard errors of the means.


Editor: F. C. Fang