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

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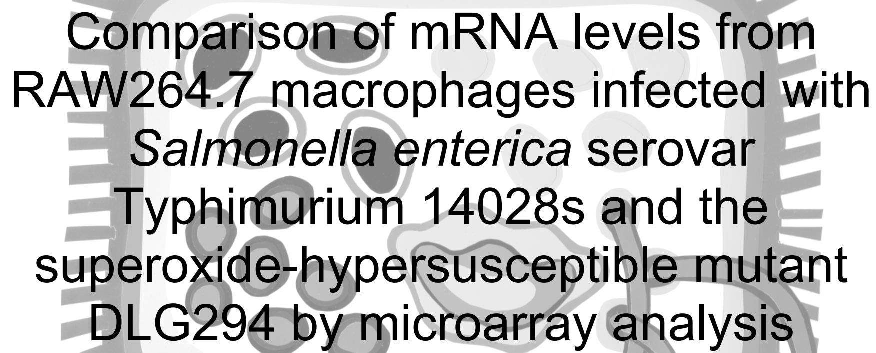

# Comparison of mRNA levels from RAW264.7 macrophages infected with *Salmonella enterica* serovar Typhimurium 14028s and the superoxide-hypersusceptible mutant DLG294 by microarray analysis

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## 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. enter*ica 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 *Salmonell*a 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 37ºC while being shaken (225 rpm).

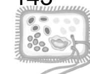
**Cells and cell culture conditions.** The mouse macrophage cell line RAW264.7 (ATCC TIB71) and the human granulocyte-like cell lines PLB-985 and X-CGD PLB-985 (26) were maintained at 37ºC with 5% $CO_2$ in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin (100 U/ml), 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 $1 \times 10^6$ 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 37ºC with 5% $CO_2$. 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, $1 \times 10^5$ cells were infected by incubating the cells together with $1 \times 10^6$ 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 dilutons of the lysate were made and plated for determination of the number of intracellular CFU.

**In vivo *Salmonella* infection.** Mice were inoculated subcutaneously with $3 \times 10^4$ 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:

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

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.

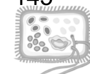
**Cytometric bead array.** At 4 h after infection, supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used in a Mouse Inflammation Cytometric Bead Array™ (CBA; BD Biosciences, San Jose, CA) according 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 polysterene beads that have been coated with capture antibodies directed against either IL-6, IL-10, MCP-1, IFNγ, TNFα, 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$^{TM}$ 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 that 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).





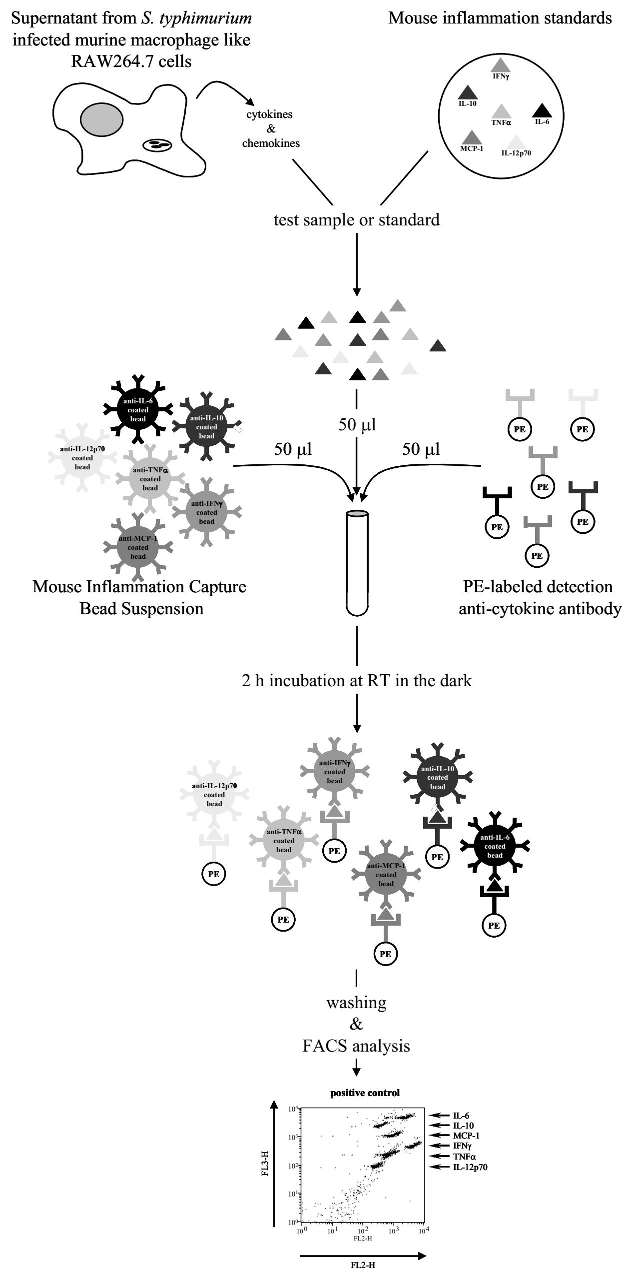



**Figure 1.** Schematic overview of the Cytometric Bead Array. Supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used according to the manufacturer's recommendations to compare the type and amount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

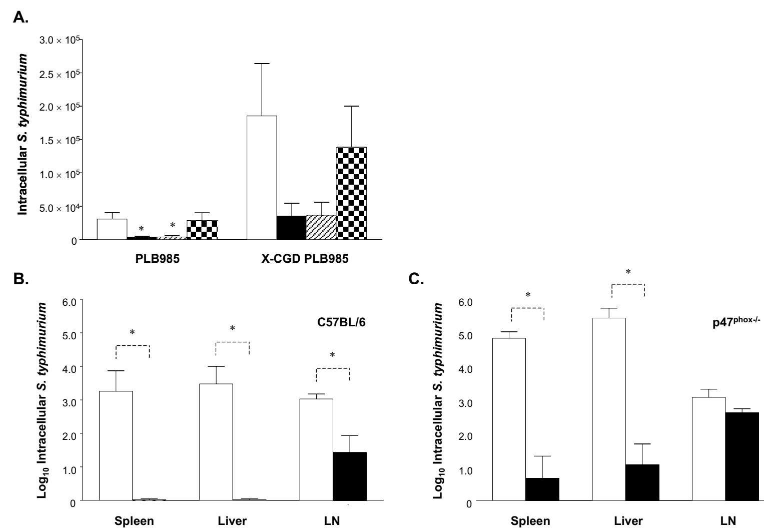
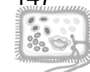



**Figure 2.** Bacterial numbers in (X-CGD) PLB985 cells after incubation with bacteria (MOI 10) (A) and in livers, spleens, and inguinal lymph nodes of C57BL/6 (B) or $p47^{phox-/-}$ mice (C) at 1 day after infection. Cells were incubated or mice were infected with wild-type *S. enterica* serovar Typhimurium 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.





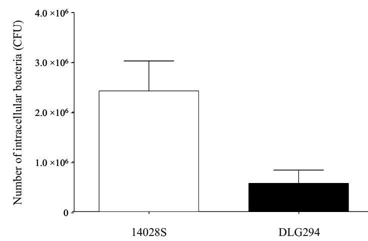



**Figure 3.** Number of intracellular bacteria in RAW264.7 macrophages at 4 h after infection (A). The cells were challenged with S. enterica serovar Typhimurium 14028s (white bars) or DLG294 (black bars) as described in Materials and Methods. Asterisks indicate that the number of intracellular bacteria 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$\gamma$ 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 be (myeloid differentiation primary-response protein Myd118) |
| **Protein Degradation and Processing** | | | | |
| U66873 | 5.73 | 6.12 | *Pla2g5* | Phospholipase A2, group V |



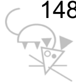


**Table 1. -continued-**

| 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 $\alpha$, lymphotoxin $\beta$, 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 | *Ninj1* | 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, Differentation, 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 domai lectin, 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 | *Psmd3* | 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 typrotein 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 | *Peli1* | 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 | *Sfpi1* | 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* | Folylpolyglytamyl synthetase |
| U05837 | 2.83 | 3.26 | *Hexa* | Hexoaminidase A |
| **Unknown Gene Function** | | | | |
| AF061346 | 2.22 | 2.86 | *Tnfaip2* | TNF$\alpha$ 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 *Il1rn* 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$\gamma$ 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 IFNγ 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 | *Ptpra* | 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 | *Waspip* | Wiskott-Aldrich syndrome protein interacting protein |
| AI505453 | 1.63 | 1.87 | *Myhg* | 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 |



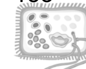


**Table 3. -continued-**

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



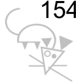


**Table 3. -continued-**

| Accession Nr. | Fold Decrease | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Other** | | | | |
| L27439 | 1.53 | 2.19 | *Pros1* | Protein S (alpha) |
| AF040252 | 2.02 | 1.75 | *Fkbp7* | FK506 binding protein 7 |
| U70674 | 2.38 | 2.22 | *Numb* | Numb gene homolog |
| X74351 | 1.83 | 1.75 | *Xpa* | Xeroderma pigmentosum, complementation group A |
| U64450 | 1.57 | 2.15 | *Npm3* | Nucleoplasmin 3 |
| AF004326 | 2.46 | 2.89 | *Agpt2* | Angiopoietin 2 |
| AF041472 | 1.62 | 1.81 | *Sca2* | Spinocerebellar ataxia 2 homolog |
| AI987985 | 2.86 | 3.26 | *Zfp288* | Zinc finger protin 288 |
| AW060819 | 1.80 | 1.80 | *Twsg1* | Twisted gastrulation homolog 1 |
| AA733664 | 2.15 | 2.52 | *Cpeb2* | Cytoplasmic polyadenylation element binding protein 2 |
| AW125218 | 1.53 | 1.76 | *Hat1* | Histidine aminotransferase |
| **Unknown Gene Function** | | | | |
| U73039 | 1.94 | 1.88 | *Nbr1* | Neighbor of Brca1 gene 1 |
| M18070 | 1.69 | 1.75 | *Prnp* | Prion protein |
| AF058797 | 1.51 | 1.68 | *Ywhab* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide |
| AW120605 | 1.83 | 1.81 | *Mllt3* | Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog |
| AI842472 | 3.19 | 2.49 | *Zdhhc14* | Zinc finger, DHHC domain containing 14 |
| AA930526 | 1.81 | 2.04 | *Mtmr13* | Myotubularin related protein 13 |
| AA968123 | 1.96 | 2.04 | *Nav1* | Neuron navigator 1 |
| AW120725 | 1.81 | 1.81 | *Ubl3* | Ubiquitin-like 3 |
| AW060827 | 2.02 | 2.00 | *Them2* | 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 *Tnip1* 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$\gamma$-induced protein 10. For *Mycobacterium tuberculosis* infected cells it has been shown that optimal expression of *cxcl10* is dependent upon TNF$\alpha$ (1). *Tnf* expression is induced in both wild-type and DLG294 infected cells (Table 1). Although not statistical 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 | *Cxcl10* | Chemokine (C-X-C- motif), ligand 10 | Inflammation, Cytokines, and Chemokines |
| L32838 | 1.6 | *Il-1rn* | IL-1 receptor antagonist | Inflammation, Cytokines, and Chemokines |
| AF037437 | -1.5 | *Gtrgeo22* | Gene trap ROSA b-geo 22 | Cytoskeleton and Membrane Proteins |
| AW213225 | -1.4 | *Ddx18* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 8 | DNA Replication |
| L17076 | 1.6 | *Raly* | HnRNP-associated with lethal yellow | RNA Processing |
| AI852592 | -1.5 | *Ndufb2* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 | Metabolism |
| U43084 | 1.5 | *Ifit1* | Interferon induced protein with tetratricopeptide repeats 1 | Immune Response |
| X14678 | 1.4 | *Zfp36* | Zinc finger protein 36 | Transcription |
| M31418 | 1.5 | *Ifi202a* | Interferon activated gene 202A | Transcription |
| L20450 | 1.6 | *Zfp97* | Zinc finger protein 97 | Transcription |
| AA981581 | 1.9 | *Hnrpu* | Heterogeneous nuclear ribonucleoprotein U | Regulatory |
| AF037437 | -1.8 | *Psap* | Prosaposin | Other |
| U82610 | -1.5 | *Lcp1* | Lymphocyte cytosolic protein 1 | Other |
| X05546 | -1.7 | *Iap2* | Intracisternal A particle 2 | Other |
| AI645561 | 1.4 | *Narg1* | NMDA receptor-regulated gene 1 | Other |

**Protein Assay as Confirmation of Microarray Analysis.** One way to validate the results form 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$\gamma$, and IL12-p70, but do secrete low amounts of MCP-1 and TNF$\alpha$.

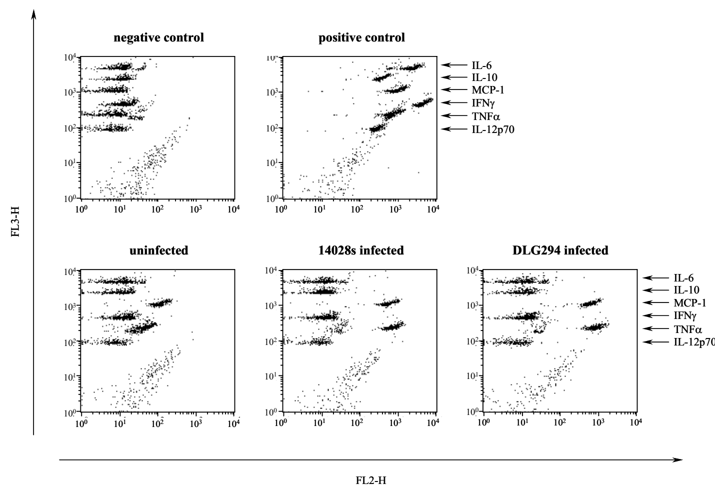


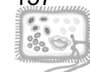

**Figure 4.** Mouse Inflammation Cytometric Bead Array analysis of supernatant from RAW264.7 cells that were left untreated or that were infected with S. enterica serovar Typhimurium 14028s or DLG294. Cells were challenged as described in Materials and Methods. At 4 h after challenge, the supernatant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

The secretion of MCP-1 and TNFα 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 TNFα, however, increased 9.5 and 10.9 fold for wild-type- and DLG294-infected cells respectively, while the secreted TNFα 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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