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Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

Diepen, A. van

Citation

Diepen, A. van. (2005, November 2). *Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence*. Retrieved from <https://hdl.handle.net/1887/4339>

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Comparison of mRNA levels from
RAW264.7 macrophages infected with
Salmonella enterica serovar
Typhimurium 14028s and the
superoxide-hypersusceptible mutant
DLG294 by microarray analysis

Angela van Diepen,¹ Riny Janssen,¹
and Jaap T. van Dissel¹

*Department of Infectious Diseases, Leiden University Medical Center,
PO Box 9600, 2300 RC Leiden, The Netherlands.¹*

Manuscript in preparation

Abstract

Upon activation, macrophages initiate the transcription of genes coding for the expression of proteins and enzymes that participate in mounting the host response against pathogens such as *Salmonella*. DLG294 is an *S. enterica* serovar Typhimurium mutant strain that is hypersusceptible to superoxide and is attenuated due to its lack of expression of *sspJ*. To further characterize DLG294 we evaluated whether macrophages responded differently upon challenge with the virulent wild type strain 14028s than upon challenge with this attenuated superoxide-hypersusceptible strain DLG294 by defining the transcript profiles of RAW264.7 cells exposed to either on of these strains.

Infection with either one of these strains resulted in altered expression of a lot of genes involved in all kinds of cellular processes and defense mechanisms. By comparing the transcript profiles of RAW264.7 macrophages exposed to wild-type *S. enterica* serovar Typhimurium 14028s or DLG294 for 4 h, however, no great differences could be observed, indicating that the intracellular presence of either of these two strains does not elicit a different host-cell response. Therefore, the differences in outgrowth between wild-type *S. enterica* serovar Typhimurium and DLG294 could only be explained by the lack of expression of *sspJ* in DLG294, although the exact function and mechanism of action of the protein encoded by *sspJ* remain unknown.



Introduction

Salmonellae are gram-negative, facultative intracellular pathogens that can cause a variety of diseases in animals and man, ranging from mild gastroenteritis to severe systemic infections like typhoid fever. *Salmonella enterica* serovar Typhimurium may cause gastroenteritis in man, but causes systemic infection in mice comparable to typhoid fever in man (15). *S. enterica* serovar Typhimurium predominantly invades mononuclear phagocytes and is able to cause persistent infections by evasion or disturbance of the host immune system (13). Despite the fact that these cells contain a multitude of antimicrobial defense mechanisms as part of the innate immune defense system, *S. enterica* serovar Typhimurium is able to enter, survive, and even replicate within these phagocytes. The exact mechanisms by which *S. enterica* serovar Typhimurium is able to survive after phagocytosis are unknown, but *S. enterica* serovar Typhimurium responds to the specific host environment by expressing factors that are necessary for intracellular survival and for resistance against the defense systems of the host (4, 8, 9, 13, 17). Like for most intracellular bacteria, this ability of *S. enterica* serovar Typhimurium to enter and replicate within phagocytic cells is essential for survival and pathogenesis, as mutants unable to do so are avirulent (7). In vitro studies have shown that *S. enterica* serovar Typhimurium is able to survive and replicate within non-activated macrophages and that this can also lead to the induction of apoptosis (18). The outcome of infection, however, strongly depends upon the interaction between the pathogen and its host.

Upon infection, the host will develop an immune response that limits bacterial growth and eventually kills and eliminates the pathogen. Although *Salmonella* resides and replicates within macrophages, these macrophages do play an important role in the host defense against *Salmonella*. In fact, interactions between the macrophage and *Salmonella* are necessary for the early control of infection, and subsequently, for the induction of acquired immunity (11, 16). Also in immune mice that developed a *Salmonella*-specific response including antigen-specific antibodies, macrophages played a major role in restricting early bacterial growth since in vivo depletion of macrophages in these mice resulted in increased susceptibility and mortality (25). In vivo, macrophages are mainly activated through T lymphocyte-dependent responses, but are also activated upon infection with live bacteria or upon contact with several bacterial components called Pathogen Associated Molecular Patterns (PAMPs) (including LPS, porins and outer membrane proteins, fimbrial proteins, flagella, lipoproteins, glycoproteins, and peptidoglycan) (10). Macrophages have evolved mechanisms to recognize such PAMPs by expressing pattern recognition receptors that recognize the PAMPs and then initiate the innate immune response to clear the infection (20). Upon activation, macrophages induce the transcription of genes coding for the expression of proteins and enzymes that participate in mounting the host response against the pathogens. The induced gene expression profile seems to be dependent upon the activation state of the macrophages (19) and might therefore give an indication on how the macrophages respond to infection with a certain pathogen.



Recently, we have described the isolation and characterization of a superoxide hypersusceptible *S. enterica* serovar Typhimurium mutant strain DLG294 (21). This mutant strain DLG294 lacks the expression of *sspJ* and is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages, but is able to grow out as much as the wild-type strain in cells and mice that cannot produce any superoxide due to a non-functional NADPH oxidase complex (21, 24). These studies showed that expression of *sspJ* in *S. enterica* serovar Typhimurium plays an important role in resistance against superoxide, but its exact function and mechanism of action remained to be elucidated. Additional experiments have shown that DLG294 is not only hyper-sensitive to superoxide, but is also more susceptible to certain antibiotics (Tahar van der Straaten, unpublished data). Although hypersusceptibility to superoxide could be the major cause of attenuated virulence of DLG294, it cannot be excluded that other factors might also play a role. Since a lot of strains that are highly susceptible to menadione, i.e. intracellular superoxide, are not attenuated in mice it is clear that superoxide sensitivity alone does not determine virulence. For example, we have recently described *S. enterica* serovar Typhimurium mutants that are much more susceptible to menadione, yet are not attenuated at all (22, 23). These data suggest that other factors play a role. For instance the macrophage response to Salmonella, which determines the level of macrophage activation, may be an important factor. Therefore we decided to study whether the macrophage responds differently to DLG294 than to wild-type *S. enterica* serovar Typhimurium 14028s. A possible difference in the activation status of the macrophages might explain the differences in virulence of DLG294 and the wild-type strain and might clarify whether attenuation of DLG294 is solely due to its hypersusceptibility to superoxide produced by the macrophages or that additional mechanisms play a role.

Materials and Methods

Bacterial strains. Single colonies of wild-type *S. enterica* serovar Typhimurium strain 14028s and superoxide-sensitive derivative DLG294 (21) were grown overnight in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm).

Cells and cell culture conditions. The mouse macrophage cell line RAW264.7 (ATCC TIB71) and the human granulocyte-like cell lines PLB-985 and X-CGD PLB-985 (26) were maintained at 37°C with 5% CO₂ in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (50 µg/ml). For granulocytic differentiation, the PLB-985 and X-CGD PLB-985 cells were exposed to 0.5% dimethylformamide for 5-6 days. Under these conditions, the PLB-985 cells acquire respiratory burst activity, while the X-CGD PLB-985 cells do not (26).



Replication of *S. enterica* serovar Typhimurium within RAW264.7 macrophages and PLB985 cells. One day before challenge RAW264.7 cells were seeded in 6-wells plates in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum, but without antibiotics at 1×10^6 cells per well. The cells were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection. To promote the uptake of the bacteria, the bacteria were spun onto the macrophages by centrifugation at $300 \times g$ for 10 minutes and the cells were allowed to internalize the bacteria for 30 minutes at 37°C with 5% CO₂. The cells were washed with phosphate-buffered saline (PBS) and were treated with 100 µg/ml gentamicin for 10 minutes to kill the extracellular bacteria and were then washed again. Medium supplemented with 10 µg/ml gentamicin was added to the cells to prevent reinfection and to kill any remaining bacteria. At 4 hours after infection, the cells were washed thoroughly with PBS and total RNA was isolated from 4 of the wells. As a control for infection, cells from duplicate wells were lysed with water and the number of intracellular bacteria was determined by plating serial dilutions. To obtain RNA from uninfected cells, the cells were treated exactly like the infected cells, but no bacteria were added. For infection of the non-adherent (X-CGD) PLB-985 cells, 1×10^5 cells were infected by incubating the cells together with 1×10^6 bacteria while rotating for 30 min at 37°C. The cells were then treated with 100 µg/ml gentamicin for another 60 min to kill the extracellular bacteria. After washing with PBS, the cells were lysed in 1 ml of distilled water. Serial dilutions of the lysate were made and plated for determination of the number of intracellular CFU.

In vivo *Salmonella* infection. Mice were inoculated subcutaneously with 3×10^4 CFU in the flanks with 0.1 ml bacterial suspension in PBS. Per group 4 mice were used to determine the bacterial load within the organs. On day 1 after infection, mice were sacrificed by carbon dioxide inhalation and the inguinal lymph nodes, livers and spleens were aseptically removed. The bacterial load within these organs was determined by preparing single-cell suspensions using 70-µm-mesh-size cell strainers (Falcon). The cells were pelleted by centrifugation for 10 min and the cells were lysed in distilled water. Serial dilutions of the lysate were made to determine the bacterial loads within the organs.

RNA sample preparation. Samples were prepared according to the protocols in the Affymetrix Gene Chip Expression Analysis technical manual (Affymetrix, Inc., Santa Clara, California) and were obtained from two independently performed in vitro replication experiments. Total RNA was isolated from the *S. enterica* serovar Typhimurium infected RAW264.7 cells at 4 hours after infection using TRIzol Reagent (Gibco BRL Life Technologies) according to the manufacturer's protocol. The RNA obtained from the uninfected cells was pooled, while the RNA samples from the infected cells were individually processed. After the ethanol precipitation in this TRIzol extraction procedure, the total RNA was cleaned up using the Qiagen RNeasy Total isolation Kit according to the manufacturer's protocol. The RNA was checked by gel electrophoresis before proceeding. Double stranded cDNA was prepared using the Gibco BRL SuperScript Choice System



and T7-(dT)₂₄ primer (Genset Corp., La Jolla, California). The cDNA was purified by phenol/chloroform extraction and ethanol precipitation. Biotin-labeled cRNA was synthesized in an in vitro transcription reaction using the ENZO Bioarray™ HighYield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc. Farmingdale, New York) according to the manufacturer's recommendations. Finally, the biotin-labeled cRNA was purified using the Qiagen RNeasy Total Isolation Kit and was fragmented in 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc at 94°C for 35 minutes.

Microarray. Microarray analysis was performed at the Leiden Genome Technology Center by Eveline Mank (Leiden, The Netherlands). The fragmented labeled cRNA (15 µg) was hybridized to GeneChip murine genome U74Av2 oligonucleotide arrays (Affymetrix, Santa Clara, CA). The chips were washed and stained with streptavidin-phycoerythrin in a GeneChip Fluidics station 400 (Affymetrix) and were then scanned using an Affymetrix GeneArray. Affymetrix Microarray Suite 5.0 (MAS5.0, Affymetrix) was used to analyze the data. The chips that were hybridized with cRNA from RAW264.7 cells infected with either *S. enterica* serovar Typhimurium 14028s or DLG294 were compared to a chip that was hybridized with pooled cRNA from uninfected cells to analyze which gene expression was changed by the infection. Difference calls were assigned as described previously (6): increased, 2; marginally increased, 1; nor changed, 0; marginally decreased, -1; decreased, -2. The sum of difference calls was calculated and a sum of ≥3 or ≤-3 was the cut off value for increase or decrease, respectively. We have converted the signal log ratio output of increased and decreased expression into fold change for convenience using the formula recommended by Affymetrix:

$$\begin{aligned}\text{Fold increase} &= 2^{\text{signal log ratio}}, \text{ if signal log ratio} > 0 \\ \text{Fold decrease} &= 2^{-\text{signal log ratio}}, \text{ if signal log ratio} < 0\end{aligned}$$

To ensure that the data are reliable, genes were considered to be differentially expressed if $P < 0.01$ (Student *t*-test) and fold increase ≥ 2.0 and fold decrease ≥ 1.75.

Cytometric bead array. At 4 h after infection, supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used in a Mouse Inflammation Cytometric Bead Array™ (CBA; BD Biosciences, San Jose, CA) according to the manufacturer's recommendations to compare the type and amount of cytokines produced by these cells and is schematically depicted in Fig. 1. The CBA assay contains six populations of polystyrene beads that have been coated with capture antibodies directed against either IL-6, IL-10, MCP-1, IFN γ , TNF α , or IL-12p70. These beads are of equal size but differ in fluorescence intensities and can be discriminated by detection in the FL3 channel of a flow cytometer. The six bead populations are mixed and 50 µl of this mixed suspension, together with 50 µl phycoerythrin(PE)-labeled detection antibodies are added to 50 µl of the test samples or cytokine standards and were incubated for 2 h at room temperature to



form the sandwich complexes. After washing the bead-cytokine-antibody-PE complexes, FACS analysis was performed on a BD FACSCalibur™ flow cytometer and data were acquired and analyzed using Becton Dickinson (BD) Cytometric Bead Array (CBA) software. Forward vs side scatter were used to gate on the beads. The fluorescence intensity detected in the FL-3 channel discriminates the six different bead populations and the mean fluorescence intensity measured with PE in the FL-2 channel is proportional to the cytokine concentration in the sample. By two-color dot plotting the FL-2 vs FL-3 the change in fluorescence intensity measured with PE for each of the six bead populations could be compared. The cytokine standards were used to make standard curves from which cytokine concentrations in the test samples could be calculated.

Results and Discussion

Early in vitro intracellular growth of *S. enterica* serovar Typhimurium. DLG294 is an *S. enterica* serovar Typhimurium mutant strain that is hypersusceptible to superoxide due to its lack of expression of *sspJ* (21). This mutant strain is attenuated in vivo in C3H/HeN mice and in vitro in RAW264.7 macrophages, but regains virulence in cells and mice that do not produce any superoxide due to a non-functional NADPH oxidase complex (21, 24). These previous studies showed that *sspJ* is involved in the defense against superoxide produced by macrophages, but its exact function and mechanism of action were still unknown. From this we concluded that attenuation of DLG294 is due to its hypersusceptibility to superoxide. However, when we examined at bacterial counts very early after infection of X-CGD PLB985 cells we observed that bacterial loads of DLG294 were lower than those of the wild-type strain both in the wild-type, superoxide producing PLB985 cells as well as in the X-CGD PLB985 cells that cannot produce any superoxide due to disruption of the *gp91^{phox}* gene (26) (Fig. 2A). Despite the fact that these X-CGD PLB-985 cells cannot produce any superoxide, DLG294, although not statistically significant, still showed lower bacterial numbers than the wild-type strain. When the cells were cultured for another 24 hours, DLG294 and the wild-type strain reached equal bacterial numbers in the X-CGD PLB985 cells while DLG294 is attenuated in PLB985 cells as described before (24). Similar data were observed in vivo, i.e. even though DLG294 reached similar bacterial numbers to wild-type 4 days after infection of mice (22), we observed that at 1 day after infection, bacterial numbers were lower than for the wild-type suggesting that early killing is different for wild-type and DLG294 (Fig. 2B and 2C). This suggests that attenuation of DLG294 is not solely due to increased susceptibility to superoxide and that additional factors such as the activation status of the cells play a role in the increased susceptibility and attenuation of DLG294 very early after infection. Once these early additional factors are overcome, DLG294 is able to grow out as much as the wild-type strain reaching comparable bacterial numbers in cells and mice that cannot produce any superoxide (24).



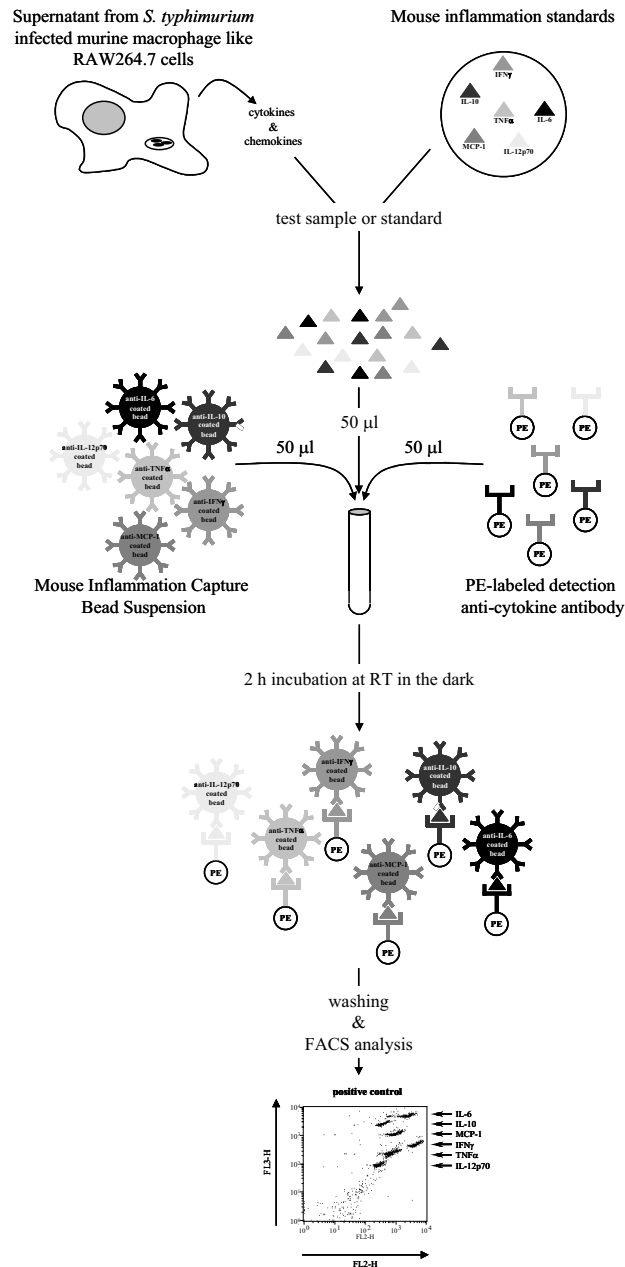


Figure 1. Schematic overview of the Cytometric Bead Array. Supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used according to the manufacturer's recommendations to compare the type and amount of cytokines produced by these cells.

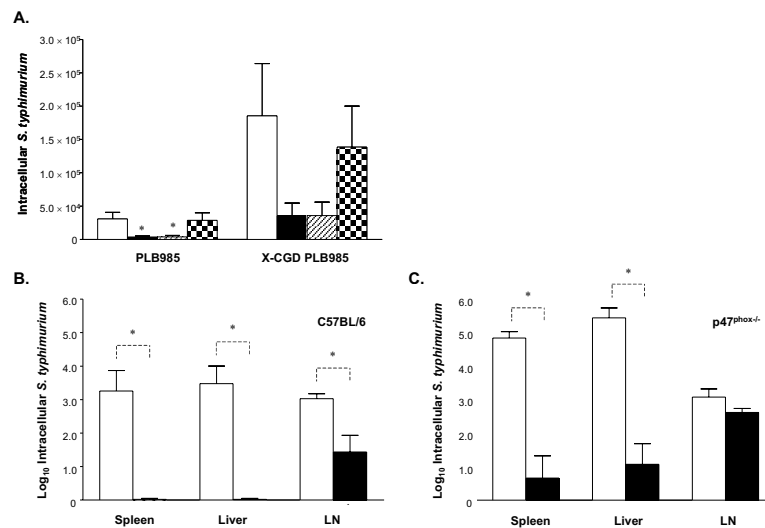


Figure 2. Bacterial numbers in (X-CGD) PLB985 cells after incubation with bacteria (MOI 10) (A) and in livers, spleens, and inguinal lymph nodes of C57BL/6 (B) or p47^{phox-/-} mice (C) at 1 day after infection. Cells were incubated or mice were infected with wild-type *S. enterica* serovar Typhimurium 14028s (white bars), DLG294 (black bars), DLG294-pWSK29 (dashed bars), or DLG294-pTS175 (checkered bars). Asterisks indicate that the number of intracellular bacteria is significantly different ($P < 0.05$) from that of wild-type *S. enterica* serovar Typhimurium 14028s.

Comparison of gene expression profiles of uninfected, wild-type, and DLG294 infected RAW264.7 cells. Next we studied the activation status of RAW264.7 macrophages infected with wild-type or DLG294 by determining gene expression profiles. Fig. 3 shows that the number of intracellular DLG294 was much lower than wild-type *S. enterica* serovar Typhimurium 14028s at 4 h after infection as shown previously (21). Bacterial numbers were equal between wild-type and DLG294 at 30 minutes after infection (data not shown), but the wild-type reached much higher bacterial counts after 24 h than DLG294, indicating that the uptake of DLG294 by the macrophages is similar to that of the wild-type strain, but its replication is severely impaired.

Infection with wild-type or DLG294 resulted in the induction of 174 genes. Only 9 of these genes were induced to a different extent in DLG294 and wild-type infected macrophages (Table 4). We will discuss genes that are highly induced, moderately induced, or that are repressed by infection and highlight the differences between wild-type and DLG294 infected cells. Genes showing down-regulated expression upon infection are shown in Table 3 that shows that the gene-expression was only moderate decreased compared to uninfected cells. The fold decreases do not exceed 3.9, while for the upregulated genes the expression increased up to more than a 100-fold for *Cxcl2* (Table 1). Genes showing decreased expression were mainly involved in cell cycle processes, signal transduction, and transcription.



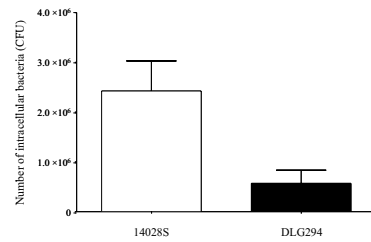


Figure 3. Number of intracellular bacteria in RAW264.7 macrophages at 4 h after infection (A). The cells were challenged with *S. enterica* serovar Typhimurium 14028s (white bars) or DLG294 (black bars) as described in Materials and Methods. Asterisks indicate that the number of intracellular bacteria is significantly different ($P < 0.05$) from that of wild-type *S. enterica* serovar Typhimurium 14028s.

Strongly induced genes. A lot of the genes that are up-regulated by the infection are involved in inflammatory processes and apoptosis, but also in signal transduction and transcription (Tables 1 and 2). The genes showing the most pronounced increase in expression are genes involved in inflammation and chemotaxis and was highest for *Cxcl2*, a gene encoding macrophage inflammatory protein 2 that is involved in the chemotaxis of leukocytes, but that does not induce chemokinesis or an oxidative burst.

Other genes showing highly increased expression are genes involved in the defense response and include cytokines, chemokines, MHC Class II, activation markers, and IFN γ or LPS induced genes involved in the immune response.

Table 1. Macrophage gene expression strongly induced by *S. enterica* serovar Typhimurium infection

Accession Nr.	Fold Increase		Gene	Gene or Protein
	14028s	DLG294		
Inflammation, Cytokines,and Chemokines				
M13926	12.06	14.06	Csf3	Colony-stimulating factor 3 (granulocyte)
M14639	8.21	10.57	Il1a	Interleukin 1 alpha
AF065947	75.41	59.18	Ccl5	Chemokine (C-C motif), ligand 5
U16985	29.27	34.63	Ltb	Lymphotoxin B
M33266	25.24	19.09	Cxcl10	Chemokine (C-X-C- motif), ligand 10
X53798	115.43	124.31	Cxcl2	Chemokine (C-X-C- motif), ligand 2
X62502	5.47	5.10	Ccl4	Chemokine (C-C motif), ligand 4
X70058	8.49	8.29	Ccl7	Chemokine (C-C motif), ligand 7
D84196	9.52	10.93	Tnf	Tumor necrosis factor
Transportation and Binding Proteins				
AI844128	6.75	7.56	Ehd1	EH-domain containing 1
AI747899	2.32	2.94	Pitpnb	Phosphatidylinositol transfer protein, beta
AF006467	12.62	6.15	Pitpnm	Phosphatidylinositol membrane-associated
Apoptosis				
U44088	36.38	21.39	Phlda1	Pleckstrin homology-like domain, family A, member 1
Cell Cycle, Differentation, and Proliferation				
M64849	3.45	5.15	Pdgfb	Platelet-derived growth factor chain B precursor (sis)
AF099973	6.56	6.83	Slfn2	Schlafen 2
Biosynthesis				
U00978	12.29	11.75	Impdh1	Inosine-5'-monophosphate dehydrogenase 1
X07888	16.00	19.75	Hmgcr	3-hydroxy-3-methylglutaryl coenzyme A reductase
Defense Response				
AF002719	6.19	6.59	Slpi	Secretory leukoprotease inhibitor
X54149	6.08	8.76	Gadd45b	Growth arrest and DNA damage-inducible protein GADD45 beta (myeloid differentiation primary-response protein Myd118)
Protein Degradation and Processing				
U66873	5.73	6.12	Pla2g5	Phospholipase A2, group V



Table 1. -continued-

Accession Nr.	Fold Increase		Gene	Gene or Protein
	14028s	DLG294		
Immune Response				
X56602	41.79	39.05	Isg15	Interferon stimulated protein
U43084	62.02	41.79	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1
AV152244	18.02	13.02	G1p2	Interferon, alpha-inducible protein
AI323667	93.76	91.38	Irg1	Immunoresponsive gene 1
L38281	76.36	57.99	Irg1	Immunoresponsive gene 1
Antigen Presentation				
X52914	10.23	10.50	H2-K	Histocompatibility 2, K region
D90146	22.06	32.48	H2-Q7	Histocompatibility 2, Q region locus 7
M27134	23.12	25.85	H2-K2	Histocompatibility 2, K region locus 2
Signalling Receptors				
U65747	7.07	4.77	Il13ra2	IL-13 receptor, alpha 2
Transcription				
X53654	6.30	6.64	Pou2f2	POU domain, class 2, transcription factor 2
Y11245	5.28	5.33	Foxm1	Forkhead box M1
X95316	5.48	4.04	Usf1	Upstream transcription factor 1
L00039	6.30	6.74	Myc	Myelocytomastosis oncogene
Regulatory				
AJ222800	8.00	8.49	Smpd2	Shingomyelin phosphodiesterase 2, neutral
M89800	10.20	11.31	Wnt6	Wingless-related MMTV integration site 6
U57524	4.64	5.33	Nfkbia	Nuclear factor of kappa light chain enhancer in B-cells inhibitor, alpha
Signal Transduction				
AB016589	13.50	21.11	Ikbke	Inhibitor of kappa-B kinase epsilon
M63659	17.93	21.93	Gna12	Guanine nucleotide binding protein, alpha 12
L35302	57.99	51.70	Traf1	TNF receptor-associated factor 1
AF053974	5.47	3.35	Swap70	Swap complex protein, 70 kDa
U58203	4.87	5.48	Arhgef1	Rho guanine nucleotide exchange factor (GEF) 1
U34960	4.09	5.28	Gnb2	Guanine nucleotide binding protein, beta 2
X61399	7.25	7.12	Mlp	MARCKS-like protein
AW120722	5.63	6.56	Mapkapk2	MAP kinase-activated protein kinase 2
AV374868	6.75	6.19	Socs3	Suppressor of cytokine signalling 3
M83380	5.78	6.08	Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B
U88328	32.48	28.08	Socs3	Suppressor of cytokine signalling 3
Other				
AF084480	6.14	8.30	Baz1b	Bromodomain adjacent to zinc finger domain, 1B
AF037437	4.15	7.56	Psap	Prosaposin
AB002136	7.32	7.84	Gpaa1	Glycosylphosphatidylinositol anchor attachment protein 1
U82610	16.24	27.00	Lcp1	Lymphocyte cytosolic protein 1
Unknown Gene Function				
AF064447	3.61	5.47	Fem1a	Feminization 1 homolog a
U50384	4.48	9.55	Smyd5	SET and MYND domain containing 5
AF073882	14.64	14.50	Mtmr7	Myotubularin related protein 7
U89434	10.06	16.15	Tbgr4	Transforming growth factor beta regulated gene 4
X67644	7.73	8.57	Ier3	Immediate early response 3
AA822413	5.89	5.86	Fbxw5	F-box and WD-40 domain protein 5
AW060657	7.73	8.59	Pmf1	Polyamine-modulated factor 1
AI837006	14.23	15.46	Cotl1	Coactosin-like 1

The production of cytokines and chemokines and the induction of activation markers are all processes involved in innate defense against *Salmonella*. Cytokines are small soluble proteins that mediate and regulate the (anti-) inflammatory responses and can have local or systemic effects on several components of the immune system. Upon infection of RAW264.7 macrophages with *S. enterica* serovar Typhimurium, expression of genes encoding cytokines involved in innate defense are induced, such as granulocyte colony-stimulating factor 3, Interleukin 1 α , lymphotoxin β , Tumor necrosis factor (encoded by *Csf3*, *Il1a*, *Ltb*, and *tnf*, respectively) (Table 1), and to a lesser extent, Interleukin 1



receptor antagonist (encoded by *Il1rn*) which regulates the expression and bioactivity of IL-1 (Table 2). Also genes encoding receptors involved in cytokine signaling such as TNF receptor, superfamily, members 5 and 1b (*tnfrsf5* and *tnfrsf1b*) and IL-13 receptor, alpha 2 (*Il13ra2*) are upregulated upon infection (Tables 1 and 2, signaling receptors).

Table 2. Macrophage gene expression moderately induced by *S. enterica* serovar Typhimurium

Accession Nr.	Fold Increase		Gene	Gene or Protein
	14028s	DLG294		
Inflammation, Cytokines, and Chemokines				
X03505	2.74	4.15	Saa3	Serum amyloid A3
L32838	2.66	1.69	Il1rn	IL-1 receptor antagonist
M88242	4.78	4.00	Ptgs2	Prostaglandin-endoperoxide synthase 2
Transportation and Binding Proteins				
U95145	3.37	3.01	Akap1	A kinase (PRKA) anchor protein 1
X57349	3.37	3.81	Trfr	Transferrin receptor
U15976	1.68	2.55	Slc27al	Solute carrier family 27 (fatty acid transporter, member 1
L23755	2.25	2.93	Slc19a1	Solute carrier family 19 (sodium/hydrogen exchanger), member 1
D21207	2.50	3.06	Bzrp	Benzodiazepine receptor, peripheral
AI852578	4.01	3.87	Slc11a2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
AI747899	2.32	2.94	Pitpnb	Phosphatidylinositol transfer protein, beta
Extracellular Matrix and Adhesion				
U91513	3.37	3.19	Nin1	Ninjurin 1
M90551	2.55	3.01	Icam1	Intercellular adhesion molecule 1
X79003	4.45	3.87	Itga5	Integrin alpha-5 (fibronectin receptor alpha)
Apoptosis				
X67914	2.86	2.47	Pdcd1	Programmed cell death 1
AF032459	4.59	3.61	Bcl2l11	BCL2-like 11 (apoptosis facilitator)
U59758	3.28	3.32	Trp53	Transformation related protein 53
L37296	2.89	2.93	Bad	Bcl-associated death promotor
M83649	4.83	4.69	Tnfrsf6	Tumor necrosis factor receptor superfamily, member 6 (Fas antigen)
AJ242778	4.04	4.30	Tnip1	TNFAIP3 interacting protein 1
Cell Cycle, Differentiation, and Proliferation				
D29678	2.52	2.75	Cdk5	Cyclin-dependent kinase 5
M95200	2.70	2.80	Vegfa	Vascular endothelial growth factor A
M64849	3.45	5.15	Pdgfb	Platelet-derived growth factor chain B precursor
AJ009862	3.66	3.62	Tgfb1	Transforming growth factor, beta 1
U09507	3.17	3.23	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21)
AI849928	2.65	2.38	Ccnd1	Cyclin D1
AW047032	3.19	4.16	Pin1	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
AW045530	2.02	2.49	Incenp	Inner centromere protein
DNA replication				
AW213225	2.61	3.77	Ddx18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
RNA processing				
AW120557	3.25	4.59	Lsm4	LSM4 homolog, U6 small nuclear RNA associated
Biosynthesis				
AB005623	2.55	2.55	Agpat1	1-acylglycerol-3-phosphate O-acetyltransferase (lysophosphatidic acid acyltransferase, alpha)
AW122653	3.26	2.65	Mvk	Mevalonate kinase
Cytoskeleton and Membrane Proteins				
J04181	2.83	3.88	Actb	Actin, beta, cytoplasmic
AJ249706	3.70	4.26	Myo10	Myosin X
AB024717	2.83	2.73	Clecsf9	C-type lectin (calcium-dependent, carbohydrate recognition domain), superfamily member 9
D14883	2.56	3.16	Kai1	Kangai 1 (suppression of tumorigenicity 6, prostate)
D00472	2.75	2.55	Cfl1	Cofilin 1, non-muscle
X56123	2.45	2.59	Tln	Talin
AI837100	3.62	3.77	Cd83	CD83 antigen
AI842889	2.49	2.75	Atp6v0b	ATPase, H ⁺ transporting, V0 subunit B



Table 2. -continued-

Accession Nr.	Fold Increase		Gene	Gene or Protein
	14028s	DLG294		
Protein Synthesis and Modification				
M76131	2.55	2.55	Eef2	Eukaryotic translation elongation factor 2
X69656	2.39	2.65	Wars	Tryptophanyl-tRNA synthetase
X05021	3.00	3.96	Rpl27a	Ribosomal protein L27a
AI265655	2.59	2.86	Ppil2	Peptidylprolyl isomerase (cyclophilin)-like 2
AV380793	2.39	2.93	Eif4g1	Eukaryotic translation initiation factor 4, gamma 1
Mitochondrion				
U85089	2.30	2.83	Txn2	Thioredoxin 2
AF043249	3.19	2.97	Tomm40	Translocase of outer mitochondrial membrane 40 homolog
D17571	2.23	2.73	Por	P450 (cytochrome) oxidoreductase
AI849904	2.17	2.74	Dlst	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxoglutarate complex)
Lipid Catabolism				
AA408341	3.10	3.52	Pla2g5	Phospholipase A2, group V
Metabolism				
AF032466	2.56	2.49	Arg2	Arginase type II
Z84471	2.30	2.75	G6pdx	Glucose-6-phosphate dehydrogenase, X-linked
AI843795	2.56	3.28	Pgls	6-phosphogluconolactonase
AW047185	2.56	3.38	Thop1	Thimet oligopeptidase 1
AI060798	3.38	4.16	Ptges	Prostaglandin E synthase
AI852592	2.18	3.07	Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2
Defense Response				
AV090497	4.44	3.66	Solpi	Secretory leukoprotease inhibitor
Superoxide Production				
AB002663	2.28	2.52	Ncf1	Neutrophil cytosolic factor 1 (p47 ^{phox})
U43384	2.55	2.93	Cybb	Cytochrome b-245, beta polypeptide
M31775	3.66	4.64	Cyba	Cytochrome b-245, alpha polypeptide
Protein Degradation and Processing				
M25149	2.49	2.89	Psmc3	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
AW124386	2.61	3.15	Ubl5	Ubiquitin-like 5
AI850365	2.38	2.65	Ubc-rs2	Ubiquitin C, related sequence 2
Regulatory				
X70764	3.46	4.09	Mark2	MAP/microtubule affinity-regulating kinase 2
AF018262	2.46	2.93	Ppp5c	Protein phosphatase 5, catalytic subunit
U20857	3.15	4.38	Rangap1	RAN GTPase activating protein 1
J02935	3.29	4.34	Prkar2a	Protein kinase, cAMP-dependent regulatory, type II-alpha
AF043070	2.30	3.04	Bckdk	Branched chain ketoacid dehydrogenase kinase
AW049387	2.56	2.86	Arl2	ADP-ribosylation factor-like 2
Intracellular Trafficking				
D87900	1.62	2.50	Arf3	ADP-ribosilation factor 3
Y13361	2.27	2.86	Rab7	RAB7, member RAS oncogene family
Signalling Receptors				
U05673	3.74	3.38	Adora2b	Adenosine A2b receptor
M83312	3.37	3.48	Tnfrsf5	TNF receptor superfamily, member 5
X62700	4.15	4.51	Plaur	Urokinase plasminogen activator receptor
X87128	4.38	4.87	Tnfrsf1b	TNF receptor superfamily, member 1b
AA608277	2.65	2.75	Adora2b	Adenosine A2b receptor
AI838195	3.46	4.26	Ogfr	Opioid growth factor receptor
Signal Transduction				
X95761	2.62	3.08	Lbcl1	Lymphoid blast crisis-like 1
X76850	3.98	2.62	Mapkapk2	MAP kinase-activated protein kinase 2
X84797	3.46	4.20	Hcls1	Hematopoietic cell specific Lyn substrate 1
X80638	3.77	4.46	Arhc	Ras homolog gene family , member C
Y17808	2.59	3.49	Ptk91	PTK9 protein tyrosine kinase 9-like (A6-related protein)
U20159	2.64	2.73	Lcp2	Lymphocyte cytosolic protein 2
U42383	2.73	2.47	Ppm1g	Protein phosphatase 1G (formarly 2C), magnesium-dependent, gamma isoform
AI642662	2.55	2.22	Dusp16	Dual specific phosphatase 16
AA153773	2.74	3.53	Tbl3	Transducin (beta)-like 3
AA764261	2.77	2.97	Pim1	Proviral integration site 1
AW124934	3.46	3.06	Pell1	Pellino 1
AA762522	4.38	4.59	Dtx2	Deltex 2 homolog



Table 2. -continued-

Accession Nr.	Fold Increase		Gene	Gene or Protein
	14028s	DLG294		
Transcription				
U09419	2.93	4.09	Nr1h2	Nuclear receptor subfamily 1, group H, member 2
AF015881	2.93	3.15	Nfe2l1	Nuclear factor, erythroid-derived 2, -like 1
AF043220	3.06	3.98	Gtf2i	Genereal transcription factor II I
AB009693	4.51	4.94	MafG	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein G
AF060076	2.62	2.66	Phc2	Polyhomeotic-like 2
X55038	2.77	3.74	Cenpb	Major centromere autoantigen B
U20735	2.66	2.77	JunB	Jun-B oncogene
X14678	4.05	2.33	Zfp36	Zinc finger protein 36
U47543	2.45	2.87	Nab2	Ngfi-A binding protein 2
M61007	2.93	3.23	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
L03215	2.59	2.84	Sfp1	SFFV proviral integration 1
AF017085	3.10	3.88	Gtf2i	General transcription factor II 1
J04103	2.73	2.55	Ets2	E26 avian leukemia oncogene 2, 3'domain
AI846152	2.74	2.70	Dscr1	Down syndrome critical region homolog 1
AW047899	2.97	2.75	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100
AI850881	2.47	2.65	Gtf2h4	General transcription factor II H, polypeptide 4
Other				
L24118	2.93	3.14	Tnfaip2	Tumor necrosis factor, alpha-induced protein 2
U60884	2.62	2.77	Bin1	Bridging integrator 1
U32197	2.86	3.62	Fpgs	Folypolyglytamyl synthetase
U05837	2.83	3.26	Hexa	Hexoaminidase A
Unknown Gene Function				
AF061346	2.22	2.86	Tnfaip2	TNFα induced protein 1
U87965	2.32	2.65	Gtpbp1	GTP-binding protein 1
AF033201	2.65	2.84	Cpsf4	Cleavage and polyadenylation specific factor 4
M59821	4.30	4.29	Ier2	Immediate early response 2
AW210320	2.62	2.59	Ptov1	Prostate tumor overexpressed gene1
AW125378	2.66	2.94	Aamp	Angio-associated migratory protein
AW122679	2.59	2.47	Prrg2	Proline-rich Gla (G-carboxyglutamic acid) polypeptide 2
AW122052	2.16	2.86	Nans	N-acetylneuraminic acid synthase (sialic acid synthase)
AW125157	2.45	2.63	Fbxw1b	F-box and WD-40 domain protein 1B
AI837492	2.39	3.14	Orf61	Open reading frame 61

These genes are upregulated upon infection with wild-type *S. enterica* serovar Typhimurium 14028s as well as by the superoxide-hypersusceptible mutant DLG294 and only the expression of *Il1m* is statistical significantly different being more expressed in wild-type 14028s-infected cells (Table 4) indicating that the inflammatory response induced by DLG294 is only slightly lower than that induced by the wild-type.

Chemokines are small molecules that are involved in chemotaxis and activation of leukocytes at the site of inflammation. These chemotactic cytokines can be divided into four subfamilies, designated C, CC, CXC, and CX3C chemokine ligands, based on the positions of their cysteine residues (2, 3). Genes encoding chemokines are induced upon infection with wild-type *S. enterica* serovar Typhimurium as well as with DLG294. The gene expression of *Ccl5*, *Cxcl10*, *Cxcl2*, *Ccl4*, and *Ccl7* are all highly induced (Table 1). Only the expression of *Cxcl10* differs significantly between 14018s and DLG294 infected cells being more expressed in 14028s infected cells (Table 4).

Isgl5, *Ifit1*, *Glp2*, and *Irg1* are genes involved in immune responses and the expression of these four genes was highly induced upon infection of RAW264.7 cells with either wild-type or DLG294 *Isgl5*, *Ifit1*, and *Glp2* are genes that are induced upon stimulation with IFN γ while *Irg1* is an LPS inducible gene (14). The expression of all four



genes seemed slightly lower for the DLG294 infected cells (Table 1). Gene expression of *Ifit1* appeared to be significantly lower for DLG294 infected cells compared to the wild-type infected cells (Table 4) suggesting that the IFN γ response may be slightly lower in DLG infected cells. Also the expression of the *H2* genes involved in antigen presentation were highly induced in the infected cells (Table 1), although no differences could be observed between the wild-type and DLG294 infected cells.

Table 3. Macrophage gene expression reduced by *S. enterica* serovar Typhimurium

Accession nr.	Fold Decrease		Gene	Gene or Protein
	14028s	DLG294		
Transportation and Binding Proteins				
AW227545	2.02	2.30	Strn	Striatin, calmodulin binding protein
Extracellular Matrix and Adhesion				
D50086	3.25	3.25	Nrp	Neuropilin
U47323	2.00	2.46	Stim1	Stromal interaction molecule 1
Apoptosis				
AI643420	1.52	1.81	Bag3	Bcl2-associated athanogene 3
Cell Cycle, Proliferation, and Differentiation				
AF003000	1.86	1.62	Terf2	Telomeric repeat binding factor 2
U95826	3.88	3.16	Ccng2	Cyclin G2
D78382	2.02	2.10	Tob1	Transducer of ErbB-2.1
D87326	1.81	2.30	Gsg2	Germ cell-specific gene 2
U42384	1.88	1.74	Fin15	Fibroblast growth factor inducible 15
AF086905	1.93	1.94	Chek2	CHK2 checkpoint homolog
M36033	1.69	1.76	Ptpa	Protein tyrosine phosphatase, receptor type A
Z35294	1.53	2.00	Mtcp1	Mature T-cell proliferation 1
M57647	2.64	3.62	Kitl	Kit ligand
AB033921	1.80	1.96	Ndr2	N-myc downstream regulated 2
Y12474	1.76	1.88	Cetn3	Centrin 3
AW121600	2.10	2.25	Ndr4	N-myc downstream regulated 4
AW209238	1.74	1.93	Tacc3	Transforming, acidic coiled-coil containing protein 2
AV349686	1.87	1.96	Ndr2	N-myc downstream regulated 2
Cytoskeleton and Membrane Proteins				
X98471	1.93	1.93	Emp1	Epithelial membrane protein 1
M58661	1.78	1.81	Cd24a	CD24a antigen
U38967	1.83	1.91	Tmsb4x	Thymosin, beta 4, X chromosome
AW121972	2.15	2.00	Wasip	Wiskott-Aldrich syndrome protein interacting protein
AI505453	1.63	1.87	Myh9	Myosin heavy chain IX
AW121840	1.99	2.23	Sel1h	Sel1 (suppressor of Lin-12) 1 homolog
DNA replication				
AI447783	2.22	2.46	Helb	Helicase (DNA) B
AA681520	1.81	2.02	Gmnn	Geminin
AW060791	1.76	1.69	Pole4	Polymerase (DNA-directed), epsilon 4 (p12 subunit)
RNA Processing				
U22262	2.75	2.66	Apobec1	Apolipoprotein B editing complex 1
Protein Synthesis				
M61215	1.64	1.93	Fech	Ferrochelatase
AF076681	1.93	1.74	Eif2ak3	Eukaryotic translation initiation factor 2 α kinase 3
AB004789	1.63	1.75	Dpm1	Dolichol-phosphate (beta-D-mannosyltransferase 1
Mitochondrion				
X51941	1.81	1.80	Mut	Methylmalonyl-Coenzyme A mutase
AF017175	2.16	2.07	Cpt1a	Carnitine palmitoyltransferase 1 (liver)
U07159	1.80	1.81	Acadm	Acetyl-Coenzyme A dehydrogenase, medium chain
AI842835	1.76	1.69	Uqcrc2	RTKubiquinol cytochrome c reductase core protein 2
Metabolism				
X77731	2.15	2.56	Dck	Deoxycytidine kinase
AI851983	1.66	1.81	Gsr	Glutathione reductase 1
AI842808	1.76	1.68	Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5



Table 3. -continued-

Accession Nr.	Fold Decrease		Gene	Gene or Protein
	14028s	DLG294		
Biosynthesis				
X86000	2.50	3.30	<i>Siat8d</i>	Sialyltransferase 8 (alpha-2, 8-sialyltransferase) D
U85414	2.55	2.30	<i>Gclc</i>	Glutamate-cysteine ligase, catalytic subunit
M26270	1.78	1.73	<i>Scd2</i>	Stearyl-Coenzyme A desaturase 2
AW060843	1.78	1.96	<i>Lias</i>	Lipoic acid synthetase
D16333	1.46	1.75	<i>Cpo</i>	Coproporphyrinogen oxidase
DefenseResponse				
M29394	1.57	2.07	<i>Cat</i>	Catalase
U77461	1.63	1.81	<i>C3ar1</i>	Complement component 3a receptor 1
Protein Degradation and Processing				
AB007139	1.52	1.81	<i>Psme3</i>	Proteasome (prosome, macropain) 28, subunit 3
AF079565	3.01	2.65	<i>Usp2</i>	Ubiquitin-specific protease 2
AW122823	2.08	2.00	<i>Ube2r2</i>	Ubiquitin-conjugating enzyme E2R2
AI844932	1.94	2.23	<i>Fbxo8</i>	F-box only protein 8
Immune Response				
AB007599	1.87	1.93	<i>Ly86</i>	Lymphocyte antigen 86
U15635	1.91	2.10	<i>Samhd1</i>	SAM domain and HD domain, 1
Intracellular Trafficking				
D49544	2.16	2.39	<i>Kifc1</i>	Kinesin family membe C1
AI847561	1.81	1.87	<i>Ap4s1</i>	Adaptor-related protein complex AP-4, sigma 1
AV059766	1.71	1.86	<i>Kif20a</i>	Kinesin family member 20a
Signalling Receptors				
D13458	2.55	2.08	<i>Ptger4</i>	Prostaglandin E receptor 4 (subtype EP4)
AF031127	1.57	1.78	<i>Itpr5</i>	Inositol 1,4,5-triphosphate receptor 5
AV012229	1.78	2.00	<i>Fcer1g</i>	Fc receptor, IgE, high affinity 1, gamma polypeptide
Regulatory				
D86344	2.47	2.47	<i>Pdcd4</i>	Programmed cell death 4
U20238	2.94	2.47	<i>Rasa3</i>	RAS p21 protein activator 3
AW122931	1.81	1.81	<i>Ikbkg</i>	Inhibitor of kappaB kinase gamma
AI835963	2.15	2.02	<i>Pias3</i>	Protein inhibitor of activated STAT3
AI851250	1.75	1.81	<i>Spred2</i>	Sprouty protein with EVH-1 domain 2, related sequence
AV335997	2.08	2.07	<i>Rgs10</i>	Regulator of G-protein signalling 10
Signal Transduction				
U37465	2.93	2.46	<i>Ptpro</i>	Protein tyrosine phosphatase, receptor type O
L11316	1.76	1.86	<i>Ect2</i>	Ect2 oncogene
AF068182	2.30	2.30	<i>Blnk</i>	B-cell linker
L21671	1.46	1.93	<i>Eps8</i>	Epidermal growth factor receptor pathway substrate 8
U67187	1.57	1.76	<i>Rgs2</i>	Regulator of G-protein signalling 2
AF079528	3.03	2.39	<i>Ier5</i>	Immediate early response 5
AF020313	1.68	1.75	<i>Apbb1ip</i>	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
AA981154	1.53	1.96	<i>Srpk2</i>	Serine/Arginine-rich protein specific kinase 2
AI317205	2.30	2.47	<i>Map3k1</i>	Mitogen activated protein kinase kinase kinase 1
AW124633	1.69	2.07	<i>Nek7</i>	NIMA (never expressed in mitosis gene-a)-related expressed kinase 7
AI849416	2.00	1.87	<i>Lats2</i>	Large tumor suppressor 2
AI847399	1.87	1.68	<i>Rgs10</i>	Regulator of G-protein signalling 10
AI835968	2.65	2.46	<i>Rin2</i>	Ras and Rab interactor 2
AI846534	1.62	1.94	<i>Nek6</i>	NIMA (never in mitosis gene-a)-related expressed kinase 6
Transcription				
M22115	1.88	1.80	<i>Hoxa1</i>	Homeo box A1
AF020200	1.88	2.35	<i>Pbx3</i>	Pre B-cell leukemia transcription factor
AF062567	1.58	1.83	<i>Sp3</i>	Trans-acting transcription factor 3
M32057	1.68	1.75	<i>Zfp239</i>	Zinc finger protein 239
AF000581	2.08	1.99	<i>Ncoa3</i>	Nuclear receptor coactivator 3
AF064088	2.00	2.07	<i>Tieg1</i>	TGFB inducible early growth response 1
AF064088	1.74	1.80	<i>Tieg1</i>	TGFB inducible early growth response 1
X64840	1.75	1.75	<i>Tcf12</i>	Transcription factor 12
AF077861	2.14	2.39	<i>Idb2</i>	Inhibitor of DNA binding 2
AI449034	2.23	1.86	<i>Rest</i>	RE1-silencing transcription factor
AI847906	1.80	1.75	<i>Tcf20</i>	Transcription factor 20



Table 3. -continued-

Accession Nr.	Fold Decrease		Gene	Gene or Protein
	14028s	DLG294		
Other				
L27439	1.53	2.19	<i>Pros1</i>	Protein S (alpha)
AF040252	2.02	1.75	<i>Fkbp7</i>	FK506 binding protein 7
U70674	2.38	2.22	<i>Numb</i>	Numb gene homolog
X74351	1.83	1.75	<i>Xpa</i>	Xeroderma pigmentosum, complementation group A
U64450	1.57	2.15	<i>Npm3</i>	Nucleoplasmin 3
AF004326	2.46	2.89	<i>Agpt2</i>	Angiopoietin 2
AF041472	1.62	1.81	<i>Sca2</i>	Spinocerebellar ataxia 2 homolog
AI987985	2.86	3.26	<i>Zfp288</i>	Zinc finger protin 288
AW060819	1.80	1.80	<i>Twsg1</i>	Twisted gastrulation homolog 1
AA733664	2.15	2.52	<i>Cpeb2</i>	Cytoplasmic polyadenylation element binding protein 2
AW125218	1.53	1.76	<i>Hat1</i>	Histidine aminotransferase
Unknown Gene Function				
U73039	1.94	1.88	<i>Nbr1</i>	Neighbor of Brca1 gene 1
M18070	1.69	1.75	<i>Pmp</i>	Prion protein
AF058797	1.51	1.68	<i>Ywhab</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide
AW120605	1.83	1.81	<i>Mllt3</i>	Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog
AI842472	3.19	2.49	<i>Zdhhc14</i>	Zinc finger, DHHC domain containing 14
AA930526	1.81	2.04	<i>Mtmr13</i>	Myotubularin related protein 13
AA968123	1.96	2.04	<i>Nav1</i>	Neuron navigator 1
AW120725	1.81	1.81	<i>Ubl3</i>	Ubiquitin-like 3
AW060827	2.02	2.00	<i>Them2</i>	Thioesterase superfamily, member 2

Moderately induced genes. Genes showing only moderate induced expression are mostly genes involved in cell cycle and cell death and apoptosis (Table 2). For all genes, no differences could be observed between wild-type and DLG294 infected cells. *Pdcd1*, *Bcl2l11*, *Trp53*, *Bad*, *Tnfrsf6* (Fas antigen), and *Tnfr1* are all genes encoding proteins that are involved in apoptosis and all genes show a 2-4 fold induction in expression. Also the expression of some genes involved in cell cycle (regulation) such as *Cdk5*, *Cdkn1a*, *Ccnd1* are induced upon infection. Again, these genes show only a slight increase in expression.

Direct comparison of gene expression profiles of 14028s and DLG294. Direct comparison of the gene expression profiles of RAW264.7 cells infected with either wild-type *S. enterica* serovar Typhimurium or the superoxide hypersusceptible mutant strain DLG294 revealed only small differences in the expression in only a few genes (Table 4). Nine genes showed increased expression in wild-type infected cells compared to DLG294 infected cells encoding proteins that are involved in inflammation and immune responses, RNA processing, transcription, and regulation. Most of these genes play a role in innate defense of the macrophages against pathogens such as *Salmonella*. *Cxcl10* encodes a chemokine (C-X-C motif) that is also known as IFN γ -induced protein 10. For *Mycobacterium tuberculosis* infected cells it has been shown that optimal expression of *cxcl10* is dependent upon TNF α (1). *Tnf* expression is induced in both wild-type and DLG294 infected cells (Table 1). Although not statistically significantly different in the direct comparison, from Table 1 it seems that *tnf* is induced even more in the DLG294 infected cells, while the induction of *cxcl10* expression is lower than for the wild-type infected cells.



Table 4. Fold change in macrophage gene expression 14028s vs DLG294

Accession Nr.	Fold Change	Gene	Gene or Protein	Function
M33266	1.4	<i>Cxcl10</i>	Chemokine (C-X-C- motif), ligand 10	Inflammation, Cytokines, and Chemokines
L32838	1.6	<i>Il-1rn</i>	IL-1 receptor antagonist	Inflammation, Cytokines, and Chemokines
AF037437	-1.5	<i>Gtge022</i>	Gene trap ROSA b-geo 22	Cytoskeleton and Membrane Proteins
AW213225	-1.4	<i>Ddx18</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 8	DNA Replication
L17076	1.6	<i>Raly</i>	HnRNP-associated with lethal yellow	RNA Processing
A1852592	-1.5	<i>Ndufb2</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	Metabolism
U43084	1.5	<i>Ifit1</i>	Interferon induced protein with tetratricopeptide repeats 1	Immune Response
X14678	1.4	<i>Zfp36</i>	Zinc finger protein 36	Transcription
M31418	1.5	<i>Ifi202a</i>	Interferon activated gene 202A	Transcription
L20450	1.6	<i>Zfp97</i>	Zinc finger protein 97	Transcription
AA981581	1.9	<i>Hnrpu</i>	Heterogeneous nuclear ribonucleoprotein U	Regulatory
AF037437	-1.8	<i>Psap</i>	Prosaposin	Other
U82610	-1.5	<i>Lcp1</i>	Lymphocyte cytosolic protein 1	Other
X05546	-1.7	<i>lap2</i>	Intracisternal A particle 2	Other
A1645561	1.4	<i>Narg1</i>	NMDA receptor-regulated gene 1	Other

Protein Assay as Confirmation of Microarray Analysis. One way to validate the results from the mRNA expression levels by microarray analysis is to determine the protein levels of certain genes involved in defense against *S. enterica* serovar Typhimurium. We decided to assess proteins secreted into the supernatant of infected RAW264.7 cells at 4 h after infection. This was done by a Mouse Inflammation Cytometric Bead Array, which allows the analysis of six proteins simultaneously. As shown in Figure 3, we observed that uninfected RAW264.7 cells secrete no detectable amounts of IL-6, IL-10, IFN γ , and IL-12-p70, but do secrete low amounts of MCP-1 and TNF α .

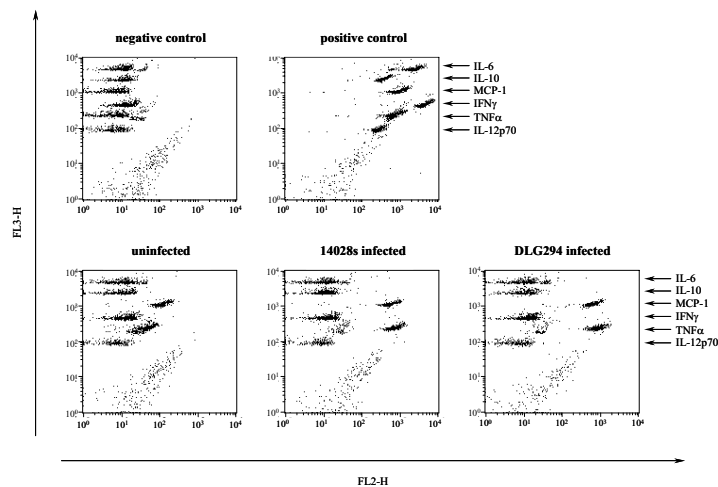


Figure 4. Mouse Inflammation Cytometric Bead Array analysis of supernatant from RAW264.7 cells that were left untreated or that were infected with *S. enterica* serovar Typhimurium 14028s or DLG294. Cells were challenged as described in Materials and Methods. At 4 h after challenge, the supernatant was taken and used for analysis in the Cytometric Bead Array according to the manufacturer's recommendations



The secretion of MCP-1 and $\text{TNF}\alpha$ is induced strongly upon infection of the cells with either wild-type *S. enterica* serovar Typhimurium 14028s or the mutant strain DLG294. MCP-1 is a chemotactic factor encoded by *Ccl2* that is secreted by the macrophages to attract monocytes, but not neutrophils. Upon infection of the cells with wild-type or DLG294 the production of MCP-1 increased 3-fold to 60.3 and 64.5 ng respectively (Fig. 4). Both strains induced the production and secretion of MCP-1 to a similar extent, which corresponds to the relative increases in mRNA expression of the gene encoding MCP-1 (*Ccl2*) in the microarray analysis (2.10 fold for wild-type and 1.94 fold for DLG294 infected cells; data not shown). Expression levels of mRNA for $\text{TNF}\alpha$, however, increased 9.5 and 10.9 fold for wild-type- and DLG294-infected cells respectively, while the secreted $\text{TNF}\alpha$ levels were induced 22.9 fold to 100.6 ng for wild-type and 33.6 fold to 147.9 ng respectively. These results show that the increase in secreted amounts of the protein does not correspond exactly to the increase in mRNA expression, but they do show that the relative induction of mRNA expression and secreted product by wild-type and DLG294-infected cells do correlate and confirms the data observed in the microarray analysis. The other four proteins were not detected within the supernatant of uninfected and infected RAW264.7 cells.

Concluding remarks. Since the gene expression profiles are dependent upon the activation status of the macrophages (19) and no clear differences in the gene expression profiles of RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 were observed, it might be concluded that the activation status of the macrophages is not altered by infection with DLG294 compared to the wild-type strain. Apparently, lack of expression of *sspJ* in DLG294 does not result in stronger or lesser activation of the macrophages, indicating that both strains are equally capable of modulating the macrophages' response. This strongly suggests that attenuation of DLG294 is not due to the hosts' macrophages, but is due to the lack of expression of *sspJ* in DLG294 causing its pleiotropic phenotype, including hypersusceptibility to superoxide. This is supported by the fact that DLG294 is able to grow out in cells and mice that do not produce any superoxide (24), although the activation statuses of these cells are unknown.

Research on *S. enterica* serovar Typhimurium mutants, including DLG294, has led to the isolation and identification of mutants that are either more susceptible or more resistant to superoxide. From these studies it became clear that *Salmonella* has evolved many mechanisms to cope with superoxide stress that may either prevent production or limit the damage that is done by these compounds (reviewed in (12)). It also became clear that mutants might share the same in vitro phenotype according to superoxide sensitivity, but they might have different in vivo phenotypes and very diverse genetic defects, ranging from defects in SOD's, glucose-6-phosphate dehydrogenase, or DNA repair mechanisms. So from the in vitro phenotype it cannot be predicted what the consequences of this mutation are for the in vivo and in vitro virulence. And reversely, from the in vivo and in vitro phenotype it cannot be predicted what the underlying genetic and proteomic defects might be. One way to understand the differences in the in vitro and in vivo phenotype is to



do research on host genes induced upon infection with *Salmonella* as is done in this study. Another approach would be to look at in vivo-regulated genes of *S. enterica* serovar Typhimurium itself during infection of host cells (5). Direct comparison of gene expression profiles of intracellular DLG294 and wild-type *S. enterica* serovar Typhimurium might reveal the role of *sspJ* in defense against superoxide stress and in virulence of *S. enterica* serovar Typhimurium.

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