



Differential Immune Phenotypes in Human Monocytes Induced by Non-Host-Adapted *Salmonella enterica* Serovar Choleraesuis and Host-Adapted *S. Typhimurium*

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ABSTRACT We studied the effects of two *Salmonella enterica* serovar Typhimurium (host-adapted) strains (14028 and 4/74) and three *S. Choleraesuis* (non-host-adapted) strains (A50, A45, and B195) in human monocytes between 2 and 24 h postinfection (p.i.) to investigate whether differences in immune response may explain the much higher prevalence of sepsis in individuals infected with *S. Choleraesuis*. Both serovars significantly increased the production of cytokines associated with acute sepsis (tumor necrosis factor alpha [TNF- α], interleukin β [IL- β], and IL-6), but temporal differences occurred between these serovars and between different *S. Choleraesuis* strains. Generally, all *S. Choleraesuis* strains induced significantly higher production of inflammatory cytokines than *S. Typhimurium* strains ($P < 0.01$ to 0.05). All *S. Choleraesuis* strains very significantly increased IL-10 production by monocytes at 6 and 24 h p.i. in comparison to *S. Typhimurium* strains ($P < 0.01$). In addition, $\sim 80\%$ of monocytes were viable at 24 h p.i. with *S. Choleraesuis* A50, compared to only $\sim 40\%$ following *S. Typhimurium* infection. Using *S. Typhimurium* 14028 and *S. Choleraesuis* A50 as examples of these two serovars, we also showed differential expression of genes within the Janus tyrosine kinase (JAK) and signal transducer and activator of transcription (STAT) (JAK/STAT) pathway via quantitative PCR (qPCR) microarray analysis. High serum IL-10 concentration and monocyte survival have been reported as markers of the development of human sepsis. We therefore conclude that high production of IL-10 by monocytes may, in part, explain the greater propensity for *S. Choleraesuis* to induce human sepsis and that this may be greater in strains such as A50, which induces both high IL-10 production and monocyte survival.

KEYWORDS salmonella, human, monocyte, sepsis

Salmonella enterica serovar Typhimurium has a broad host range, and in humans, although it can cause bacteremia and sepsis, disease due to *S. Typhimurium* is usually seen as a self-limiting gastrointestinal infection. In contrast, *Salmonella enterica* serovar Choleraesuis is host adapted to pigs and in humans is most likely to cause bacteremia and sepsis with little gastrointestinal pathology (1). In humans, the mortality rate due to *S. Choleraesuis* infection may be as high as 30% (2), but predisposing factors such as underlying disease are also known to contribute to such a high rate (3). However, in comparison to *S. Typhimurium*, very little has been published regarding *S. Choleraesuis* pathogenesis in humans, and no comparisons have been reported regarding the effect of these *Salmonella* serovars in human immune cells.

The fact that *S. Choleraesuis* is much more likely to cause sepsis than *S. Typhimurium* may not be due simply to increased virulence and the ability to escape the intestinal barrier. For example, survival in blood cells may prolong infection, increasing the concentration of inflammatory cytokines produced by these cells and the development of the acute phase of sepsis.

Cytokine receptors which signal via the Janus tyrosine kinase/signal transducer and

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activator of transcription (JAK/STAT) pathway include gamma interferon receptor 1 (IFN- γ R1), interleukin 6 receptor (IL-6R), and IL-10R (4), and the cytokines which signal via these receptors (IFN- α , IL-6, and IL-10) have been studied as prognostic markers during the systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) phases of sepsis (5–7). The JAK/STAT pathway is not only activated by a range of different cytokines but is also responsible for the transcription of many different cytokine genes (4). The pathway includes three JAK proteins (JAK1 to JAK3), a JAK-related protein, tyrosine kinase 2 (TYK2), and seven STAT proteins (STAT1 to STAT4, STAT5a, STAT5b, and STAT6) (8). In postoperative human sepsis, mortality has been associated with monocyte resistance to IFN- γ , which fails to increase the release of crucial monocyte cytokines (9), and pneumonia-induced sepsis has been reported to be associated with single-nucleotide polymorphisms (SNPs) in *IFNG* genes (10). Many other experimental studies have now shown the essential requirement for IFN- γ in the killing of *Salmonella* by monocytes and macrophages, both *in vitro* and *in vivo* (11).

The aim of this study was to compare the pathogenesis of *S. Typhimurium* with that of *S. Choleraesuis* in human monocytes, with the goal of identifying possible differences that may explain the greater propensity of *S. Choleraesuis* to cause sepsis in humans.

RESULTS

At 2 and 6 h postinfection (p.i.), comparable numbers of *S. Typhimurium* and *S. Choleraesuis* bacteria were isolated from monocytes ($\sim 1 \times 10^4$ CFU/ml); although higher numbers of *S. Choleraesuis* B195 bacteria were isolated at 2 h p.i., the difference was not significant (Fig. 1A). At 24 h p.i., a significantly higher number of *S. Choleraesuis* A50 bacteria were isolated than either *S. Typhimurium* 14028 or 4/74 or other *S. Choleraesuis* strains ($P < 0.05$) (Fig. 1A). We then examined the effect of these bacteria on the monocytes themselves. At 6 h p.i., monocyte viability was $\sim 70\%$ but fell to only $\sim 40\%$ at 24 h p.i. when infected with *S. Typhimurium* 14028 or 4/74. In contrast, monocyte viability following *S. Choleraesuis* A50 infection was slightly higher, at $\sim 79\%$, at 6 h p.i. and was almost completely maintained ($\sim 77\%$) at 24 h p.i. (Fig. 1B). This effect was not seen in monocytes infected with *S. Choleraesuis* A45 or B195, which had a viability similar to that of monocytes infected with *S. Typhimurium* (Fig. 1B).

Only *S. Choleraesuis* A50 induced significantly higher production levels of tumor necrosis factor alpha (TNF- α) by monocytes than the levels induced by *S. Typhimurium* 14028 or 4/74 (6 h p.i.) ($P < 0.05$) (Fig. 2A). However, at 6 h p.i., *S. Choleraesuis* strains A45 and B195 induced significantly higher expression of IL- β ($P < 0.05$ and $P < 0.01$, respectively) than either *S. Typhimurium* serovar (Fig. 2B). At 24 h p.i., *S. Choleraesuis* A50 induced significantly higher IL- β production by monocytes than *S. Typhimurium* ($P < 0.05$), and this production level was further increased by *S. Choleraesuis* A45 or B195 ($P < 0.01$) (Fig. 2B).

The temporal production levels of IL-6 were also different in *S. Typhimurium*- and *S. Choleraesuis*-infected monocytes. Monocytes infected with *S. Choleraesuis* A50 or B195 produced significantly more IL-6 at 6 h p.i. ($P < 0.05$) than monocytes infected with *S. Typhimurium* 14028 or 4/74 (Fig. 2C). However, at 24 h p.i., monocytes infected with *S. Choleraesuis* A45 or B195 (but not A50) produced significantly more IL-6 ($P < 0.05$) than monocytes infected with *S. Typhimurium* 14028 or 4/74 (Fig. 2C).

The most striking difference in cytokine production that we measured was that of IL-10. At both 6 h and 24 h p.i., monocytes infected with *S. Choleraesuis* A50, A45, and B195 induced significantly ($P < 0.01$) more IL-10 than did those infected with *S. Typhimurium* 14028 or 4/74. However, it was the scale of difference that was most surprising, with around a 10-fold difference at 24 h p.i. (Fig. 2D).

Using *S. Choleraesuis* A50 and *S. Typhimurium* 14028 as examples of these two serovars, we compared expression of *TNFR1*, *IL-10R*, and genes within the JAK/STAT pathway, all of which have been shown to be required for signaling during different phases of sepsis. Although both *S. Typhimurium* 14028 and *S. Choleraesuis* A50 increased TNFR1 protein expression on the surface of monocytes, this was much

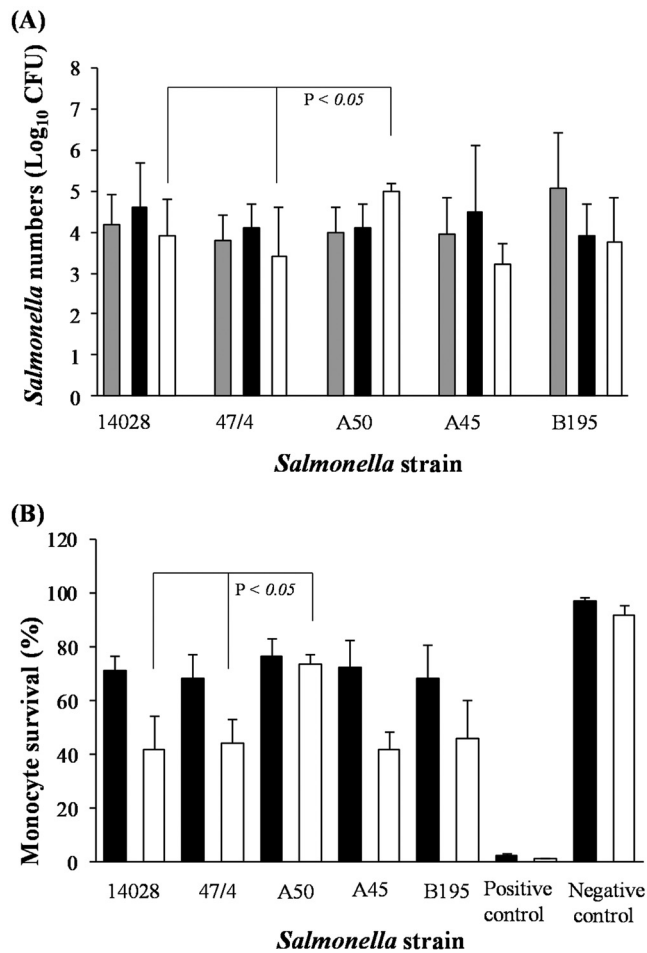


FIG 1 *Salmonella* bacterial numbers and human monocyte survival are increased following *S. Choleraesuis* A50 infection in comparison to *S. Typhimurium* infection. (A) Numbers of viable CFU of *S. Choleraesuis* (A50, A45, and B195) and *S. Typhimurium* (14028 and 4/74) recovered from monocytes at 2, 6, and 24 h p.i. (B) Monocyte viability was measured by propidium iodide uptake and FACS analyses following infection with *S. Choleraesuis* or *S. Typhimurium* strains after 6 and 24 h. Monocyte viability was compared with that of negative controls (uninfected monocytes cultured in medium for the same time period) and positive controls (monocytes immersed in ice-cold [-20°C] methanol for 30 min). Error bars represent standard deviations from the mean, and linkage bars at the top of the graph indicate significant differences ($P = 0.05$). Results shown are mean values calculated from triplicate experiments performed on five separate occasions. Gray bars, 2 h p.i.; black bars, 6 h p.i.; white bars, 24 h p.i.

greater in *S. Choleraesuis*-infected monocytes (Fig. 3A to D). However, neither serovar affected the expression of *IL-10R* (Fig. 3E to G).

S. Choleraesuis A50 induced differential expression of 19 JAK/STAT genes, with 17 upregulated and 2 downregulated (Fig. 4A and C; Table 1). *S. Typhimurium* 14028 infection induced differential expression of 19 JAK/STAT genes, with 15 upregulated and 4 downregulated (Fig. 4B and C; Table 2). In monocytes infected with *S. Typhimurium*, expression of *IFNG*, which encodes IFN- γ protein, was significantly increased ($P < 0.05$) by >30 -fold compared to that in uninfected cells (Fig. 4B). However, expression of the gene which encodes IFN- γ receptor 1 (*IFNGR1*) was significantly downregulated ($P < 0.05$) in *S. Typhimurium*-infected monocytes (Fig. 4C). No changes in expression of either *IFNG* or *IFNGR1* was measured in *S. Choleraesuis*-infected monocytes, but *S. Choleraesuis* infection did significantly increase ($P < 0.05$) expression of the interferon regulatory factor 1 (*IRF1*) gene (Fig. 4A), which was not altered by *S. Typhimurium* infection. Both *Salmonella* serovars significantly upregulated ($P < 0.05$) *IL-20* and *IL-2RA* genes, the latter of which encodes the IL-2 receptor and was upregulated >40 -fold by *S. Typhimurium* but almost 80-fold by *S. Choleraesuis* (Fig. 4A and B).

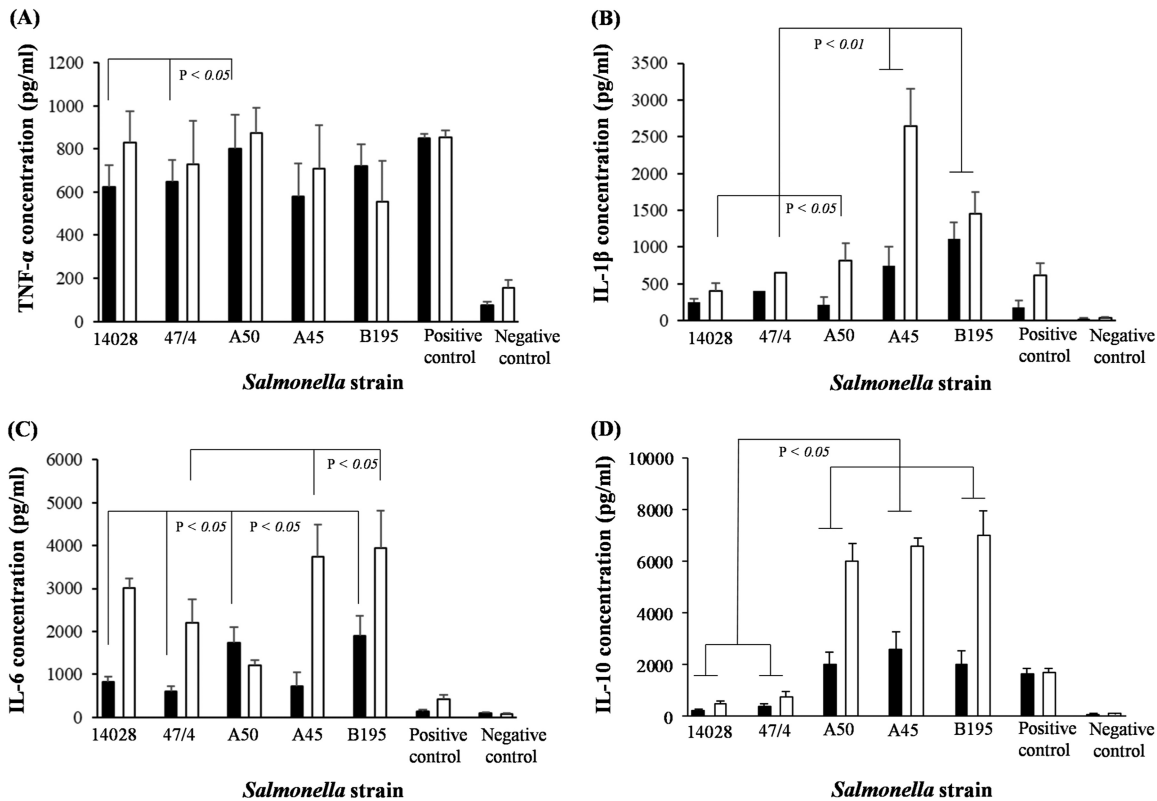


FIG 2 Hyperexpression of IL-10 by human monocytes is associated with *S. Choleraesuis* infection. ELISA analyses were performed to measure the production of proinflammatory cytokines TNF- α (A), IL-1 β (B), and IL-6 (C) and anti-inflammatory IL-10 (D) by monocytes infected with *S. Choleraesuis* (A50, A45, and B195) or *S. Typhimurium* (14028 and 4/74). These production levels were compared with those of monocytes cultured with PMA (positive control) or of monocytes cultured only in medium for the same experimental time period. Error bars represent standard deviations from the mean, and linkage bars indicate significant differences ($P = 0.05$). Results shown are mean values calculated from triplicate experiments performed on five separate occasions. Black bars, 6 h p.i.; white bars, 24 h p.i.

Genes which encode transcription factors of many different cytokines (*JUNB*, *NF- κ B*, and *CEBPD*) were also upregulated by both *S. Typhimurium* and *S. Choleraesuis* (Fig. 4A and B). Both serovars also significantly upregulated ($P < 0.05$) the *STAT4* gene, but this was most pronounced in *S. Choleraesuis*-infected monocytes, which showed a >40-fold increase (Fig. 4A), in comparison with about a 12-fold increase in *S. Typhimurium*-infected monocytes (Fig. 4B). In addition, *S. Choleraesuis* also significantly upregulated ($P < 0.05$) *STAT5A* and *JAK3* genes (Fig. 4A), neither of which were changed by *S. Typhimurium* infection.

Differences were also measured in the expression of genes which encode suppressor of cytokine signaling 1 to 3 (*SOCS1* to *SOCS3*). Both serovars induced significant increases in expression of *SOCS1* and *SOCS3* genes, and in the case of *SOCS3*, the expression levels were similar (~20-fold increase); but, in the case of *SOCS1*, *S. Choleraesuis* induced double that induced by *S. Typhimurium* (Fig. 4A and B) and only *S. Choleraesuis* infection induced significant upregulation ($P < 0.05$) of *SOCS2* (Fig. 4A). *S. Choleraesuis* infection also significantly upregulated the gene which encodes glucocorticoid receptor (*NR3C1*) (Fig. 4A), but neither serovar affected expression of the IL-10 receptor gene (*IL-10RA*).

Expression of other genes within the JAK/STAT pathway, which may have a direct effect on the ability of *Salmonella* to survive in host cells, was differentially affected by *Salmonella* infection. These included genes which encode the interferon-stimulated gene 15 protein (*ISG15*), BCL2 protein (*BCL2L1*), and protein tyrosine-protein phosphatase nonreceptor type 1 (*PTPN1*). All three genes were significantly upregulated ($P < 0.05$) by *S. Choleraesuis* and *S. Typhimurium* (Fig. 4A and B) infection. In *S. Typhimurium*-infected monocytes, expression of the gene which encodes colony-stimulating factor 1 receptor (*CSF1R*) was downregulated by >6-fold (Fig. 4C) but was

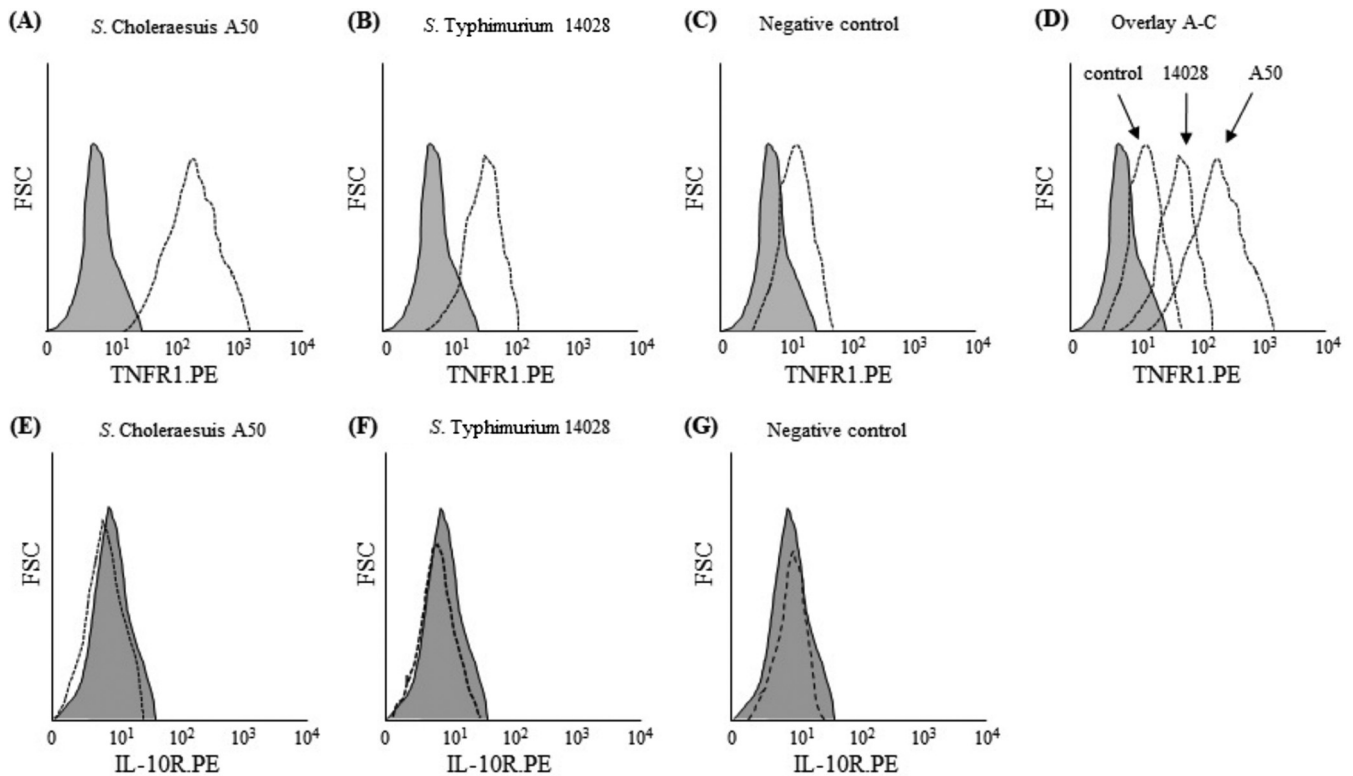


FIG 3 *S. Choleraesuis* A50 increases TNFR1 protein on the surface of human monocytes in comparison to *S. Typhimurium* 14028. FACS analyses were used to measure TNFR1 and IL-10R proteins on the surface of monocytes infected with *S. Choleraesuis* A50 and *S. Typhimurium* 14028. (A) TNFR1 expression by *S. Choleraesuis*-infected monocytes; (B) TNFR1 expression by *S. Typhimurium*-infected monocytes; (C) TNFR1 expression by uninfected monocytes; (D) overlay of panels A to C showing increased TNFR1 expression in *S. Choleraesuis*-infected monocytes compared to *S. Typhimurium*-infected monocytes; (E) IL-10R expression by *S. Choleraesuis*-infected monocytes; (F) IL-10R expression by *S. Typhimurium*-infected monocytes; (G) IL-10R expression by uninfected monocytes. Broken lines represent cytokine receptor expression relative to that of isotype controls (gray-shaded area). Histograms are representative of those obtained in triplicate experiments performed on five separate occasions.

not changed during *S. Choleraesuis* infection. Differential upregulation of JAK/STAT gene expression due to *S. Choleraesuis* A50 and *S. Typhimurium* 14028 infection in human monocytes is shown as a Venn diagram in Fig. 5.

DISCUSSION

We report differences in the immune responses to host-adapted *S. Typhimurium* and non-host-adapted *S. Choleraesuis* in human monocytes, which may have direct relevance to the greater propensity for *S. Choleraesuis* to cause human sepsis. Our study also indicates that some strains of *S. Choleraesuis* may be more likely to cause sepsis than others. First, *S. Choleraesuis* A50 infection prolonged monocyte viability much more so than infection with *S. Typhimurium* 14028 or 4/74 and at 24 h p.i., but this was not the case for *S. Choleraesuis* A45 or B195, which had results similar to *S. Typhimurium*. Previous studies have shown that increased apoptosis of blood monocytes correlates with increased survival of septic patients (12), and conversely, elevated monocyte numbers have been reported to correlate with sepsis (6). *S. Typhimurium* is known to induce apoptosis in human monocyte-derived macrophages (13) and, in a study by Watson et al. (14), *S. Typhimurium* bacteria damaged porcine macrophages that they invaded, but this was not the case for *S. Choleraesuis*. In pigs, as in humans, *S. Choleraesuis* causes a systemic infection and sepsis, and our results in combination with previously published work may indicate that in both humans and pigs, *S. Choleraesuis* A50-induced survival of monocytes may influence the development of sepsis. Susceptibility to the development of sepsis (and poor prognosis) in humans is also associated with a high serum IL-10 concentration (15–17). Our study shows that all strains of *S. Choleraesuis* induced ~10-fold more IL-10 than *S. Typhimurium*. However,

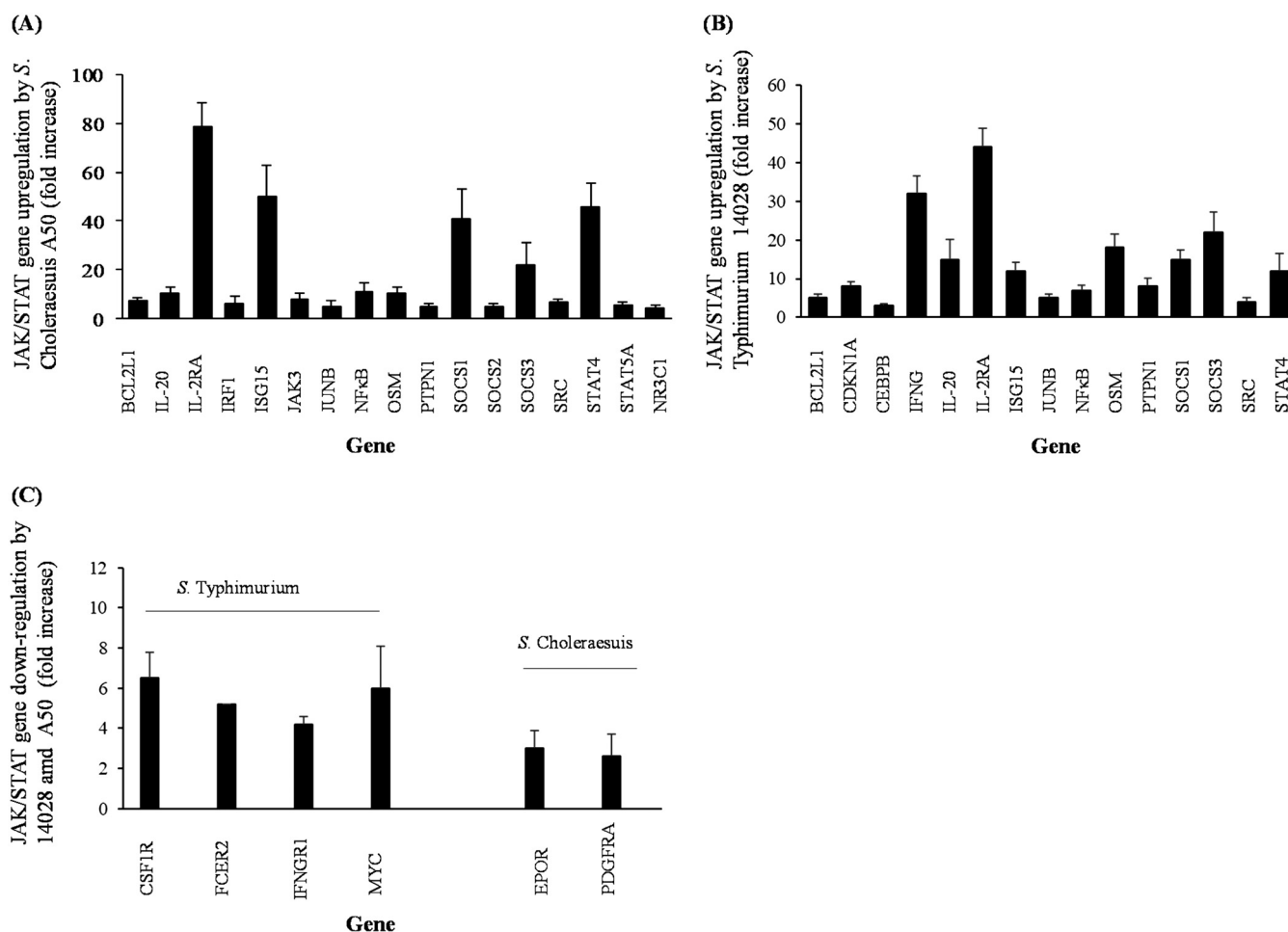


FIG 4 Differential expression of JAK/STAT genes by monocytes infected with *S. Choleraesuis* A50 and *S. Typhimurium* 14028. (A) Expression of JAK/STAT genes upregulated by *S. Choleraesuis* infection; (B) expression of JAK/STAT genes upregulated by *S. Typhimurium* infection; (C) expression of JAK/STAT genes downregulated by *S. Typhimurium* or *S. Choleraesuis* infection. Error bars represent standard deviations from the mean. Results shown are mean values calculated from triplicate experiments performed on three separate occasions. All bars indicate significant changes ($P < 0.05$) in threshold cycle (C_T) values in comparison to gene expression in uninfected (control) monocytes.

using *S. Typhimurium* 14028 and *S. Choleraesuis* A50 as examples, neither serovar increased IL-10R protein on the monocyte surface, which indicates that although *S. Choleraesuis* induces copious amounts of IL-10, this may not have an autocrine effect. High concentrations of IL-10, however, may inhibit the production of proinflammatory cytokines by Th1 lymphocytes (18) and/or induce peripheral tolerance via the differentiation of tolerogenic dendritic cells (19).

S. Choleraesuis A50 also induced a significant increase in TNF- α and IL-6 above that measured in *S. Typhimurium* 14028- or 4/74-infected monocytes after 6 h, but at 24 h p.i., TNF- α concentrations induced by the two serovars were comparable and the IL-6 concentration was significantly increased by *S. Typhimurium* infection above that induced by *S. Choleraesuis* A50 infection. Comparable levels of IL-1 β were produced by monocytes infected with *S. Choleraesuis* A50 and both *S. Typhimurium* strains at 6 h p.i., but IL-1 β production was increased at 24 h p.i. by *S. Choleraesuis* A50. However, *S. Choleraesuis* A45 and B195 infection induced the highest production levels of IL-6 and IL-1 β by any *Salmonella* serovar or strain measured at 6 and 24 h p.i. Although all three cytokines are prominent in sepsis, TNF- α infusion in experimental animals induces septic shock and infusion of either TNF- α or IL-1 β in humans induces symptoms very similar to those of the SIRS phase of acute sepsis (20). TNFR1 protein was increased on the surface of monocytes infected with either *S. Choleraesuis* A50 or *S. Typhimurium*

TABLE 1 Reputed functions of JAK/STAT genes differentially expressed in human monocytes infected with *S. Choleraesuis* A50^a

Gene	GenBank accession no.	Protein encoded	Gene function
Upregulated genes			
<i>BCL2L1</i>	NM_138578	BCL2-like1	Anti- or proapoptotic regulators that are involved in a wide variety of cellular activities
<i>IL-20</i>	NM_018724	Interleukin 20	Encodes a cytokine that has been shown to transduce its signal through signal transducer and activator of transcription 3 (STAT3) in keratinocytes
<i>IL2RA</i>	NM_000417	Interleukin 2 receptor alpha	Encodes IL-2 receptor protein
<i>IRF1</i>	NM_002198	Interferon regulatory factor 1	Transcription factor which activates IFN- α and IFN- β
<i>JAK3</i>	NM_000215	Janus kinase 3	Intracellular signaling molecule downstream of activated cytokine receptors
<i>ISG15</i>	NM_005101	ISG15 ubiquitin-like modifier	Encodes a ubiquitin-like protein that is conjugated to intracellular target proteins upon activation by interferon-alpha and interferon-beta
<i>JUNB</i>	NM_002229	JunB proto-oncogene	Transcription factor involved in regulating gene activity following the primary growth factor response
<i>NFκB</i>	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	Transcription regulator that is activated by various intra- and extracellular stimuli, such as cytokines, oxidant-free radicals, UV irradiation, and bacterial or viral products
<i>OSM</i>	NM_020530	Oncostatin M	Encodes a growth regulator which inhibits the proliferation of a number of tumor cell lines
<i>PTPN1</i>	NM_002827	Protein tyrosine phosphatase, non-receptor type 1	Encodes a PTP that was also reported to dephosphorylate JAK2 and TYK2 kinases, which implicated the role of this PTP in cell growth control and cell response to interferon stimulation
<i>SOCS1</i>	NM_003745	Suppressor of cytokine signaling 1	SOCS1 functions downstream of cytokine receptors and takes part in a negative feedback loop to attenuate cytokine signaling
<i>SOCS2</i>	NM_003877	Suppressor of cytokine signaling 1	Inhibitor of JAK/STAT pathway and IGF1R-mediated signaling
<i>SOCS3</i>	NM_003955	Suppressor of cytokine signaling 3	Encodes a protein that can bind to JAK2 kinase and inhibit the activity of JAK2 kinase
<i>SRC</i>	NM_005417	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	This proto-oncogene may play a role in the regulation of embryonic development and cell growth
<i>STAT4</i>	NM_003151	Signal transducer and activator of transcription 4	Known to induce Th1 development and IFN- γ production following stimulation of Th0 cells with IL-12
<i>STAT5A</i>	NM_003152	Signal transducer and activator of transcription 4	Transcriptional regulator of many different cytokine genes
<i>NR3C1</i>	NM_000176	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	Encodes glucocorticoid receptor proteins
Downregulated genes			
<i>EPOR</i>	NM_000121	Erythropoietin receptor	Encodes the erythropoietin receptor, a member of the cytokine receptor family; upon erythropoietin binding, this receptor activates Jak2 tyrosine kinase
<i>PDGFRA</i>	NM_006206	Platelet-derived growth factor receptor, alpha polypeptide	Encodes a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family

^aThe protein encoded by each gene, the gene's known function, and the GenBank accession number are shown. Upregulated genes are genes that were upregulated by *S. Choleraesuis* infection. Downregulated genes are genes that were downregulated by *S. Choleraesuis* infection.

14028. Expression of *TNFR1* was, however, much greater in *S. Choleraesuis* A50-infected monocytes, which may suggest that both infected monocyte populations are receptive to TNF- α but that this is possibly increased by *S. Choleraesuis* infection.

Analysis of gene expression in the JAK/STAT pathway also highlighted differences between *S. Choleraesuis* A50 and *S. Typhimurium* 14028. For example, although *ISG15* expression was upregulated in monocytes infected with both serovars, *S. Choleraesuis* A50 induced a 50-fold increase compared to a 12-fold increase for *S. Typhimurium* 14028-infected monocytes. *ISG15* has a number of different target proteins but is removed from these by ubiquitin-specific protease 18 (USP18) (21, 22), and USP18 knockout mice are susceptible to infection with both *Salmonella* and *Mycobacterium* and subsequently to Gram-negative sepsis (23, 24). We cannot say whether increased *ISG15* expression was compensated for by a parallel increase in USP18, but it is possible

TABLE 2 Reputed functions of JAK/STAT genes differentially expressed in human monocytes infected with *S. Typhimurium* 14028^a

Gene	GenBank accession no.	Protein encoded	Gene function
Upregulated genes			
<i>BCL2L1</i>	NM_138578	BCL2-like 1	Anti- or proapoptotic regulators that are involved in a wide variety of cellular activities
<i>CDKN1A</i>	NM_000389	Cyclin D1	Cyclins function as regulators of CDK kinases
<i>CEBPB</i>	NM_005194	CCAAT/enhancer binding protein (C/EBP) beta	Encodes a protein whose activity is important in the regulation of genes involved in immune and inflammatory responses, among other processes
<i>IFNG</i>	NM_000619	Interferon gamma	Encodes a soluble cytokine with antimicrobial, immunoregulatory and antitumor properties and is a potent activator of macrophages
<i>IL-20</i>	NM_018724	Interleukin 20	Encodes a cytokine that has been shown to transduce its signal through signal transducer and activator of transcription 3 (STAT3) in keratinocytes
<i>IL2RA</i>	NM_000417	Interleukin 2 receptor alpha	Encodes IL-2 receptor protein
<i>ISG15</i>	NM_005101	ISG15 ubiquitin-like modifier	Encodes a ubiquitin-like protein that is conjugated to intracellular target proteins upon activation by interferon-alpha and interferon-beta
<i>JUNB</i>	NM_002229	Jun B proto-oncogene	Transcription factor involved in regulating gene activity following the primary growth factor response
<i>NFκB</i>	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	NFκB is a transcription regulator that is activated by various intra- and extracellular stimuli, such as cytokines, oxidant-free radicals, UV irradiation, and bacterial or viral products
<i>OSM</i>	NM_020530	Oncostatin M	Encodes a growth regulator which inhibits the proliferation of a number of tumor cell lines
<i>PTPN1</i>	NM_002827	Protein tyrosine phosphatase, non-receptor type 1	Encodes a PTP that was also reported to dephosphorylate JAK2 and TYK2 kinases, which implicated the role of this PTP in cell growth control and cell response to interferon stimulation
<i>SOCS1</i>	NM_003745	Suppressor of cytokine signaling 1	SOCS1 functions downstream of cytokine receptors and takes part in a negative feedback loop to attenuate cytokine signaling
<i>SOCS3</i>	NM_003955	Suppressor of cytokine signaling 3	Encodes a protein that can bind to JAK2 kinase and inhibit the activity of JAK2 kinase
<i>SRC</i>	NM_005417	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	This proto-oncogene may play a role in the regulation of embryonic development and cell growth
<i>STAT4</i>	NM_003151	Signal transducer and activator of transcription 4	Known to induce Th1 development and IFN-γ production following stimulation of Th0 cells with IL-12
Downregulated genes			
<i>CSF1R</i>	NM_005211	Colony-stimulating factor 1 receptor	Encodes a protein that is the receptor for colony-stimulating factor 1, a cytokine which controls many monocyte functions
<i>FCER2</i>	NM_002002	Fc fragment of IgE, low affinity II, receptor for CD23	Encodes a protein that is a B cell-specific antigen and a low-affinity receptor for immunoglobulin E (IgE)
<i>IFNGR1</i>	NM_000416	Interferon gamma receptor 1	The initial receptor subunit of IFN-γ receptor to bind IFN-γ
<i>MYC</i>	NM_002467	V-myc myelocytomatosis viral oncogene homolog (avian)	Encodes a multifunctional, nuclear phosphoprotein that plays a role in apoptosis and cell cycle progression

^aThe protein encoded by each gene, the gene's known function, and the GenBank accession number are shown. Upregulated genes are genes that were upregulated by *S. Typhimurium* infection. Downregulated genes are genes that were downregulated by *S. Typhimurium* infection.

that elevated *ISG15* expression may be associated with increased numbers of *S. Choleraesuis* A50 bacteria measured in monocytes at 24 h p.i.

Expression of IFN-γ and IFN-γ receptor 1 genes (*IFNG* and *IFNGR1*, respectively) was also very different in monocytes infected with *S. Choleraesuis* A50 or *S. Typhimurium* 14028. *S. Typhimurium* 14028 upregulated expression of *IFNG* but downregulated *IFNGR1*, while neither gene was differentially expressed by *S. Choleraesuis* A50. In murine J774 macrophages, IFN-γ is required to kill wild-type *S. Typhimurium* 14028 (25), and human monocytes cultured with lipopolysaccharide (LPS) also produce IFN-γ (26), which may have an autocrine or paracrine effect on intracellular killing pathways.

However, viable bacteria are much more complex than LPS, since they express an array of different proteins required for intracellular infection and survival, and although we did not measure any additional effect due to IFN-γ in culture, the results may

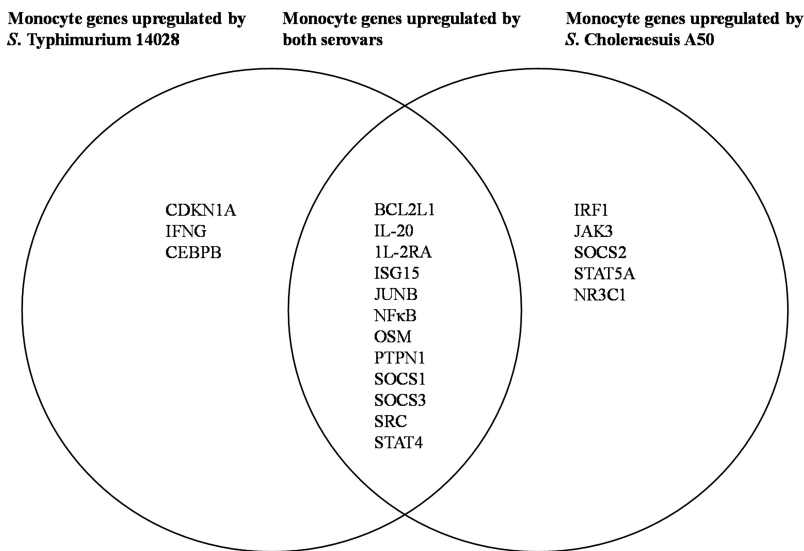


FIG 5 Venn diagram to show JAK/STAT genes upregulated in human monocytes by infection with *S. Choleraesuis* A50, *S. Typhimurium* 14028, or both serovars. The overlapping portion shows JAK/STAT genes upregulated in human monocytes following infection by both *S. Choleraesuis* A50 and *S. Typhimurium* 14028. Nonoverlapping portions show differences in genes upregulated by each serovar.

suggest that the effect of IFN- γ on intracellular *S. Typhimurium* may be reduced by the reduction in *IFNGR1* expression in *S. Typhimurium*-infected monocytes and in *S. Choleraesuis*-infected monocytes in which neither *IFNG* nor *IFNGR1* was differentially expressed.

In addition to a high level of IL-10 production, *S. Choleraesuis* A50-infected monocytes upregulated the expression of genes associated with immunosuppression. This included *NR3C1*, which encodes a glucocorticoid receptor, and *SOCS1* to *SOCS3*, although *S. Typhimurium* 14028 also upregulated the expression of *SOCS1* and *SOCS2*. SOCS proteins downregulate many inflammatory cytokines by influencing JAK/STAT signaling and downstream transcription (27), and overall, there was a lack of response in JAK and STAT genes in monocytes infected with either serovar. *STAT4* genes were upregulated by both serovars, while *S. Choleraesuis* also induced upregulation of *STAT5A* and *JAK3*, but we do not know whether SOCS expression and production of SOCS proteins may have influenced JAK and STAT gene expression by these monocytes.

In conclusion, our study shows a differential immune phenotype expressed by human monocytes infected with either *S. Choleraesuis* or *S. Typhimurium*. Importantly, all strains of *S. Choleraesuis* stimulate much greater concentrations of IL-10 than *S. Typhimurium*, and one strain (*S. Choleraesuis* A50) also significantly prolonged monocyte survival. These traits are associated with human sepsis and may, in part, explain the much higher prevalence of sepsis in humans infected with *S. Choleraesuis*.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Poole, Dorset, United Kingdom. PCR and microarray reagents were purchased from Qiagen, Manchester, United Kingdom.

Bioethics. Human blood was purchased from the blood transfusion service (Sheffield, United Kingdom). Blood used in this study was obtained with patient consent, and all studies were conducted following approval by local ethics committees.

Isolation of peripheral blood monocytes. Whole blood was diluted with sterile phosphate-buffered saline (PBS) and then gently poured onto Histopaque-1077 prior to isolation of the buffy coat, as standard procedure. After appropriate washing steps, buffy coat supernatants were resuspended with appropriate amounts of cold MACS buffer and anti-CD14 antibody-coated micromagnetic beads (Miltenyi Biotec, Biscy, Surrey, United Kingdom) according to the manufacturer's instructions. The viability of isolated monocytes was assessed using trypan blue (10% vol/vol) and was found to be >90% prior to

use. Experiments were performed in triplicate using blood from five individual (healthy) patients (five separate occasions).

Bacterial strains. *S. Typhimurium* 14028 is virulent in mice (28) and in *ex vivo* human monocytes (29). *S. Typhimurium* 4/74 was originally isolated from a diseased calf and is highly virulent for pigs (30, 31) and in *ex vivo* human monocytes (32). *S. Choleraesuis* A50 was isolated from diseased pigs and is virulent *in vivo* (30) and in *ex vivo* systems (33). *S. Choleraesuis* A45 was isolated from diseased pigs but has not been previously reported. *S. Choleraesuis* B195 was originally isolated from diseased pigs but is virulent for many mammalian species (34). All strains were cultured for 24 h in 10 ml of Luria-Bertani broth (Difco Laboratories, Detroit, MI) in a shaking incubator (150 rpm) at 37°C. They generally reached densities of 3×10^9 to 5×10^9 cells/ml.

Salmonella invasion assays. Monocytes were first washed with sterile PBS prior to the addition of *S. Typhimurium* or *S. Choleraesuis* at a multiplicity of infection (MOI) of 10:1 at 37°C, in 5% CO₂, for 60 min. The cells were then washed and cultured with RPMI medium containing 100 µg/ml gentamicin and incubated for a further 60 min. The monocytes were washed again, and the medium was replaced with RPMI medium containing 25 µg/ml of gentamicin for a further 6 or 24 h in total. The cells were then washed three times with PBS at room temperature and then lysed using 1% Triton X (Fisher Scientific LTD, Loughborough, United Kingdom) for 15 min at 37°C. Intracellular bacterial counts were determined by serial dilution at different time points of 2, 6, and 24 h p.i. Viable bacterial cell counts were measured in CFU per ml. All counts were performed in triplicate on five separate occasions.

Monocyte survival assay. The uptake of the fluorescent restriction dye propidium iodide (PI) was used to measure the viability of cells under the experimental procedures described above. At 6 and 24 h postculture, the monocytes were incubated in PBS containing PI (10 µg/ml) for 10 min. The number of nonviable cells (PI positive) was assessed using a FACSCanto II analyzer (Becton Dickinson, USA). Samples were acquired using BD FACSDiva (BD Biosciences, USA) and analyzed using CyFlogic 2.8 software, licensed to Nottingham University. Monocytes which had been immersed in ice-cold (−20°C) methanol for 30 min were used as a positive control, and monocytes cultured in medium only, for the same experimental time periods, were used as a negative control. All experiments were performed in triplicate on five separate occasions.

Cytokine concentration in monocyte supernatants. An enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, United Kingdom) was used to determine the concentration of cytokines known to be highly important during the SIRS phase (IL-6, IL-1β, TNF-α) and the CARS phase (IL-10) of sepsis. Supernatants were analyzed (in accordance with the manufacturer's instructions) at 6 h and 24 h p.i. from *S. Typhimurium*- and *S. Choleraesuis*-infected monocytes and compared to uninfected (negative) monocytes and supernatants from monocytes cultured with phorbol myristate acetate (PMA; 10 µg/ml) (positive control).

Expression of cytokine receptors on monocyte membranes. TNF-α and IL-10 have been proposed as possible biological markers of the SIRS and CARS phases of sepsis, respectively. Therefore, in addition to measuring TNF-α and IL-10 output, fluorescence-activated cell sorting (FACS) analyses were performed to compare the effects of *S. Typhimurium* and *S. Choleraesuis* infection on the expression of TNF-α receptor type 1 (TNFR1) and IL-10 receptor (IL-10R) protein on the surface of monocytes. Due to differential IL-10 profiles and monocyte survival associated with *S. Choleraesuis* A50 and *S. Typhimurium*, we chose *S. Choleraesuis* A50 and *S. Typhimurium* 14028 as examples of these two serovars. At 6 h p.i., 1×10^6 monocytes, from each group, were washed three times in FACS buffer (2 mM EDTA, 1% [wt/vol], bovine serum albumin [BSA]) at $300 \times g$ for 10 min per wash step. The cell pellets were then incubated with human TruStain FcX (Biolegend, San Diego, CA, USA) for 15 min to block FC receptors. After washing three times in FACS buffer, the cell pellets were resuspended in FACS buffer containing rat anti-human IL-10R.PE (where PE is phycoerythrin; 0.5 µg/ml) or an isotype control (rabbit anti-rat Ig2a.PE) (Biolegend, USA) or containing mouse anti-human TNFR1.PE (0.5 µg/ml) or an isotype control (rat anti-mouse IgG2b.PE) (eBioscience, Cambridge). After antibody incubation, for 45 min in the dark at 4°C on an end-to-end shaker, the cells were washed three times in FACS buffer and then resuspended in 0.2 ml of FACS buffer prior to analysis on a FACSCanto II analyzer (BD Biosciences, USA). Samples were acquired using BD FACSDiva (BD Biosciences, USA) and analyzed using the CyFlogic 2.8 software. Each experiment was performed in triplicate on five separate occasions.

Multiplex PCR array analysis. Following the results obtained for survival and cytokine production, we chose *S. Choleraesuis* strain A50 and *S. Typhimurium* strain 4/74 as examples of these two serovars to study gene regulation in the JAK/STAT pathway.

Monocytes were isolated at 6 h p.i. with *S. Typhimurium* 14028 or *S. Choleraesuis* A50 or unstimulated controls incubated with gentamicin for the same time period. The monocytes were washed briefly with ice-cold PBS, and the cell pellet was immediately harvested in 100 µl RNeasy lysis reagent, according to the manufacturer's recommendations. QIAshredder spin columns (Qiagen, Manchester, United Kingdom) were used to homogenize the cell lysate by centrifugation at 13,000 rpm ($>10,000 \times g$) for 5 min. Total RNA was extracted using an RNeasy plus minikit (Qiagen, United Kingdom). RNA quality was measured with a Nanodrop (ND 1000) spectrophotometer (Thermo Scientific, Loughborough, United Kingdom).

RT2 SYBR green master mix and an RT2 first-strand DNA kit were used, as per the manufacturer's instruction, to obtain monocyte DNA prior to multiplex PCR analysis using an RT2 profiler PCR array (Qiagen, United Kingdom). Changes in the expression of 84 genes of the JAK/STAT pathway were examined and compared to the mean expression of two reference genes, the beta-actin and glyceraldehyde 3-phosphate dehydrogenase genes. Mean fold changes in results from three replicate experiments performed on three separate occasions were recorded. Agarose gel electrophoresis was also used to verify quantitative PCR (qPCR) amplicon JAK/STAT products in infected monocytes.

Statistical analysis. Analysis of variance (ANOVA) analyses with a one-way classification were performed to determine the significance between experimental groups. Tukey's *post hoc* test was used to determine significant differences between groups at the 95% confidence limit ($P = 0.05$).

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