Interaction Differences of the Avian Host-Specific *Salmonella enterica* Serovar Gallinarum, the Host-Generalist *S. Typhimurium*, and the Cattle Host-Adapted *S. Dublin* with Chicken Primary Macrophage

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ABSTRACT Most *Salmonella* serovars cause disease in many host species, while a few serovars have evolved to be host specific. Very little is known about the mechanisms that contribute to *Salmonella* host specificity. We compared the interactions between chicken primary macrophages (CDPM) and host-generalist serovar *Salmonella enterica* serovar Typhimurium, host-adapted *Salmonella enterica* serovar Dublin, and avian host-specific *Salmonella enterica* serovar Gallinarum. *S. Gallinarum* was taken up in lower numbers by CDPM than *S. Typhimurium* and *S. Dublin*; however, a higher survival rate was observed for this serovar. In addition, *S. Typhimurium* and *S. Dublin* caused substantially higher levels of cell death to the CDPM, while significantly higher concentrations of NO were produced by *S. Gallinarum*-infected cells. Global transcriptome analysis performed 2 h postinfection showed that *S. Gallinarum* infection triggered a more comprehensive response in CDPM with 1,114 differentially expressed genes (DEGs) compared to the responses of *S. Typhimurium* (625 DEGs) and *S. Dublin* (656 DEGs). Comparable levels of proinflammation responses were observed in CDPM infected by these three different serovars at the initial infection phase, but a substantially quicker reduction in levels of interleukin-1 β (IL-1 β), CXCLi1, and CXCLi2 gene expression was detected in the *S. Gallinarum*-infected macrophages than that of two other groups as infections proceeded. KEGG cluster analysis for unique DEGs after *S. Gallinarum* infection showed that the JAK-STAT signaling pathway was top enriched, indicating a specific role for this pathway in response to *S. Gallinarum* infection of CDPM. Together, these findings provide new insights into the interaction between *Salmonella* and the host and increase our understanding of *S. Gallinarum* host specificity.

KEYWORDS *S. Gallinarum*, *S. Typhimurium*, *S. Dublin*, primary macrophages, transcriptome analysis

Salmonella enterica is a highly diverse Gram-negative pathogen, which includes more than 2,600 different serovars differentiated on the basis of antigen variation in somatic O, capsular (Vi), and flagellar H antigens (1). Some serovars infect a wide range of host species (host generalists). The most notable members of this group are *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis, which annually account for around 94 million cases of self-limiting gastroenteritis in humans and which also cause diarrhea in livestock (2, 3). Other serovars, such as *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Gallinarum, have evolved to be host specific. *S. Typhi* causes a systemic and life-threatening disease in humans known as typhoid fever. This disease is estimated to be responsible for over 200,000 deaths annually (4). *S. Gallinarum* bv. Gallinarum is the causative agent of fowl typhoid, a disease which often leads to high morbidity and mortality in chickens and

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which affects both young and adult birds and represents a great burden for the chicken industry, in particular in low- and middle-income countries (5, 6). Some *Salmonella* serovars appear to be in between these two groups; they are often referred to as host adapted since they generally cause disease in one particular host species but occasionally infect other species (7). *Salmonella enterica* serovar Dublin is a member of this group, as it typically causes systemic disease in cattle; however, sporadically, it also infects other hosts, including humans (8, 9). Genome comparisons indicate that host specificity has been accompanied by genome degradation with a high level of pseudogene accumulation, causing defective metabolic pathways and virulence profile differences between serovars (10, 11).

After surviving in the acid environment of the stomach and reaching the intestine, broad-host-range *Salmonella* strains invade the enterocytes mediated by a type III secretion system (T3SS) encoded from *Salmonella* pathogenicity island 1 (SPI-1) (12–15). This triggers a robust intestinal inflammation, which favors multiplication of *Salmonella* in the intestine (13). The host-specific serovars do not colonize the intestine to the same extent as host-generalist serovars but tend to cause serious systemic infection (16). Upon crossing the intestinal epithelial barrier, they are engulfed by phagocytic cells, such as dendritic cells and macrophages. Inside these cells, translocation of several effector molecules through a second type III secretion system encoded from *Salmonella* pathogenicity island 2 (SPI-2) is induced. Together, the effectors facilitate bacterial survival and replication within a vacuole in the phagocytic cell by subversion or exploitation of the unfavorable intracellular microenvironment (17–19).

It is generally accepted that the intracellular survival and replication within phagocytic cells are essential for the onset of systemic *Salmonella* infection (13, 18), and it has been proposed that through hiding within macrophages, *Salmonella* can evade the attack of the complement system and antibodies and be transported around the host (13, 20). This contributes to bacterial colonization and multiplication in different organs, including the spleen and liver, leading to life-threatening systemic disease, particularly in immune-suppressed individuals (13, 20).

Very little is known with regard to differences in the interaction between phagocytic cells and the different types of *Salmonella* serovars; for example, how interaction between phagocytic cells and host-specific serovars in the preferred host of that serovar differs from interaction between phagocytic cells and broad-host-range serovars in the same host. Therefore, in this study, we employed chicken monocyte-derived primary macrophages and analyzed their interaction with the chicken host-specific serovar *S. Gallinarum* bv. *Gallinarum* compared to their interaction with the host-generalist serovar *S. Typhimurium* and the cattle-adapted serovar *S. Dublin*. The kinetics of infection, cytokine profiles of the infected macrophages, and differences in global transcriptome after infection with these three serovars were determined. The results obtained increase our understanding of the mechanisms behind *S. Gallinarum* bv. *Gallinarum* host specificity.

RESULTS

Phenotype of cultured chicken primary macrophages before and after infection. After isolation by Ficoll, a mix of mononuclear cells including lymphocytes, monocytes, and thrombocytes was obtained. The nonadherent lymphocytes and dead thrombocytes were removed by vigorous washing using Dulbecco's phosphate-buffered saline (DPBS). After 7 days of culture, monocytes were highly enriched. These were developed and differentiated into primary macrophages with an obvious reduction of the nucleocytoplasmic ratio and formation of irregular shape (Fig. 1A). To further confirm the cell population purity and type, the cells were detached and stained by the chicken monocyte/macrophage marker KUL01 and an isotype control antibody IgG1. The flow cytometer results indicated that a pure cell population was obtained in the forward scatter/side scatter (FSC/SSC) analysis. A significantly higher mean fluorescence intensity of the primary macrophages (gated cell) incubated with KUL01 than of the cells stained by the isotype control antibody IgG1 or blank cells was observed (Fig. 1B),

FIG 1 Phase contrast image and flow cytometer characterization of chicken monocyte-derived primary macrophages. Mononuclear cells were isolated from heparinized wing blood using Ficoll-Paque premium 1.084 solution. (A) Phase contrast image of cultured chicken peripheral blood mononuclear cells. The green arrows indicate the monocytes, and the red arrows depict the lymphocytes. The blue and purple arrows denote the thrombocytes and macrophages, respectively. (B) Flow cytometer characterization of chicken primary macrophages. The primary macrophages were positively stained with the monocyte/macrophage marker KUL01 (blue) compared with the isotype control (IgG1, yellow) and blank cells (red).

suggesting the successful generation of pure monocyte-derived chicken primary macrophages.

After incubating the macrophages with bacterial strains at a multiplicity of infection (MOI) of 5:1, the *S. Typhimurium* 4/74 and *S. Dublin* 3246 strains were rapidly taken up, and this induced the formation of numerous vacuoles in the primary macrophages. The phagocytized *Salmonella* bacteria were located inside these vacuoles (Fig. 2). In contrast, a significantly lower number of visible vacuoles was observed after *S. Gallinarum*

FIG 2 Phase contrast image of primary macrophage morphology development after infection with *Salmonella*. The cells were infected at a rate of 5 to 1 by *Salmonella*. (A to C) The morphological change of chicken primary macrophages infected with *S. Typhimurium* (A), *S. Gallinarum* (B), and *S. Dublin* (C) at 20 min incubation time. *S. Typhimurium* and *S. Dublin* were taken up rapidly and triggered the primary macrophages to form irregular flat shapes and numerous vacuoles. *Salmonella* was trapped into the vacuoles (red arrow).

FIG 3 Uptake and intracellular survival of *Salmonella* strains in chicken primary macrophages. The primary cells were infected at an MOI of 5:1. After 30 min incubation, the cells were washed and fresh medium containing 100 μ g gentamicin was added. This time point was defined as T_0 hours postinfection. (A and B) The intracellular survival curves of strains of three *Salmonella* serovars without and with opsonization (op) before infections. (C and D) The fold net replication without and with opsonization. Asterisks indicate significance between the *S. Typhimurium* or *S. Dublin* infection group with the *S. Gallinarum* group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

G9 infection (Fig. 2), though extension of incubation time to 2 h induced the macrophages to gradually form more vacuoles (data not shown).

Uptake and survival of *Salmonella* in chicken primary macrophages. The infection study showed that both *S. Typhimurium* 4/74 and *S. Dublin* 3246 were taken up by macrophages in significantly higher numbers than *S. Gallinarum* G9 (T_0), irrespective of whether the strains were opsonized or not (Fig. 3A and B). After uptake into macrophages, strains of all three serovars were quickly killed and cleared; particularly, *S. Typhimurium* 4/74 and *S. Dublin* 3246 showed a substantial decrease of viable intracellular bacteria with only marginally more viable bacteria present in infected macrophages at T_4 hours postinfection than for *S. Gallinarum* (Fig. 3). Thus, a higher survival rate was observed for *S. Gallinarum* than for *S. Typhimurium* and *S. Dublin* during this initial infection phase, though only significant to *S. Dublin* and only significant in the experiment without opsonization (Fig. 3C and D).

Cytotoxicity and nitric oxide production after *Salmonella* infection. *Salmonella* can induce cell death in both epithelial and phagocytic cells (21, 22). At T_{12} hours postinfection, *S. Typhimurium* 4/74 and *S. Dublin* 3246 induced a significantly higher level of cell death than *S. Gallinarum* G9, regardless of whether the bacteria were opsonized or not (Fig. 4A). Within the serovars, no statistically significant difference in cell death was observed between opsonized and nonopsonized bacteria, although an increased tendency was seen when bacteria were opsonized (Fig. 4A).

Macrophage cell death/lysis was also compared at T_4 by microscopy, as by this time point, the lactate dehydrogenase (LDH) levels were below detection level (data not shown). No obvious differences in cell death/lysis were observed between the three infection groups at this stage, and the majority of macrophages were still morphologically complete.

No significant accumulation of nitric oxide was detected at T_6 and T_{12} hours postinfection (data not shown). At T_{24} hours postinfection, a significantly higher NO

FIG 4 Cytotoxicity toward chicken primary macrophages and induction of NO production. (A) The cytotoxicity level was determined by measuring the released LDH (lactate dehydrogenase) in the supernatant at T_{12} hours postinfection. (B) The NO concentration in the supernatants of primary macrophages was estimated at T_{24} hours postinfection.

concentration was measured from *S. Gallinarum* G9-infected macrophages than from *S. Typhimurium* 4/74 and *S. Dublin* 3246 infected cells, regardless of opsonization or no opsonization prior to infection (Fig. 4B).

Analysis of global gene expression in *Salmonella*-infected monocyte-derived primary macrophages. In sequencing, between 47,904,008 and 63,214,490 reads were obtained per sample, generating between 6.98 GB and 9.32 GB clean data. The clean reads were mapped to the reference chicken genome, with $73.64\% \pm 1.94\%$ of total reads mapped. In the *S. Typhimurium* 4/74-infected cells, a total of 625 differentially expressed genes (DEGs) were identified, with 443 DEGs being upregulated and 182 downregulated compared to those of the uninfected primary macrophages (Fig. 5A). A similar differential expression level was observed for *S. Dublin* 3246 infection, with 451 DEGs upregulated and 205 DEGs downregulated (Fig. 5C). In contrast, the strain of the avian-specific serovar *S. Gallinarum* G9 stimulated a total of 1,114 DEGs, with 699 DEGs being upregulated compared with the noninfected control macrophages (Fig. 5B).

To validate the RNA sequencing (mRNA-seq) results, five DEGs that were common to all three serovars were analyzed by reverse transcription-quantitative PCR (qRT-PCR), namely, interleukin-1 β (IL-1 β), IL-18, CXCL12, CH25H, and ADTA1. Four of these genes were upregulated and one was downregulated (ADTA1) in all infected cells. The comparison between qRT-PCR results and mRNA-seq was performed using the Pearson R approach. Results showed that the correlation between gene expression levels resulting from qRT-PCR and mRNA-seq was high, with an R value of 0.9838 ($P < 0.001$), indicating the reliability and reproducibility of the gene expression data in the study (Fig. 6).

Analysis of differentially expressed genes, Gene Ontology, and KEGG pathway analysis. A total of 367 DEGs were common to all three serotypes, accounting for around 50% of DEGs in both *S. Typhimurium* 4/74- and *S. Dublin* 3246-infected macrophages. A deeper look at the DEGs with the highest fold changes in macrophages

FIG 5 GenesVolcano plot showing the overall distribution of DEGs. The threshold of DEG is set as $|\log_2(\text{fold change})| > 1$ and P value < 0.05 . The y axis indicates the statistical significance of the difference. The red and green dots represent statistically significantly upregulated and downregulated genes, respectively. *S. Typhimurium* (Sty)-infected (A), *S. Gallinarum* (Sga)-infected (B), and *S. Dublin* (Sdu)-infected (C) cells. CK indicates uninfected control cells.

FIG 6 Correlations between the expression levels in three *Salmonella* serovars of five randomly selected DEGs measured by the RNA-seq and qRT-PCR. The x and y axes show the log₂ fold change of IL-1 β , IL-18, CXCLi2, CH25H, and ADTA1 levels in *S. Typhimurium*-, *S. Gallinarum*-, and *S. Dublin*-infected macrophages that were measured by the RNA-seq and qRT-PCR approaches.

infected with strains of the three serovars revealed that DEGs primarily comprised immune-response-related genes like IL-1 β , IL-6, CXCLi1, and CXCLi2, which are typical proinflammation cytokines and chemokines for phagocytic cells. Moreover, a comparable upregulation of these genes was observed in the *S. Gallinarum* G9-infected cells and in the *S. Typhimurium* 4/74- and *S. Dublin* 3246-infected macrophages. In addition, the genes F3 (coagulation factor III), CCL20 (C-C motif chemokine 20 precursor), CCL4, and NF κ BIZ (NF- κ B inhibitor zeta) were also found to be highly upregulated in all three groups. The top 20 DEGs for the primary macrophages infected with the three strains are presented in Files S1, S2, and S3 in the supplemental material. Only 146 and 43 DEGs, respectively, were found to be unique to macrophages infected with *S. Typhimurium* 4/74 and *S. Dublin* 3246. This was considerably less than the 449 genes that were specific DEGs in *S. Gallinarum* G9-infected macrophages (Fig. 7). The top 10 unique DEGs in macrophages infected with the three serovars are shown in Table 1, Table 2, and Table 3. The specific DEGs in *S. Gallinarum*-infected cells included *IL23R*, *TRAF3IP2*, and *TNFAIP2* genes, which play an important role in downstream immune response after pathogen recognition. The complete lists of DEGs from macrophages infected with the three serovars are shown as the Files S4, S5, and S6 in the supplemental material.

For the sake of a better understanding of the molecular and biological functions associated with the specific DEGs, a Gene Ontology (GO) analysis was performed to categorize the functions of these genes. The most significantly enriched GOs were from the biological process category and included immune system processes (GO: 0002376), biological regulation (GO: 0065007), response to stimulus (GO: 0048583), cellular process (GO: 0009987), signaling (GO: 0023052), and single-organism process (GO: 0044699). In addition, the cell surface (GO: 0009986) and receptor complex (GO: 0043235) and cytokine activity (GO: 0005125) and receptor binding (GO: 0005102) groups were also enriched among cellular component and molecular function in all three macrophage infection groups. The 30 most significantly enriched GOs in the *S. Typhimurium* 4/74-, *S. Gallinarum* G9-, and *S. Dublin* 3246-infected macrophages are shown in File S7 in the supplemental material (pages labeled Files S7, S8, and S9, respectively).

For identification of the involved cellular processes, we performed a KEGG pathway enrichment analysis of DEGs. The most enriched pathways in all three infection groups

FIG 7 Venn diagram of number of DEGs in macrophages infected with three different *Salmonella* serovars. The Venn plot shows the numbers of shared and unique differential expressed genes in the primary macrophages infected with *S. Typhimurium* (Sty), *S. Gallinarum* (Sga), and *S. Dublin* (Sdu).

were the recognitions of pathogen-associated molecular patterns. This included the Toll-like receptor, the NOD-like receptor, and the RIG-like receptor pathways. Moreover, other immune reaction pathways, including the *Salmonella* infection, mitogen-activated protein kinase (MAPK), JAK-STAT, P53, cytokine-cytokine receptor interaction, and apoptosis pathways, were also commonly enriched in three serovar-infected macrophages. The top 20 enriched pathways in macrophages infected with *S. Typhimurium* 4/74, *S. Gallinarum* G9, and *S. Dublin* 3246 are presented in File S7 in the supplemental material (pages labeled Files S10, S11, and S12, respectively).

In order to understand what constituted the response to host-specific infection, a KEGG analysis was also conducted for the DEGs specifically regulated in *S. Gallinarum*-infected macrophages. Results showed that the top two enriched pathways were the JAK-STAT signal pathway and the cytokine-cytokine receptor interaction pathway. For instance, *CCND2* (cell cycle regulation function), *BCL2L1* (apoptosis regulator), and *SOCS1* (macrophage polarization regulator) from the JAK-STAT signal pathway were found to be only differentially expressed in *S. Gallinarum* G9-infected cells (Table 4). Downregulation of *SOCS1* has been reported to promote polarization of macrophages into M1 type (23), while a significant upregulation (3.3 fold) was exclusively observed in *S. Gallinarum*-infected cells.

IL-1 β , gamma interferon (IFN- γ), CXCL11, and CXCL12 expression levels were surprisingly upregulated in all three serotypes, which contradicts the previous observation

TABLE 1 Top 10 specific DEGs in *S. Gallinarum* G9-infected primary macrophages

Gene name	Log ₂ fold change	P value	Gene description/product
Heme	7.1089	0.00032684	Heme peroxidase/epidermal growth factor-like domain
C2H2	5.383396	0.0019573	Krueppel-associated box/zinc finger, C ₂ H ₂
LOC101750112	5.0655	0.023076	Polyketide synthase PksM-like
BDKRB2	4.867	0.0027819	Bradykinin receptor B
ENSGALG00000006449	4.6375	2.11E-06	PDZ domain
TNFAIP2	4.5719	0.017822	Tumor necrosis factor alpha-induced protein 2
RNF182	4.382	0.0037421	Ring finger protein 182
IL23R	4.3439	6.94E-06	Interleukin-23 receptor
TRAF3IP2	4.0532	0.0049788	TRAF3 interacting protein 2
SLC5A10	3.8758	4.12E-06	Solute carrier family 5 member 10

TABLE 2 Top 10 specific DEGs in *S. Typhimurium* 4/74-infected primary macrophages

Gene name	Log ₂ fold change	P value	Gene description/product
ENSGALG00000009006	6.5713	8.90E-06	Ferric reductase transmembrane component-like domain
IRF-4	6.3236	2.82E-05	Interferon regulatory factor 3
FAM150B	5.1906	2.37E-14	Ligands for anaplastic lymphoma kinase
TNN	4.9059	0.000291	Fibronectin type III
PIM1	4.2767	4.13E-08	Lipopolysaccharide kinase
ENSGALG00000010101	4.246	2.51E-11	Zinc finger, C ₂ H ₂
TLE3	4.1461	0.001466	Groucho/transducin-like enhancer
ENSGALG00000026069	4.065	0.00231	Sushi/SCR/CCP domain
ACKR4	4.0414	0.000869	CC chemokine receptor like 1
UNG	-4.0657	0.046906	Uracil-DNA glycosylase-like

that avian host-specific serovars induce less of a proinflammatory response than broad-host-range serovars (16). In order to clarify whether this was a consequence of the time point used for analysis ($T = 2$ h), we determined the expression of the same genes by reverse transcriptase PCR, this time including additional time points. In line with the transcriptome results, comparable levels of IL-1 β , CXCLi1, and CXCLi2 gene expression were observed for the three infection groups at the initial infection phase. As the infections proceeded, a marked decrease in the expression was seen in all three infection groups. For IL-1 β , CXCLi1, and CXCLi2 expression, the *S. Gallinarum*-infected cells showed significantly lower expression than the two other serovars at $T = 6$ h (Fig. 8). A significant downregulation of TLR4 was also detected in all three infection groups as the infection proceeded further. In contrast, TLR5 was downregulated at the initial infection phase for all three infection groups ($T = 2$ h) and then increased; at $T = 6$ h, expression in the *S. Gallinarum*- and *S. Dublin*-infected macrophages was significantly above that in the *S. Typhimurium*-infected group (Fig. 8).

DISCUSSION

Serovars of *Salmonella* can be grouped into three groups based on the host range (3, 7). The mechanisms that contribute to this host specificity remain unclear. It is well appreciated that intracellular survival and replication within macrophages are essential for systemic *Salmonella* infection and probably, therefore, of particular relevance to *Salmonella* host specificity, which has systemic infection as a hallmark (13, 20, 24). Thus, in this study, interaction between the avian host-specific serovar *S. Gallinarum* and primary macrophages derived from peripheral blood monocytes of chicken was characterized. In order to understand what was unique in the interaction between this serotype and its preferred host, responses were compared to those when the macrophages were infected with *S. Typhimurium* (host generalist) or *S. Dublin* (host adapted to cattle). By using primary macrophages, as opposed to the commonly used macrophage-like cell cultures, we anticipated to obtain a more realistic picture of the

TABLE 3 Top 10 specific DEGs in *S. Dublin* 3246-infected primary macrophages

Gene name	Log ₂ fold change	P value	Gene description/product
IL17C	3.8997	0.000309	Interleukin-17, chordata
PRDM8	3.4158	0.046237	Zinc finger
STC2	3.177	0.049835	Stanniocalcin
GUCY2C	2.9633	1.64E-05	Serine/threonine/dual specificity protein kinase
SLCO4A1	2.9351	0.001344	Major facilitator superfamily
ALDH6	2.8701	0.010882	Aldehyde/histidinol dehydrogenase
ARG2	2.7598	0.048739	Ureohydrolase/arginase
CRYGN	2.5778	0.01416	Gamma-crystallin-related
CACNB2	-3.3866	0.030191	Guanylate kinase/L-type calcium channel beta subunit
Aiolos	-4.1144	0.034209	Zinc finger, C ₂ H ₂ -like

TABLE 4 Unique DEGs from the JAK-STAT signal pathway in *S. Gallinarum*-infected primary chicken macrophage

Gene name	Log ₂ fold change	P value	Gene description/function
CCND3	−1.051	0.018707	Cyclin, cell cycle progression
IL13RA1	2.1785	6.52E-05	Interleukin-13 receptor
BCL2L1	−0.98025	0.024562	Apoptosis regulator
IL2RA	2.3875	3.21E-10	Interleukin-2 receptor
SOCS1	1.7821	3.75E-06	Suppressor of cytokine signaling 1, macrophage polarization regulator
IL23R	4.3439	6.94E-06	Interleukin-23 receptor
IL3RB	−1.1474	0.007875	Interleukin-3 receptor
IFN- α/β	2.6493	0.028586	Interferon alpha/beta/delta
IFNAR1	−1.2097	0.002606	Interferon alpha/beta receptor

differences in the macrophage responses to the interaction with these *Salmonella* types.

A significantly higher uptake of *S. Typhimurium* and *S. Dublin* than of *S. Gallinarum* by chicken primary macrophages was observed. However, the majority of the phagocytized bacteria were cleared by primary macrophages already at T_4 hours postinfection, where the numbers of intracellular bacteria were similar for all of the three serovars. Thus, intriguingly, *S. Gallinarum* showed a higher survival rate than the other two serovars regardless of opsonization or not. A weaker invasion ability of *S. Gallinarum* than of the host generalist *S. Enteritidis* has previously been reported using both chicken and human epithelial cell lines (25), and it seems to be the general property of *S. Gallinarum* that it is found in low numbers in the intracellular environment. Thus, host specificity of this serovar seems to be related not to an increased ability to enter cells of its preferred host but rather to a better survival ability of the few bacteria that enter cells. Others have proposed that the weak invasion or uptake of *S. Gallinarum* is related to mutations in its SPI-1 T3SS effectors (10, 25, 26). It would be interesting to investigate what happens when such mutations are introduced into the same genes in *S. Typhimurium* and *S. Enteritidis*. It has been observed, however, that SPI-1 genes are not essential for the ability of *S. Gallinarum* to cause fowl typhoid (20). Other factors like the lack of flagella and mutation in certain fimbria genes may also contribute to this phenotype.

Some T3SS effectors of SPI-1 and SPI-2 mediate host cell death, with the characteristics of both apoptosis and necrosis, via caspase-1 or caspase-3 activation (27–31). This

FIG 8 Expression of immune-response-related genes at different time points in chicken primary macrophages infected with three different serovars of *Salmonella* compared to uninfected cells. *, $P < 0.05$; **, $P < 0.01$.

includes the SPI-1 T3SS effector *sipB*, which mediates a quick cell death and which also stimulates the release of inflammation cytokines IL-1 β and IL-18 (28). Some effectors from SPI-2 T3SS, conversely, have also been reported to be involved in a delayed onset of macrophage apoptosis in the extraintestinal tissues, in which infected cells can be engulfed by incoming macrophages leading to a cycle of cell-to-cell dissemination mode (28, 32–34). In this study, a substantially higher level of macrophage cell death was induced by *S. Typhimurium* and *S. Dublin* than by *S. Gallinarum* infections at 12 h postinfection. We, therefore, suggest that the low uptake coupled with high survival capacity and long-term intracellular persistence due to lower cell death is beneficial to *S. Gallinarum* during system disseminations. This may be because it protects it from extracellular peptides, complement molecules, and antibodies. The low uptake but long-term persistence due to low cell death was also observed for the human host-specific serovar *S. Typhi* during interaction with human macrophages (35), and it may be that this is a common and essential feature of host-specific serovars.

Activated macrophages release proteases and reactive oxygen species (ROS) like NO and superoxide to boost killing of intracellular pathogens (36, 37). In this study, NO production was observed in macrophages infected with strains of all three serovars, but only at very late time points. Interestingly, *S. Gallinarum* triggered significantly higher NO production than the two other serovars. Activation of macrophages for induction of ROS and NO is mainly initiated and achieved through the signals of IFN- γ and the CD40 ligand CD154 (38). Interestingly, a higher differential expression of IFN- γ and CD40 was observed in *S. Gallinarum*-infected cells than in macrophages infected with the other two serovars; this might explain the observed results. Conversely, the higher production of NO in the *S. Gallinarum*-infected cells was also probably associated with a lower level of cell death (and, therefore, a higher number of live macrophages) induced by this serovar than by *S. Typhimurium* and *S. Dublin* at the late infection phase.

In order to obtain an overall picture of what constitutes a host-specific response to infection with *S. Gallinarum* in chicken primary macrophages, a full transcriptome analysis was conducted at 2 h postinfection. This time point was chosen, as a quick morphological response to *Salmonella* infection in primary macrophage was observed (Fig. 2). Therefore, we suspected macrophages had adapted expression levels to fighting the specific bacteria at this time point. Interestingly, despite the fact that *S. Gallinarum* was taken up in lower numbers than *S. Typhimurium* and *S. Dublin* and caused a delayed morphological response in the macrophages to infection, *S. Gallinarum* challenge induced almost double the number of DEGs compared to that of the *S. Typhimurium* and *S. Dublin* infection groups. These results may be influenced by the variation between repeats within each serovar; however, the very high difference observed compellingly indicates that there is no correlation between the magnitude of response in the form of DEGs and the number of intracellular *Salmonella* bacteria as has also been reported in previous research (39). The unique DEGs detected in *S. Gallinarum*-infected cells primarily consist of genes involved in immune response and metabolism pathways, such as the JAK-STAT signaling pathway, cytokine-cytokine receptor interaction, porphyrin and chlorophyll metabolism, and glycerophospholipid metabolism. The JAK-STAT signaling pathway has been reported to be involved in the polarization of macrophages into the M2 type, the cell type in which *Salmonella* prefers to reside and replicate (23, 40). Interestingly, in a previous study, the JAK-STAT signaling pathway was also found to be enriched in spleen tissues of chicken infected with *S. Gallinarum* bv. Pullorum, which is a close relative of *S. Gallinarum* bv. *Gallinarum* and is also host specific to chicken (41). The JAK-STAT signaling pathway has been documented to regulate cell apoptosis, suggesting a possible link to the low induction of macrophage death by *S. Gallinarum* infection. The exact role of the JAK-STAT pathway during *S. Gallinarum* infection in chicken deserves further studies. Also, the inhibitor of apoptosis protein BIRC2, which inhibits cell death by interfering with caspase 6 and caspase 3 activities, was upregulated in *S. Gallinarum*-infected macrophages compared to levels in *S. Dublin*- and *S. Typhimurium*-infected cells. Additionally, the BCL2L1 genes, which act as proapoptotic regulators, were specifically downregulated in *S.*

Gallinarum-infected macrophages. Together, these results suggested that the observed lower macrophage cell death caused by *S. Gallinarum* infection was at least partly attributable to these two signal pathway regulations.

In previous reports, host-specific serovars such as *S. Typhi* and *S. Gallinarum* bv. *Gallinarum* and *S. Gallinarum* bv. *Pullorum* have been reported to induce a lower proinflammatory response than that of the host-generalist serovars during *in vitro* infections of epithelial cell lines and *in vivo* infection of the chicken intestine (11, 16, 24). In our study, however, the results of the transcriptome analysis showed comparable proinflammation gene expression levels between *S. Gallinarum*-, *S. Typhimurium*-, and *S. Dublin*-infected macrophages. Since this contrasted previous observations, a further monitoring of the expression of immune-response-related genes in chicken primary macrophages was carried out with a qRT-PCR approach. The results showed a substantial and significantly higher decrease of transcription of IL-1 β , CXCL1, and CXCL2 in the *S. Gallinarum*-infected cells at T_6 hours postinfection, indicating that some immune modulating factors may exist in *S. Gallinarum* that can cause downregulation of the transcription of these proinflammation immune genes or that *S. Gallinarum* lacks a factor that sustains the proinflammatory response at a higher level. It should be noted that the transcription of these genes was also decreased in *S. Typhimurium*- and *S. Dublin*-infected cells compared to that of uninfected cells from T_2 to T_6 but not as dramatic as that detected in the *S. Gallinarum*-infected cells. These results clearly show that the intracellular bacteria alter the macrophage gene expression as the intracellular infection phase proceeds.

The Toll-like receptor 15 (TLR15) is unique in chicken species (42). Although its ligand specificity has not yet been conclusively determined, some microbial components have been reported to be able to activate it (43, 44). This chicken unique Toll-like receptor was found to be upregulated in all infected primary macrophages. This is in line with previous studies, where an upregulation of the TLR15 gene was observed in chicken cecum cells and heterophils after *S. Typhimurium* and *S. Enteritidis* infections (42, 45). These results collectively corroborate the possibility that TLR15 can recognize certain conserved components from the *Salmonella* genus and play a pivotal role in chicken defense against *Salmonella* infections, although its specific ligand has not yet been defined in detail.

In summary, in this study, we observed for the first time comparable proinflammation immune responses between host-specific *S. Gallinarum* and host-generalist serovar *Salmonella* at the initial chicken primary macrophage infection phase, though a substantial decrease of expression was observed in *S. Gallinarum*-infected cells as the infection proceeded. Host-specific *S. Gallinarum* showed a different infection mode with a lower uptake, higher survival capacity, and low induction of cell death, leading to long-term intracellular niche persistence. In addition, host-specific *S. Gallinarum* triggered a more comprehensive global differential gene expression than the other two *Salmonella* serovars in the infected chicken macrophages. These results provide us new insights into the interactions of *Salmonella* with the host immune cells and advance our understanding and knowledge of *S. Gallinarum* pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. Typhimurium* 4/74 (46), *Salmonella* *Gallinarum* bv. *Gallinarum* (herein denoted *S. Gallinarum*) G9 (47), and *S. Dublin* 3246 (48) were used as representative strains of the three serovars under study. Strains were routinely cultured overnight in Luria broth (LB) (Oxoid, Denmark) with shaking at 200 rpm or on LB agar plates at 37°C.

Isolation of primary chicken macrophages and cell culture conditions. Chicken monocyte-derived primary macrophages were obtained following a previously reported method with minor modifications (49, 50). Briefly, heparinized chicken blood was taken from the wing vein of *Salmonella*-free Bovans Brown 16-week-old chickens and mixed with an equal volume of DPBS buffer (1:1) (Gibco, Denmark). A 50-ml centrifuge tube containing 15 ml Ficoll-Paque premium 1.084 solution (GE Healthcare, Denmark) was carefully and slowly layered with 15 ml of half-diluted blood on the top of the Ficoll solutions and then centrifuged at $1,200 \times g$ for 40 min (acceleration = 1, brake = 0, horizontal centrifuge). Blood mononuclear cells were collected from the ring interface and then washed with DPBS buffer 3 times and centrifuged. The cell pellet was resuspended and cultured in RPMI 1640 medium (Gibco, Denmark) supplemented with 5% chicken serum (Sigma, Denmark), 5% fetal bovine serum (FBS)

(Gibco, Denmark), 25 μ g/ml gentamicin, and 200 μ M L-glutamine (GE Healthcare, Denmark) as previously reported (50). The cell concentration was determined by counting using a hemocytometer. The cell culture medium was changed every 24 h for the first 3 days to remove nonadherent cells. Phase contrast microscopy was used to monitor and record the cell population and its morphological development.

Flow cytometer analysis. Flow cytometry assays were used to determine the proportion of macrophages, as previously reported (50). Briefly, after 7 days of culture, the monocyte-derived macrophages were washed three times with DPBS buffer, detached by adding Accutase following instructions (Sigma), and adjusted to a final concentration of 1×10^6 cells/ml. Subsequently, cells were labeled with 1 μ g chicken macrophage/monocyte-specific marker antibody KUL01 (Abcam, Denmark), which was conjugated with fluorescein isothiocyanate (FITC) on ice for 30 min. The FITC-conjugated antibody IgG1 kappa (Abcam, Denmark) was used as the isotype control. After wash with precold DPBS, the cells were resuspended in DPBS buffer supplemented with 2% FBS and subjected to flow cytometer analysis using the FACSCalibur (BD Biosciences) instrument. Data were analyzed using FlowJo software.

Opsonization of *Salmonella* strains and infection of macrophages. For preparation of inocula, single colonies of *Salmonella* were grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 and collected by centrifugation (5,000 rpm for 5 min), washed by DPBS, and eventually resuspended into RPMI 1640 medium. For opsonization, the bacterial suspensions were incubated with DPBS containing 20% inactivated *Salmonella* antibody-free chicken serum (Sigma, USA) for 45 min at 37°C with gentle shaking as previously reported (51). Following incubation, the strains were washed three times with DPBS buffer and then resuspended in RPMI 1640 medium. The inoculum concentration was verified by plating on LB agar plates.

The monocyte-derived macrophages were grown and differentiated on 6- or 12-well plates (TPP, Switzerland). The exact number of cells per well was determined by counting using a hemocytometer 2 h before infection. Cells were washed twice with DPBS, and new fresh medium without gentamicin was added. Macrophage infection was performed as previously described (52) with minor modifications. The bacteria were added to the macrophages at a MOI of approximately 5:1, and the macrophage morphology change was monitored by microscopy analysis. After 30 min of infection, the supernatants were removed, and the cells were washed three times with DPBS. For killing of the extracellular bacteria, macrophages were further incubated with fresh RPMI 1640 medium containing 100 μ g/ml gentamicin. To monitor the uptake and intracellular survival of *Salmonella*, macrophages were washed with DPBS and lysed with 0.9% NaCl supplemented with 0.1% Triton X-100. Ten-fold dilutions of the lysates were then prepared and plated on LB agar plates to determine the intracellular bacteria at T_0 , T_2 , and T_4 hours postinfection. The time point 30 min after addition of bacteria (bacterial inoculation), where the extracellular *Salmonella* was washed away by DPBS and fresh medium containing 100 μ g gentamicin was added to kill extracellular bacteria, was defined as T_0 postinfection. The average CFU per cell was calculated based on the bacterial enumerations at each time point and macrophage cell counting results.

Macrophage cytotoxicity assay. The level of cell death was assessed by measuring the release of cytosolic lactate dehydrogenase (LDH) (53). Briefly, at 12 h postinfection, the supernatants from both infected and noninfected macrophages were collected. LDH activities were measured by using a colorimetric CytoTox 96 kit (Promega, Denmark). The percentage of cytotoxicity was calculated using the following formula where the maximum release is the value obtained for the cell lysates:

$$\% \text{ Cytotoxicity} = \frac{(\text{infected cell release} - \text{uninfected cell release})}{(\text{maximum release} - \text{uninfected cell release})} \times 100$$

Quantification of nitric oxide production by macrophages. Production of nitric oxide from macrophages was measured using the Griess reagent (Sigma-Aldrich) as previously described (54). In brief, at the desired time point postinfection (T_6 , T_{12} , and T_{24} hours postinfection), aliquots of 500 μ l supernatant of infected cell cultures were mixed with the same volume of Griess reagent and incubated at room temperature for 15 min. The nitrite concentration was assessed by measuring the absorbance at 540 nm. Serial 10-fold dilutions of sodium nitrite (Sigma-Aldrich) were used as standards to yield a calibration curve.

RNA extraction, library preparation, and sequencing. After opsonization, bacteria were added to primary macrophages at a MOI of approximately 5:1 as described above. Uninfected macrophages were included as controls. At T_2 , T_6 , and T_{12} hours postinfection, the cells were collected and subjected to RNA extraction, and three independent repeats were conducted. Total RNA was extracted from macrophages using the RNeasy minikit (Qiagen, Denmark) according to the manufacturer's instructions. RNA degradation and contamination were monitored by agarose gel electrophoresis; the RNA purity and quality were assessed using the NanoDrop and Agilent 2100 Bioanalyzer, respectively. High qualities of mRNA samples were used as indicated by both $A_{260/280}$ and $A_{260/230}$ values over 2.0 and RNA integrity numbers (RIN) over 9. Subsequently, a sequencing library was constructed using the NEBNext Ultra RNA library prep kit for Illumina according to the manufacturer's recommendations for the RNA sample obtained at T_2 hours postinfection. After testing the quality by Agilent 2100, the libraries were sequenced on the Illumina HiSeq 2500 platform with 100-bp paired-end reads.

Mapping to reference genome and differential expression analysis. Raw sequencing data were filtered to remove the adaptors and low-quality reads, and the resulting clean reads were mapped to the *Gallus gallus* genome (Gallus_gallus-4.0) using TopHat 2 (55). Gene expression levels were measured by transcript abundance based on the fragments per kilobase of exon model per million mapped read (FPKM) values method, which takes into account the effects of both sequencing depth and gene length (56). HTSeq software was utilized to analyze the gene expression levels with the union mode (57). The differential gene expression (DEG) analysis between infected groups and an uninfected control group

was performed using the DESeq package (58). For the differentially expressed gene analysis, a threshold of $\log_2$ (fold change) > 1 with a P_{adj} of <0.05 was used to identify differentially regulated genes.

Gene ontology and KEGG pathway enrichment analysis of DEGs. GO enrichment analysis was performed using the Goseq software (59), which is based on Wallenius' noncentral hypergeometric distribution. Analysis was performed for enrichment in biological processes, cellular component, and molecular function. In all three groups of infected cells, DEGs were enriched in biological processes, cellular component, and molecular function. KEGG pathway analysis was performed to identify signaling and metabolic pathways that were overrepresented in the chicken DEG lists. The P value of each signal pathway was calculated using a hypergeometric test, and pathways with corrected P values of less than 0.05 were considered significantly enriched in the analysis.

Validation of the RNA-seq results and measurement expressions of DEGs at T_6 and T_{12} by qRT-PCR. The RNA extracted from the infection assays was treated with DNase and then transcribed into cDNA using the GoScript reverse transcription system (Promega, Denmark) following the manufacturer's instructions. For validation of the RNA-seq results, five DEGs (IL-1 β , IL-18, CXCLi2, CH25H, and ADTA1) were randomly chosen for qRT-PCR analysis using the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control. In addition, the expression of immune-response-related genes IL-1 β , IL-18, IFN- γ , CXCLi1, CXCLi2, TLR4, and TLR5 at T_6 and T_{12} hours postinfection was also monitored using the same RT-PCR approaches. The primer list is present in File S1 in the supplemental material. The quantitative PCR (qPCR) was performed using the SYBR FastStart essential DNA green master kit (Roche, Denmark) on the Roche LightCycler 96 real-time PCR system. The relative gene expression value was calculated using the $2^{-\Delta\Delta C_t}$ method (60).

Statistical analysis. Unless otherwise stated, the results are presented as mean values $\pm$ standard deviations (SD) of at least three independent experiments, and comparisons were performed using one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons posttest applying GraphPad Prism 7 software. The correlations between RNA-seq and RT-PCR results were analyzed by calculating the Pearson correlation coefficient R using GraphPad Prism 7. A difference was considered statistically significant when the P value was lower than 0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00552-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 7, PDF file, 1.3 MB.

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J.E.O. and K.H. conceived and designed the study. K.H. and I.T. performed the experiments. K.H., A.H.-F., I.T., S.S., and J.E.O. provided critical advice and contributed to the analysis of data and commented on the manuscript. All authors read and approved the final manuscript.

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