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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*

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**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 *SALI* (7, 11, 13–15, 21). Previous studies of inbred White Leghorn chickens have shown that, of these factors, *SALI* 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 7<sub>2</sub>-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  $\beta$ 4 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 to *in vitro* challenge.

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

\* 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.

TABLE 1. *Salmonella* uptake and survival in monocyte-derived macrophages from *Salmonella*-resistant and -susceptible inbred lines of chickens<sup>a</sup>

Time postinfection	Mean log <sub>10</sub> CFU (SEM) of <i>Salmonella</i> for:			
	W1 (resistant)		7 <sub>2</sub> (susceptible)	
	Serovar Gallinarum 9	Serovar Typhimurium F98	Serovar Gallinarum 9	Serovar Typhimurium F98
20 min	2.74 (0.17)	4.71 (0.07)	3.31 (0.33)	4.69 (0.09)
1 h	4.28 (0.11)	5.05 (0.29)	3.42 (0.05)	4.63 (0.08)
4 h	3.38 (0.21)	4.13 (0.14)	3.91 (0.23)	4.64 (0.09)

<sup>a</sup> Results shown are as determined by a gentamicin protection assay ( $n = 6$ ).

*Salmonella*-resistant or -susceptible chickens of 8 to 12 weeks of age. Specific-pathogen-free line W1 *Salmonella*-resistant and line 7<sub>2</sub> *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  $1 \times 10^6$  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  $-70^\circ\text{C}$  and grown for 18 h in Luria-Bertani broth at  $37^\circ\text{C}$  in an orbital shaking incubator at 150 rpm. Macrophages were challenged with nonopsonized *Salmonella* serovar Gallinarum 9 or serovar Typhimurium F98 at a multiplicity of infection (MOI) of 10 *Salmonella* bacteria per macrophage as described previously (8, 22). The numbers of *Salmonella* bacteria that were taken up by or surviving within macrophages at 20 min and 1 and 4 h postchallenge were determined by a gentamicin protection assay as previously described (21). To obtain macrophage RNA, macrophages were challenged in parallel as described above. At 20 min, 1 h, and 4 h postinfection, supernatants were removed and then 350  $\mu\text{l}$  of RLT lysis buffer from a QIAGEN RNeasy mini kit was added to each well and agitated to homogenize the cell sheet. The cell homogenates were removed and stored at  $-70^\circ\text{C}$  prior to isolation of macrophage RNA. At each time point, cell homogenates were produced from unchallenged cells as controls. Each challenge experiment was performed in triplicate using different batches of isolated macrophages from different birds for three repeats for RNA expression. RNA was isolated from cell homogenates in RLT buffer by using RNeasy mini kits following the manufacturer's instructions. Isolated RNA was stored at  $-70^\circ\text{C}$  until required. The expression levels of cytokine mRNA in control and *Salmonella*-challenged macrophages for the proinflammatory cytokines IL-1 $\beta$  and IL-6 (9), the Th1 cytokine IL-18 (10), the chemokine CXCL1

(K60), and the MIP family CC chemokine CCLi2 (24, 25) were determined using previously described probes, primers, and conditions (9, 24, 25). Differences in RNA levels between samples were corrected against 28S rRNA levels as previously described (9). Results are expressed as differences ( $n$ -fold) between *Salmonella*-challenged samples and uninfected controls. Statistical analysis of mean values between groups was determined by analysis of variance using Microsoft Excel 2002 SP3. Significance was taken as  $P$  of  $<0.05$ .

As described previously (21), *Salmonella* bacteria were taken up by both resistant- and susceptible-line macrophages in similar numbers, though significantly fewer *Salmonella* serovar Gallinarum bacteria were phagocytosed by cells from the susceptible 7<sub>2</sub>-line relative to cells from the resistant line by 1 h after challenge ( $P = 0.02$ ) (Table 1). *Salmonella* numbers declined significantly ( $P \geq 0.05$ ) in the resistant W1 macrophages between 1 and 4 h postinfection in resistant W1 cells, but macrophages from susceptible 7<sub>2</sub> line chickens remained at the same level or increased, which was consistent with previous studies (21). The expression levels of cytokines and chemokines were markedly different between chicken lines in both magnitude of expression and kinetics. Although macrophages from both resistant- and susceptible-line chickens showed similar levels of expression for IL-1 $\beta$ , with up to 70-fold increases in expression after 1 h (Fig. 1A) following *Salmonella* serovar Gallinarum challenge, expression following *Salmonella* serovar Typhimurium challenge was more rapid, with an increase of greater than 60-fold after 20 min and one higher still in macrophages from the *Salmonella*-resistant W1 line chickens after 1 h (Fig. 1B). The expression of IL-6 was significantly greater ( $P = 0.022$ ) in macrophages from resistant-line W1 chickens at 1 h postchallenge with both serovars (Fig. 1C), though there appeared to be significant down-regulation of both IL-6 and IL-1 expression at 4 h postinfection following *Salmonella* serovar Typhimurium challenge in macrophages from the resistant line (Fig. 1B and D). Both CCLi2 and CXCLi1 chemokines were expressed at significantly higher levels by macrophages from the *Salmonella*-resistant line than by macrophages from the susceptible line following challenge by both serovars ( $P < 0.05$ ). Expression of CXCLi1 mRNA was also quicker in macrophages from the *Salmonella*-resistant line (Fig. 2A and C), with significant expression detected at 20 min postchallenge with both serovars, while there was also more rapid expression of the MIP family CC chemokine CCLi2 by macrophages from the resistant line following *Salmonella* serovar Typhimurium challenge (Fig. 2B and D). The differences in these responses are consistent with the phenotype of in-

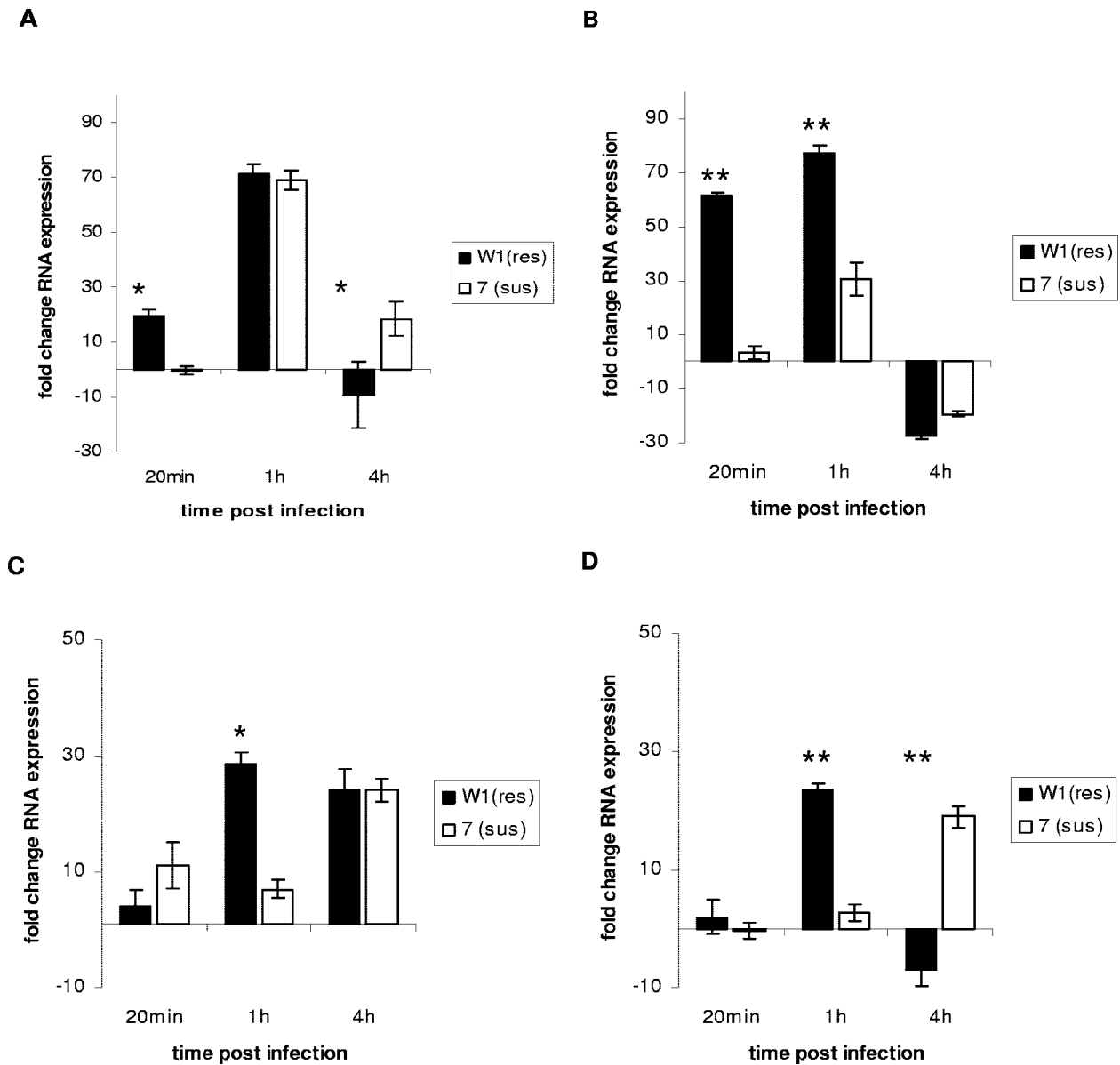


FIG. 1. Expression of interleukin-1 $\beta$  (A and B) and interleukin-6 (C and D) by monocyte-derived macrophages from *Salmonella*-resistant (res) and -susceptible (sus) inbred chicken lines challenged with *Salmonella* serovar Gallinarum (A and C) or *Salmonella* serovar Typhimurium (B and D) at an MOI of 10. Expression was determined by quantitative reverse transcriptase (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 ( $P \leq 0.05$ ) are indicated by an asterisk; highly significant differences ( $P \leq 0.01$ ) are indicated by a double asterisk ( $n = 9$ ). Error bars indicate standard errors of the means.

creased 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-1 $\beta$ , IL-6, and CXCL12 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.

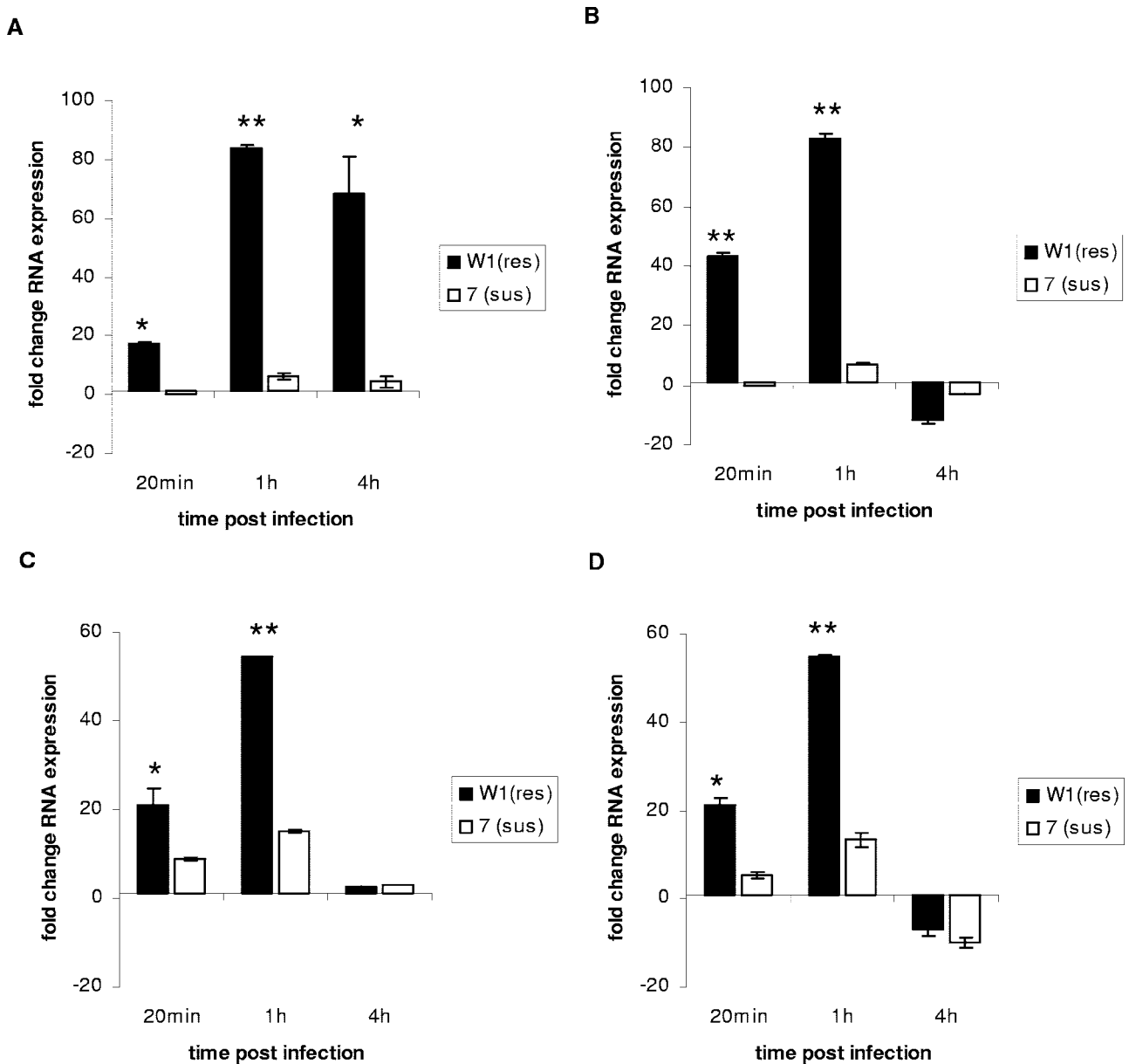


FIG. 2. Expression of the CXC chemokine CXCLi1 (K60) (A and B) and the MIP-family CC chemokine CCLi2 (C and D) by monocyte-derived macrophages from *Salmonella*-resistant and -susceptible inbred chicken lines challenged with *Salmonella* serovar Gallinarum (A and C) or *Salmonella* serovar Typhimurium (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 ( $P \leq 0.05$ ) are indicated by an asterisk; highly significant differences ( $P \leq 0.01$ ) are indicated by a double asterisk ( $n = 9$ ). Error bars indicate standard errors of the means.

Limited expression of IL-18 was found in both lines at 1 h postchallenge, with higher levels of expression following *Salmonella* serovar Typhimurium challenge (Fig. 3) at 4 h postchallenge. Significantly higher expression was observed in macrophages from the resistant line W1 ( $P = 0.019$ ). This was most pronounced in the macrophages challenged with *Salmonella* serovar Gallinarum. In contrast, a decrease in expression was observed in macrophages from the susceptible line 7<sub>2</sub>. These differences in IL-18 (a cytokine expressed particularly by activated macrophages) expression is particularly interesting. The roles of IL-12 and IL-18 are pivotal in the immunity to primary

*Salmonella* serovar Typhimurium infections of mice in the initiation of gamma interferon (IFN- $\gamma$ ) production by Th1 lymphocytes and NK cells (16). Initiation of such a response is crucial in the clearance of intracellular pathogens, including *Salmonella*, mycobacteria, and trypanosomes (6). Recently, the roles of T cells and IFN- $\gamma$  in the clearance of primary *Salmonella* serovar Typhimurium systemic infections of chickens (3, 4, 24) and in the clearance of the live attenuated *Salmonella* serovar Gallinarum vaccine strain 9R (23) have been determined. The data here indicate that macrophages from the *Salmonella*-resistant line express significantly higher levels of

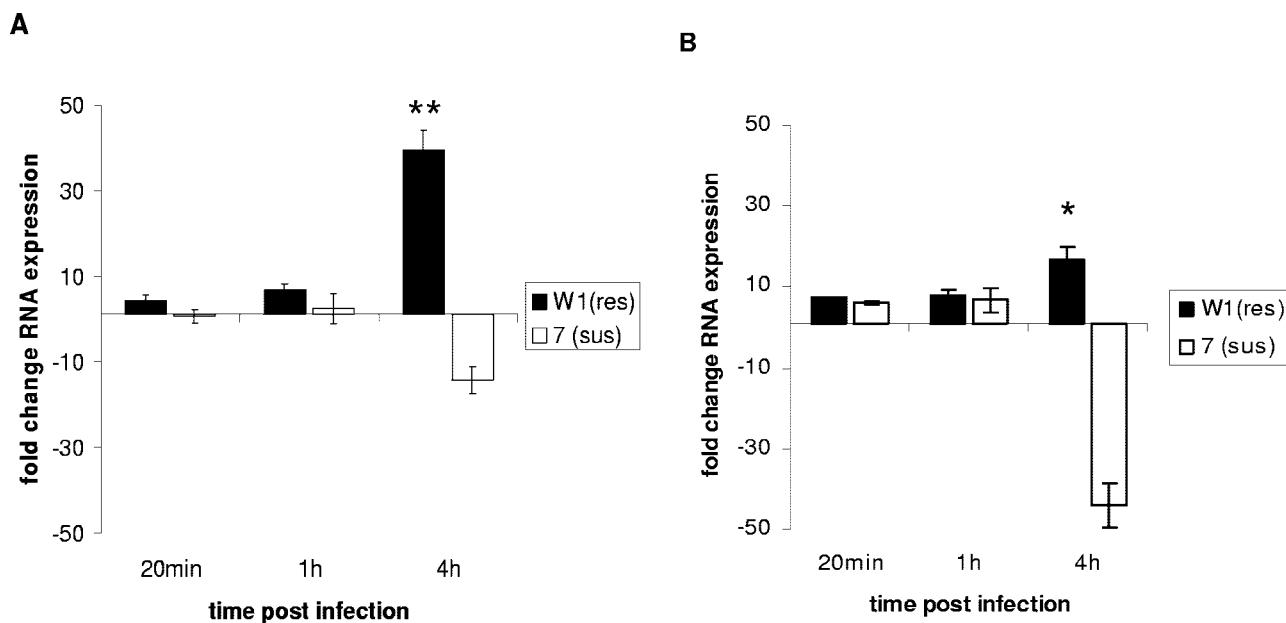


FIG. 3. Expression of interleukin-18 by monocyte-derived macrophages from *Salmonella*-resistant and -susceptible inbred chicken lines challenged with *Salmonella* serovar Gallinarum (A) or *Salmonella* serovar Typhimurium (B) at an MOI of 10. Expression was determined by qRT-PCR from RNA isolated in 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 ( $P \leq 0.05$ ) are indicated by an asterisk; highly significant differences ( $P \leq 0.01$ ) are indicated by a double asterisk ( $n = 9$ ). 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- $\gamma$ -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.

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