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3 # "Transcriptome analysis of murine macrophages in response to infection

4 # with *Streptococcus pyogenes* reveals an unusual activation program"

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6 ***Running title:*** *Unusual activation of S. pyogenes-infected macrophages*

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ABSTRACT

The complex response of murine macrophages to infection with *Streptococcus pyogenes* was investigated at the level of gene expression using a high-density oligomer microarray. More than 400 genes were identified as being differentially regulated. Many of the up-regulated genes encoded molecules were involved in immune response and inflammation, transcription, signalling, apoptosis, cell cycle, electron transport and cell adhesion. Of particular interest was the up-regulation of proinflammatory cytokines, typical of the classically activated macrophages (M1 phenotype) such as TNF-α, IL-1 and IL-6, and also the up-regulation of anti-inflammatory mediators such as IL-1ra and IL-10 associated with macrophage alternative activation (M2 phenotype). Furthermore, the gene encoding inducible nitric oxide synthase (iNOS), an enzyme typically implicated in classical activation was not induced in infected macrophages. Instead, the gene encoding arginase, a competitor for the iNOS substrate arginine and involved in the alternative activation pathway was up-regulated in *S. pyogenes*-infected cells. Thus, the microarray-based gene expression analysis demonstrated that *S. pyogenes* induced an atypical activation program in macrophages with some but not all features of classically or alternatively activation phenotypes. The microarray data also suggested that the bactericidal activity of macrophages against *S. pyogenes* is mediated by phagocyte oxydase since *p47phox* was up-regulated in infected cells. Indeed, the *in vivo* and *in vitro* killing of *S. pyogenes* was markedly diminished in the absence of functional phagocyte (p47$^{phox-/-}$) but not in the absence of iNOS (iNOS$^{-/-}$). Understanding how macrophages respond to *S. pyogenes* at the molecular level may facilitate the development of new therapeutic paradigms.

INTRODUCTION

S. pyogenes (group A streptococcus) is a prevalent human pathogen responsible for a broad spectrum of clinical manifestations including infections of the skin and upper respiratory tract, bacteremia and occasionally sepsis and septic shock (9). Streptococcal septic shock is the most severe form of streptococcal disease and is characterized by an intense inflammatory reaction (25). The severity and outcome of the infections caused by *S. pyogenes* is likely to depend on the ability of the host innate immune mechanisms to control bacterial growth and to limit further spread of the pathogen beyond the site of infection.

Previous studies examining host responses to *S. pyogenes* in a mouse model of infection have shown the importance of resident macrophages for controlling infection (17, 18). Macrophages are capable of recognizing, phagocytozing, and destroying *S. pyogenes* in order to eliminate the invading pathogen, while also producing cytokines and chemokines which are crucial in controlling recruitment and activation of inflammatory cells at the site of infection (17, 18). Although it is assumed that the activation of macrophages is directed toward the elimination of the invading pathogens, it is equally likely that the excessive and unregulated stimulation of macrophages can lead to a continuous release of inflammatory mediators that act synergistically and thus lead to sepsis and septic shock (12). Therefore, the functional activities of macrophages during *S. pyogenes* infection may greatly influence the character, course, and outcome of the pathogenic process.

To improve our understanding of the complex response of macrophages to *S. pyogenes* and to identify new targets at which therapeutic options might be possible, we have analysed the global gene expression profile of murine resident peritoneal macrophages after *in vivo* infection with this pathogen using gene array technology. We have identified more than 400 genes differentially transcribed in macrophages following 1 h of infection with *S. pyogenes.* Infection-induced genes fell into several functional categories, including immune response

and inflammation, transcription, signalling, apoptosis, cell cycle, electronic transport, cell adhesion, and other genes with unknown function.

Macrophages are plastic cells that respond to microenvironmental signals with distinct activation programs (19, 20, 30). Classically activated macrophages (M1 phenotype) are induced by inflammatory molecules such as LPS and IFN-γ. These M1 macrophages produce proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-12, and MIP-1α and generate reactive nitrogen species such as nitric oxide (NO) via expression of iNOS (19, 20, 30). Alternatively activated macrophages (M2 phenotype) are generated after exposure to certain stimuli such as IL-4, IL-13, TGF-β, or glucocorticoids (19, 20). The M2 macrophages express anti-inflammatory molecules, such as IL-10 and IL-1 decoy receptor (IL-1ra), and metabolize arginine through arginase rather than iNOS (19, 20). Arginase blocks iNOS activity by a variety of mechanisms, including competing for the arginine substrate that is required for NO production (5). Classically activated M1 macrophages are potent effector cells integrated in Th1 responses which kill microorganisms and tumor cells and produce copious amounts of proinflammatory cytokines. In contrast, M2 macrophages tune the inflammatory responses, promote angiogenesis, tissue remodeling and repair. However, the M1 and M2 phenotypes seem to represent the two extremes of a spectrum of possible forms of macrophage activation and different versions of the M2 phenotype, M2a, M2b and M2c, have been described with different functional properties. That is, M2a macrophages are induced by IL-4 or IL-13 and are involved in promotion of Th2 responses; M2b macrophages are induced by exposure to agonists of Toll-like receptors (TLRs) or IL-1 receptor and they play a role in suppression and regulation of inflammation and immunity; and the M2c phenotype, induced by IL-10 and glucocorticoid hormones, participate in matrix deposition and tissue remodelling (1, 26, 27).

The phenotype of macrophages activated by *S. pyogenes* is currently unknown but may be important in understanding the contribution of these phagocytic cells to the disease

1 pathogenesis. In this regard, we have shown here that *S. pyogenes* induces an atypical
2 activation phenotype in macrophages that includes markers characteristic of M1 as some of
3 the M2 activation pathways.
4

MATERIALS AND METHODS

Bacteria. The *S. pyogenes* strains used in this study were the *S. pyogenes* strain A20 (M-type 23), a human isolate obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 2071) and the sequenced M-type 1 strain SF370 (14). Stocks were maintained at -70°C and were routinely cultured at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, UK), supplemented with 1% yeast extract. Bacteria were collected in mid-log-phase, washed twice with sterile PBS, diluted to the required inoculum and the number of viable bacteria determined by counting CFU after diluting and plating in blood agar plates (GIBCO, Karlsruhe, Germany) containing 5% sheep blood.

Mice. Inbred female C3H/HeN and BALB/c mice were purchased from Harlan-Winkelmann, (Borchen, Germany). Mice with either a targeted disruption in the iNOS gene (B6.129P2-*Nos2*^{tm1Lau}/J; iNOS$^{-/-}$) or with a targeted deletion in the cytosolic p47phox gene (B6(Cg)-*Ncf1*^{m1J}/J; p47$^{phox-/-}$), as well as their wild-type control mice (C57BL/6J) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Animals were housed in microisolator cages and given food and water ad libitum. All studies were approved by the local Ethical Board.

In vivo infection of peritoneal macrophages. Mice were intraperitoneally infected with 5 × 10^7 CFU of *S. pyogenes,* euthanized 1 h thereafter, and their peritoneum was lavaged with sterile PBS. Macrophages present in the lavage samples were labeled with anti-F4/80 antibodies, further purified by positive selection using miniMACS magnetic microbeads according to the manufacture's instructions (Miltenyi Biotec Inc., Germany) and used for the cDNA microarray analysis or RT-PCR.

For macrophage killing assays, peritoneal macrophages isolated from infected mice (1 h postinoculation) were seeded in 48-wells microtiter plates and cultured at 37°C, 5% CO2 in Dulbecco's modified Eagle medium (DMEM) (GIBCO) containing 10 mM HEPES, 2 mM L-

1 glutamine, and 100 μg/ml of gentamicin. At several time points, the macrophages were lysed
2 with dH_2O and surviving bacteria were enumerated by plating serial dilutions in blood agar.
3 In some experiments, peritoneal macrophages were stimulated with 1 μg/ml of LPS
4 from *Salmonella typhimurium* (Sigma-Aldrich, Taufkirchen, Germany) plus 100 U/ml of
5 recombinant murine IFN-γ (PeproTech, Rocky Hill,USA).
6
7 **Array analysis.** Total RNA was isolated from highly purified F4/80+ cells obtained from the
8 peritoneal cavity of BALB/c or C3H/HeN infected and uninfected control mice using
9 peqGold TriFast^{TM} (Peqlab) according to the manufacturer's instructions and hybridized to an
10 Affymetrix GeneChip MOE430A using standard Affymetrix protocols as described elsewhere
11 (36). Two replicate chips per group were used with pooled macrophages harvested from 8-10
12 mice. The data set used in this study is available in a MIAME compliant format at the NCBI
13 Gene Expression Omnibus (GEO) under accession number GSE7769 (GEO, Gene Expression
14 Ominibus, http://www.ncbi.nlm.nih.gov/geo/). Normalized gene expression intensities were
15 compared and genes were considered as differentially expressed between infected and
16 uninfected samples when their fold change was greater than or equal to 2 or less than or equal
17 to –2. The statistical parameter used to define significant change was less than 0.001 (change
18 *p*-Value) and the difference between compared signal intensities of a certain gene was more
19 than 200.
20
21 **RT-PCR.** To independently confirm the microarray results, RT-PCR was carried out on
22 arbitrarily selected genes that were found up-regulated in infected macrophages in the
23 microarray analysis (*Il-1α*, *Il-1β*, *Il-6*, *Csf2*, *Tnf-α*, *Mip-1α*, *Mip-1β*) and 2 unaffected genes
24 (*Ifn-γ*, *Il-12p40*). Total RNA was prepared as described above. RNA was reverse transcribed
25 with Reverse Transcriptase (Hoffman La Roche) and cDNA synthesis was performed using a
26 Gibco RT-PCR kit following the manufacturer's instructions. The single-stranded cDNA was

1 then subjected to PCR under standard reaction conditions. The PCR primer sequences for
2 these genes as well as the housekeeping genes *β-actin* or *Rsp9* are described in Table 1. The
3 resultant PCR products were electrophoresed on a 2% agarose gel, stained with ethidium
4 bromide and photographed.
5
6 **Detection of IL-6 production by ELISA.** The determination of IL-6 was performed by
7 specific ELISA. In brief, 96-well microtiter plates were coated overnight at 4°C with purified
8 rat anti-mouse anti-IL-6 capture antibody (Pharmingen, San Diego, CA) at 2 µg/ml in sodium
9 bicarbonate buffer. The wells were washed and then blocked with 2% bovine serum albumin-
10 PBS before the supernatant samples and the appropriate standard were added to each well.
11 Biotinylated rat monoclonal anti-IL-6 (Pharmingen) at 2 µg/ml was added as the second
12 antibody. Detection was carried out with streptavidin-peroxidase and the plates were
13 developed using ABTS. A standard curve was generated using recombinant murine IL-6
14 (Pharmingen).
15
16 **Measurement of nitric oxide (NO).** The Greiss reaction was used to determine NO
17 concentrations in supernatants of *S. pyogenes*-infected macrophages as previously described
18 (11). Briefly, supernatant from cultured uninfected or *S. pyogenes*-infected macrophages was
19 mixed with an equal volume of Griess's reagent (1% sulfanilamide, 0.1%
20 naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). Absorbance at 550 nm was
21 recorded. Serial dilutions of sodium nitrite were used to construct a standard curve.
22
23 **Determination of reactive oxygen radicals (ROS).** For detection of ROS generated by *S.*
24 *pyogenes*-infected macrophages, mice were intraperitonenally infected as described above
25 with either unlabelled *S. pyogenes* of *S. pyogenes* labeled green with carboxyfluorescein
26 (Molecular Probes, Göttingen, Germany). For labeling, a suspension of 5 x10^8 bacteria was

centrifugated, resuspended in 1 ml of Hanks balanced salt solution (HBSS) containing 0.2 mg/ml of carboxyfluorescein, and incubated 30 min 4°C in the dark. After incubation, labeled bacteria were washed several times to remove unbound dye. Infected mice were euthanized 1 h after bacterial inoculation and subjected to peritoneal lavage. Mice intraperitoneally injected with PBS were used as control. Lavage samples were added to wells containing glass coverslips and incubated for 1 h at 37°C, 5% CO2. After washing to remove non-adherent cells, macrophages were incubated with 1 mg/ml of Nitro blue tetrazolium (NBT) dissolved in krebs-Ringer phosphate glucose buffer (KRPG: 144 mM NaCl, 5 mM KCl, 8.5 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1.3 mM $MgSO_4$, % mM glucose, 10 mM HEPES, pH 7.4) for 45 min at 37°C. After incubation, the cells were washed twice with KRPG buffer, fixed in 4% paraformaldehyde and counterstained with Giemsa stain. Samples were examined by light and fluorescence microscopy for the presence of green-fluorescence bacteria and blue-black formazan precipitate.

Experimental infection of mice. A previously described murine model of *S. pyogenes infection* was used (16). In brief, mice were inoculated with 10^5 CFU of *S. pyogenes* in 0.2 ml of PBS via a lateral tail vein. Viable bacterial counts were determined in blood of infected mice by collecting blood samples from the tail vein at 24 h post-inoculation and plating serial dilutions in blood agar.

Statistical Analysis. Statistical significance between samples was determined by ANOVA analysis and the Mann-Whitney (Wilcoxon) W- test with $p < 0.05$ considered significant.

RESULTS

Tanscriptome analysis of murine macrophages infected with *S. pyogenes*. Analysis of the relative expression levels of 22623 genes showed that more than 400 genes were differentially expressed in resident peritoneal macrophages after *in vivo* infection with *S. pyogenes* (Fig. 1A). Approximately 70% of the differentially induced genes were up-regulated and 30% down-regulated (Fig. 1B). To facilitate subsequent analysis, the differentially expressed genes with known function were divided into several categories based on their biological activities. Table 2 lists some of the genes that demonstrated at least a twofold increase or decrease in expression of infected macrophages as compared with uninfected control cells ($p < 0.001$). After 1 h of infection many of the up-regulated genes encoded pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, and IL-6), chemokines (MCP-1, MCP-3, MIP-1α, MIP-1β, MIP-2α), and growth factors (GM-CSF, G-CSF). Genes encoding anti-inflammatory molecules (*Ilrn* and *Il10*) were also induced in macrophages upon stimulation with *S. pyogenes.* Also, the gene encoding type II arginase (*Arg2*) and the gene encoding the p47phox component of the NADPH oxidase (*Ncf1*), were both up-regulated after infection.

A set of genes up-regulated in response to the infection encoded for cell surface receptors involved in the recognition of gram-positive cell wall components such as TLR-2 and CD14 (47), co-stimulatory molecules involved in the induction of antigen-specific immune responses (CD80 and CD86) (7), and cell-adhesion molecules critical for the entry of immune cells into sites of infection (ICAM-1 and CD44) (31).

Other genes whose expression was greatly increased were associated with cell cycle (*Gadd45a*, *Cdkn1a*, *Fosb*), transcription (*Maff*, *Egr2*, *Relb*, *Nfkb1*) or cell signalling (*Rab20*, *Socs3* or *Plaur*). Elevated mRNA levels were also observed for apoptosis-regulating genes (*Gadd45b*, *Bcl2a1a*, *Birc2*, *Cflar*) as well as apoptosis-associated genes (*Pmaip1*, *Casp4*, *Bcl211).*

1 Among the down-regulated genes were transcription factors such as *Gata 6* which has
2 been shown to regulate innate immune responses (39) or *Tieg* (TGFB inducible early growth
3 response) and signalling genes such as *Il16*.
4 To independently confirm the microarray results, RT-PCR was carried out on 7
5 arbitrarily selected genes that were found up-regulated in infected macrophages in the
6 microarray analysis (*Il-1*α, *Il-1*β, *Il-6*, *Csf2*, *Tnf-*α, *Mip-1*α, and *Mip-1*β) and 2 unaffected
7 genes (*Ifn-*γ and *Il-12p40*). As shown in Fig. 2A, expression of mRNA was detected by RT-
8 PCR for IL-1α, IL-1β, IL-6, TNF-α, MIP-1α, MIP-1β, and GM-CSF in infected macrophages
9 but not in uninfected cells. The levels of mRNA for IL12p40 and IFN-γ were undetectable in
10 both *S. pyogenes*-infected and uninfected control macrophages in the microarray data and
11 were also undetectable by RT-PCR (Fig. 2A). Similar results were obtained when the
12 transcription of these mentioned genes was investigated in resident macrophages after
13 exposure to a different strain of *S. pyogenes* (M type 1 strain SF370) (data not shown).
14 The array data were further demonstrated by detection of protein expression. The gene
15 encoding IL-6 (*Il6*) was found strongly up-regulated in the microarray analysis (Table 2).
16 Accordingly, high levels of IL-6 were detected in the supernatant of *S. pyogenes*-infected
17 macrophages (Fig. 2B).
18
19 **Activation phenotype of *S. pyogenes*-infected macrophages.** Some markers of classical
20 activation (M1 phenotype) and some markers compatible with the alternative activation (M2
21 phenotype) were induced in macrophages after exposure to *S. pyogenes*. Thus, the levels of
22 transcripts encoding cytokines and chemokines such as TNF-α, IL-1, IL-6, IP-10, MIP-
23 1α, and MCP-1 implicated in the classical activation phenotype were significantly increased
24 after infection (Table 2). On the other hand, several transcripts typical of the alternative
25 activation phenotype (*e.g.* IL-1ra and IL-10) were also up-regulated in infected macrophages.

1 As classically activated macrophages are developed in response to IFN-γ, along with exposure
2 to a microbe or microbial product such as LPS (30), peritoneal macrophages stimulated with
3 IFN-γ+LPS were used for comparison. Treatment of murine macrophages with LPS+IFN-γ
4 also resulted in up-regulation of inflammatory genes such as TNF-α, Mip-1α, and Mip-
5 1β typical of the classical activation pathway (Fig. 3A).

6 Classical activation and alternative activation have also been associated with the
7 activities of the enzymes iNOS and arginase, respectively (30). While *S. pyogenes*-infected
8 macrophages showed increased expression of the gene encoding arginase (*Arg2*) after 1 h of
9 infection, *Nos2* was not induced. We then determined whether *Nos*2 was induced in *S.*
10 *pyogenes*-infected macrophages at later times after infection. Results in Fig. 3B show that
11 while stimulation of macrophages with IFN-γ and LPS resulted in induction of *Nos2* within 1
12 h of exposure, *Nos2* transcripts were undetectable in macrophages neither at 1h nor at 4 h or
13 16 h after infection. For that reason, NO was undetectable in the supernatant of cultured *S.*
14 *pyogenes*-infected macrophages at the selected time points (data not shown).

15 The up-regulation of *Arg2* observed in the array data was then confirmed by RT-PCR.
16 As shown in Fig. 3C, *Arg2* was induced in infected macrophages as early as 1 h of infection
17 and the gene transcription is maintained after 4 h and 16 h of infection (Fig. 3C). *Arg2* gene
18 was not induced in macrophages stimulated with IFN-γ and LPS (Fig. 3C).
19
20 **NADPH oxidase is involved in *S. pyogenes*-killing activity of murine macrophages.** Since
21 NO produced by iNOS and ROS produced by phagocyte oxydase (phox) are the major anti-
22 microbial mechanisms involved in host defence (4, 22, 34), we hypothesized that production
23 of oxygen radicals may play a major role in elimination of phagocytosed *S. pyogenes* by
24 murine macrophages. To confirm this hypothesis, the production of ROS by *S. pyogenes*-
25 infected macrophages was determined using the NBT reaction. Uninfected macrophages were

1 used as a control (Fig. 4A). As shown in Fig. 4B, significantly high levels of NBT-reducing
2 activity (dark blue precipitate) were detected in infected macrophages (red arrows). Similar
3 experiments were performed using *S. pyogenes* labelled with fluoresceine to determine
4 whether production of ROS takes place in macrophages that are associated with bacteria. Fig.
5 4C shows that the oxidative response (black precipitate) largely occurred in macrophages with
6 associated *S. pyogenes* (green). Co-localization of ROS and *S. pyogenes* was also evident in
7 infected macrophages (Fig. 4D, red arrows).

8 The contribution of phagocyte oxidase to macrophage-mediated *in vitro* killing of *S.*
9 *pyogenes* was further demonstrated using macrophages from p47$^{phox-/-}$ mice. As shown in Fig.
10 5A, macrophages from p47$^{phox-/-}$ mice exerted less antimicrobial activity to *S. pyogenes* than
11 macrophages from wild-type control animals. In fact, while macrophages from wild-type
12 animals eliminated >99.9% of the original inoculum during the first 7 h of infection,
13 macrophages lacking phagocyte oxidase activity did not reduce the original inoculum, but
14 were still able to maintain the bacterial burden at a steady level over time (Fig. 5A). In
15 contrast, macrophages deficient in iNOS were as efficient at killing *S. pyogenes* as the wild-
16 type macrophages (Fig. 5B). These results clearly indicate that the antimicrobial activity of
17 macrophages is dependent of the phagocyte oxidase but not of iNOS.
18
19 **NADPH oxidase is involved in *S. pyogenes*-killing during *in vivo* infection.** The *in vivo*
20 relevance of these findings was determined by evaluating the ability of iNOS$^{-/-}$ and p47$^{phox-/-}$
21 mice to control bacterial growth after intravenous infection with *S. pyogenes*. The amount of
22 bacteria recovered from the blood of infected p47$^{phox-/-}$ mice was significantly higher than the
23 amount of bacteria present in blood of wild-type control mice (Fig. 6A). No significant
24 differences were found between the level of bacteria in the blood of iNOS$^{-/-}$ and wild-type
25 mice (Fig. 6B).

DISCUSSION

The results reported here show that *S. pyogenes* induced an unusual activation phenotype in murine macrophages. The type of macrophage activation initiated upon phagocytosis of a particular pathogen is important since it strongly influences the pathogenesis and outcome of the infection. Thus, in the current paradigm of macrophage polarization, the proinflammatory properties of classically activated M1 macrophages directed to promote inflammation and kill the invading pathogens are in contrast with the anti-inflammatory activities of alternatively activated M2 macrophages, which provide regulatory signals to protect the host from overzealous inflammatory responses (18, 20, 30). Interestingly, the transcriptional response of *S. pyogenes*-infected macrophages revealed features of classically activated M1 macrophages since an increased expression of genes involved in the recruitment and activation of inflammatory cells such as those encoding pro-inflammatory cytokines, chemokines, and colony-stimulation factors were observed. On the other hand, several transcripts implicated in anti-inflammatory responses typical of alternatively activated M2 macrophages (e.g. IL-1ra, and IL-10) were also up-regulated.

Another important difference between M1 and M2 macrophages involves the balance between arginase and iNOS activities. Classically activated murine macrophages undergo a respiratory burst and express iNOS, whereas alternatively activated macrophages metabolize arginine through arginase rather than iNOS. Arginase and iNOS both utilize L-arginine as a substrate (46). However, while iNOS generates reactive NO species with microbicidal and proinflammatory effects important in immune responses, arginase competes with iNOS for arginine as a substrate and generates L-ornithine, an important precursor for proline that enhances collagen biosynthesis, promoting cell growth and tissue repair (21). The induction of either iNOS or arginase is usually associated with the suppression of the opposing enzyme, indicating a competitive nature in these alternative states of macrophage metabolism. We report in this study that during experimental *S. pyogenes* infection arginase II mRNA but not

iNOS mRNA was up-regulated in macrophages. These results also suggested that the bactericidal activity of macrophages against *S. pyogenes* was mediated by phagocyte oxidase and not by iNOS. This assumption was further confirmed by the impaired capacity of macrophages with dysfunctional phagocyte oxidase (p47$^{phox-/-}$) to kill *S. pyogenes*. In contrast, this capability was preserved in macrophages deficient in iNOS expression. Besides *S. pyogenes*, oxygen radical formation has been shown to participate in the killing of a diverse group of pathogens, including the promastigote form of *Leishmania donovani* (32), *Toxoplasmam gondii* (33), *Plasmodium falciparum* (45) and *Staphylococcus aureus* (24). The critical role of the phagocyte oxidase for control of *S. pyogenes* infection in humans is reflected by the enhanced susceptibility of patients with chronic granulomatous disease, a inherited disease characterized by deficient functional activity of phagocyte oxidase complex, to recurrent pyogenic infections (15, 37).

Little is known regarding the interaction of *S. pyogenes* with human macrophages. Thulin *et al.* (42) recently reported that some *S. pyogenes* microorganisms were capable of surviving intracellularly in human macrophages during acute invasive infection as well as in *in vitro* infected human monocytes/macrophages. In contrast to this observation, murine macrophages infected *in vivo* with *S. pyogenes* were refracting to bacterial persistence. However, Thulin *et al.* (42) also showed that the percentage of human monocytes associated with viable microorganisms was reduced to almost 50% between 4 and 12 h after *in vitro* infection. This observation clearly indicates that, although some human monocytes allowed *S. pyogenes* persistence, other infected monocytes were also capable of efficiently eliminating ingested *S. pyogenes*. Whether the killing of *S. pyogenes* by human monocytes/macrophages is mediated by the phagocyte oxidase system or by iNOS remains to be elucidated.

The phagocyte oxidase system produces superoxide after bacteria phagocytosis, which is rapidly converted to other potent ROS, such as hydrogen peroxide within forming phagosomes (3). In addition to participating in bacterial killing, ROS released in high levels

by overstimulated immune cells have been implicated in inflammation and tissue injury (13). In blood, ROS can be neutralized by the antioxidant activity of red cell and plasma components (44). However, local generation of ROS can cause tissue injury as it has been shown in *Pseudomonas aeruginosa* pneumonia (41), pneumococcal meningitis (2), and *Helicobacter pylori* gastritis (35). Therefore, immune cells also require adequate levels of anti-oxidants in order to avoid the harmful effect of an excessive production of ROS. The excess of oxygen radicals can be neutralized by a wide array of antioxidant molecules including superoxide dismutase (SOD) (28). In this regard, our array data shows that the genes encoding metallothioneins (*Mt1* and *Mt2*), thioredoxin reductase (*Txnrd1*), and superoxidismutase 2 (*Sod2*), which are reactive-oxygen scavengers and play an important role in the detoxification of free radicals (38, 43) were up-regulated in *S. pyogenes*-infected macrophages. The up-regulation of these genes may be critical for protection against the potential harmful effect for the cells of high levels of oxygen radicals.

The gene encoding prostaglandin-endoperoxidase synthase 2 (*Ptgs2*) was also strongly induced following *S. pyogenes* infection, with an average increase of more than 1000-fold. While the role of *Ptgs2* expression in response to *S. pyogenes* is unknown, it may serve as a potent regulator of inflammation. Up-regulation of this gene is responsible for the increased production of inflammatory prostaglandins implicated in the pathogenesis of many inflammatory diseases, including sepsis (6).

Of particular interest was the up-regulation of Gadd45 family proteins, which have been implicated in DNA repair following stress (40), and the cytoprotective Tnfaip3 protein, which is antiapoptotic through inhibition of the caspase cascade at the level of the initiator caspase 8 (10). These cytoprotective molecules may be critical for maintenance of cellular homeostasis under the severe stress conditions associated with streptococcal infection. Interestingly, the gene encoding the suppressor of cytokine signalling 3 (Socs3) was also strongly up-regulated. Socs3 protein is a key negative regulator of cytokine signalling (8). Up-

regulation of *Socs3* may constitute a negative feedback mechanism for the maintenance of homeostasis.

Taken together this data strongly suggests that resident macrophages activate an array of specific survival pathways after infection with *S. pyogenes* directed to maintain cell integrity and ensure survival. In contrast to this survival program activated in macrophages, exposure of human neutrophils to *S. pyogenes* results in the induction of apoptotic genes and acceleration of apoptosis (23). This divergence in the response between these two phagocytic cells may most probably reflect fundamental differences either in the cellular receptors recognizing *S. pyogenes* or in their phagocytic pathways.

In summary, the results of our study indicated that *S. pyogenes* induces an uncommon activation profile in murine macrophages that does not strictly fit either of M1 or M2 activation phenotype. This profile can be explained by the high plasticity of the mononuclear phagocyte system. Thus, the activation response of macrophages to *S. pyogenes* infection may be the result of exposure to a multiplicity of polarizing signals emerging from the pathogen (e.g. cell-wall components, exotoxins, etc.) to yield either a mixed M1/M2 activation phenotype or a mixed population of macrophages belonging to M1 and M2 phenotypes. The characterization of the transcriptional profile adds a new dimension to the analysis of the macrophage response to *S. pyogenes* with the identification of potential fingerprints useful to define states within the complexity of the infection process.

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24

1 **FIGURE LEGEND**

2 **Figure 1.** Transcriptional profile of resident murine macrophages after 1 h of infection with *S.*
3 *pyogenes*. (A) Expression pattern of cDNAs representing differentially regulated genes
4 analyzed by microarray. Gene regulation was expressed as signal log ratios (SLR values
5 calculated by the Affymetrix MAS5 software) from the comparison of infected vs. non
6 infected control macrophages. Induced gene expression by *S. pyogenes* are indicated in red,
7 whereas suppressed gene expression are indicated in green. The degree of red intensity
8 represents the level of induction, whereas that of green intensity represents the level of
9 repression. (B) After 1 h infection with *S. pyogenes* more than 400 genes were found to be
10 differentially expressed with respect to the uninfected state. 70% were induced while 30%
11 were repressed.

12

13 **Figure 2.** Confirmation of microarray data by RT-PCR and protein expression. (A) RT-PCR
14 analysis of selected gene transcription in resident macrophages uninfected or infected with *S.*
15 *pyogenes*. Uninfected samples are loaded in lane 1 and infected samples in lane 2. *β-actin*
16 expression served as a control. (B) IL-6 protein expression by *S. pyogenes*-infected
17 macrophages. Resident macrophages were isolated from the peritoneal cavity of mice after 1
18 h of infection with *S. pyogenes* and cultured *in vitro* for 2 h. Non-infected macrophages were
19 used as a control. The levels of IL-6 in the supernatants were determined by ELISA. Each
20 column represents the mean ± SD of triplicate samples obtained from three independent
21 experiments. $P < 0.0001$, by analysis of variance.

22

23 **Figure 3.** Effect of *S. pyogenes* in the regulation of *Nos2* and *Arg2* gene transcription. (A)
24 RT-PCR analysis of inflammatory genes transcription (TNF-α, Mip-1α, and Mip-1β) in
25 resident macrophages unstimulated (0 h) or stimulated with IFN-γ + LPS for 1 h, 4 h, 6h, or
26 16 h. *Rsp9* expression served as an internal control (B) RT-PCR analysis of *Nos2* gene

1 transcription in resident macrophages uninfected or after 1 h, 4 h, or 16 h of infection with *S.*
2 *pyogenes*. Macrophages stimulated with IFN-γ + LPS were used as a positive control. *Rsp9*
3 expression served as an internal control. (C) RT-PCR analysis of *Arg2* gene transcription in
4 resident macrophages uninfected or after 1 h, 4 h, or 16 h of infection with *S. pyogenes.*
5 Macrophages stimulated with IFN-γ + LPS were used as a negative control *Rsp9* expression
6 served as an internal control.
7
8 **Fig. 4.** Production of oxygen radicals by peritoneal macrophages after infection with *S.*
9 *pyogenes*. Uninfected (A) or *S. pyogenes*-infected macrophages (B) were incubated with
10 nitroblue tetrazolium (NBT) for 45 min and examined by light microscopy. NBT precipitates
11 as a blue/purple formazan when reduced by superoxide (red arrows). Macrophages infected
12 with green fluorescence-labelled *S. pyogenes* and incubated with NBT for 45 min is shown in
13 (C). Production of ROS by macrophages is evidenced by the black precipitation. Co-
14 localization of ROS and *S. pyogenes* is shown in (D) (red arrows). Bars represent 15 µm in A-
15 C and 5µm in D.
16
17 **Figure 5.** Role of iNOS and phagocyte oxidase in the killing of *S. pyogenes* by peritoneal
18 macrophages. (A) Killing activity of *S. pyogenes* by peritoneal macrophages isolated from
19 wild-type (■) or $p47^{phox-/-}$ (▲) mice. (B) Killing activity of *S. pyogenes* by peritoneal
20 macrophages from wild-type (■) or $iNOS^{-/-}$ (▲) mice. Macrophages were isolated from the
21 peritoneal cavity of mice after 1 h of intraperitoneal infection with 5×10^7 CFU of *S.*
22 *pyogenes* and cultured at 37°C, 5% CO2. At several time points, the macrophages were lysed
23 with dH_2O and surviving bacteria were enumerated by plating serial dilutions in blood agar.
24 Data presented are the mean ± SD for triplicate samples from one experiment representative
25 of three independent determinations.
26

1 **Figure 6.** Levels of bacteria in the blood of mice at 24 h after intravenous infection with *S.*
2 *pyogenes*. Wild-type, p47$^{phox-/-}$ (A) or iNOS$^{-/-}$ (B) mice were inoculated with 10^5 CFU *S.*
3 *pyogenes* in 0.2 ml of PBS via a lateral tail vein. Viable bacterial counts were determined in
4 blood of infected mice by collecting blood samples from the tail vein at 24 h post-inoculation.
5 Each column represents the mean ± SD of 10 mice per group. $P < 0.0001$, by analysis of
6 variance.

1 **Table 1.** Primers used for reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Primers sequence	PCR product
IL-1α	Sense: 5’ CAGTTCTGCCATTGACCATC 3’	218 bp
	Antisense: 5’ TGGATAAGCAGCTGATGTGAAGTA 3’	
IL-1β	Sense: 5’ ACTACAGGC TCCGAGATGAACAAC 3’	163 bp
	Antisense: 5’ CCCAAGGCCACAGGTATTTT 3’	
IL-6	Sense: 5’ CTGGTGACAACCACGGCCTTCCCTA 3’	600 bp
	Antisense: 5’ ATGCTTAGGCATAACGCACTAGGTT 3’	
IL-12p40	Sense: 5’ CGTGCTCATGGCTGGTGCAAAG 3’	280 bp
	Antisense: 5’ CTTCATCTGCAAGTTCTTGGGC 3’	
Tnf-α	Sense: 5’ AGCCCACGTCGTAGCAAACCACCAA 3’	446 bp
	Antisense: 5’ ACACCCATTCCCTTCACAGAGCAAT 3’	
Mip-1α	Sense: 5’ CTCCCAGCCAGGTGTCATTTTC 3’	110 bp
	Antisense: 5’ CTCAGGCATTCAGTTCCAGGTCAG 3’	
Mip-1β	Sense: 5’ GCAAACCTAACCCCGAGCAACA 3’	127 bp
	Antisense: 5’ AGCAGGAAGTGGGAGGGTCAGAG 3’	
Inf-γ	Sense: 5’ AGGAACTGGCAAAAGGATGGTGA 3’	106 bp
	Antisense: 5’ TGTTGCTGATGGCCTGATTGTCTT 3’	
Csf2	Sense: 5’ CATTGTGGTCTACAGCCTCTC 3’	278 bp
	Antisense: 5’ GGCAGTATGTCTGGTAGTAGC 3’	
Nos	Sense: 5’ CCCTTCCGAAGTTTCTGGCAGCAGC 3’	496 bp
	Antisense: 5’ GGCTGTCAGAGCCTCGTGGCTTTGG 3’	
Arg2	Sense: 5’ CGC ACA GAA GAA GCT AGG AG 3’	174 bp
	Antisense: 5’ CCCACT GAA CGA GGA TAC AC 3’	
β-actin	Sense: 5’ TGGAATCCTGTGGCATCCATGAAAC 3’	318 bp
	Antisense: 5’ TAAAACGCAGCTCAGTAACAGTCCG 3’	
Rsp9	Sense: 5’ CTGGACGAGGGCAAGATGAAGC 3’	143 bp
	Antisense: 5’ TGACGTTGGCGGATGAGCACA 3’	

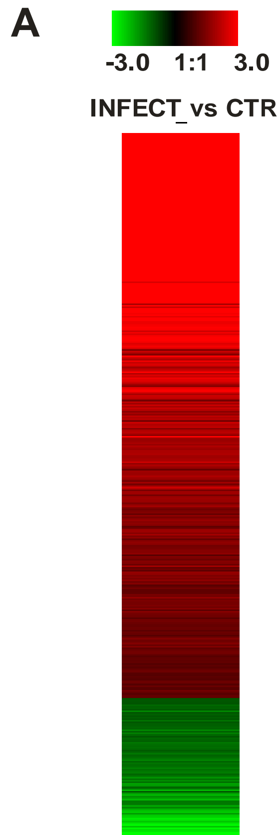
1 **Table 2.** Genes differentially expressed in resident macrophages at 1 h post-infection with *S.*

2 *pyogenes.*

Gene Symbol	GI-Number	Description	GeneBank ID	Fold changes
Immune response and inflammation				
Ptgs2	19225	prostaglandin-endoperoxide synthase 2	M88242	1357.75
Il6	13624310	interleukin 6	NM_031168	427.86
Tnfsf9	141803209	tumor necrosis factor (ligand) superfamily, member 9	NM_009404	366.33
Csf2	51100	colony stimulating factor 2 (granulocyte-macrophage)	X03019	141.24
Ccl7	6652905	chemokine (C-C motif) ligand 7	AF128193	110.66
Il1b	15030320	interleukin 1 beta	BC011437	108.84
Ccl2	6531370	chemokine (C-C motif) ligand 2	AF065933	79.89
Il1a	13277631	interleukin 1 alpha	BC003727	72.35
Ccl3	126432552	chemokine (C-C motif) ligand 3	NM_011337	71.36
IL23a	133892789	interleukin 23, alpha subunit p19	NM_031252	69.50
Ccl4	6652955	chemokine (C-C motif) ligand 4	AF128218	68.55
Il1rn	145301622	interleukin 1 receptor antagonist	NM_031167	53.82
Cxcl1	141802720	chemokine (C-X-C motif) ligand 1	NM_008176	45.73
Csf3	6753535	colony stimulating factor 3 (granulocyte)	NM_009971	35.53
Lif	16354736	leukemia inhibitory factor	BB235045	29.88
Tnf	133892368	tumor necrosis factor	NM_013693	26.35
Il10	6754317	interleukin 10	NM_010548	21.53
Cxcl2	118130527	chemokine (C-X-C motif) ligand 2	NM_009140	19.04
Tlr2	31981332	toll-like receptor 2	NM_011905	15.61
Cxcl10	10946575	chemokine (C-X-C motif) ligand 10	NM_021274	8.19
Cd80	2412318	CD80 antigen	AA596883	7.73
Icosl	118131092	icos ligand	NM_015790	6.97
Ncf1	3061281	neutrophil cytosolic factor 1	AB002663	5.14
Cd14	118129882	CD14 antigen	NM_009841	3.85
Irf1	6680466	interferon regulatory factor 1	NM_008390	3.63
Cd86	15489434	CD86 antigen	BC013807	3.61
Ccl24	11181621	chemokine (C-C motif) ligand 24	AF281075	2.98
Transcription				
Maff	18605754	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	BC022952	113.06
Egr2	52812	early growth response 2; protein containing zinc fingers	X06746	66.63
Crem	4320936	cAMP responsive element modulator	AI467599	48.00
Nr4a1	6754215	nuclear receptor subfamily 4, group A, member 1	NM_010444	47.31
Ets2	13529535	E26 avian leukemia oncogene 2, 3' domain	BC005486	25.02
Egr1	76559936	early growth response 1	NM_007913	24.37
Rel	112181203	reticuloendotheliosis oncogene	NM_009044	17.56
Tgif	31982824	TG interacting factor	NM_009372	11.03
Atf3	18044779	activating transcription factor 3	BC019946	10.85
Nr4a2	7305324	nuclear receptor subfamily 4, group A, member 2	NM_013613	8.88
Jundm2-pending	31982607	Jun dimerization protein 2	NM_030887	8.66
Relb	31982052	avian reticuloendotheliosis viral (v-rel) oncogene related B	NM_009046	6.41
Junb	6680511	Jun-B oncogene	NM_008416	3.81
Sra1	40106182	steroid receptor RNA activator 1	BG074964	3.49
Nfe2l2	76573877	nuclear, factor, erythroid derived 2, like 2	NM_010902	3.21
Nfkb1	468361	nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	L28118	2.76
Copeb	4092799	core promoter element binding protein	AF072403	2.63
Cebpd	17009389	CCAAT/enhancer binding protein (C/EBP), delta	BB831146	2..31
Cebpg	31486229	CCAAT/enhancer binding protein (C/EBP), gamma	BM228675	-2.24
Gilz	116517341	glucocorticoid-induced leucine zipper	NM_010286	-2.68
Gata6	31537073	GATA binding protein 6	BM214048	-4.41
Tieg	118130846	TGFB inducible early growth response	NM_013692	-5.27
Cebpa	15029793	CCAAT/enhancer binding protein (C/EBP), alpha	BC011118	-5.02
Klf2	6680579	Kruppel-like factor 2 (lung)	NM_008452	-6.95
Signalling				
Rab20	40013469	RAB20, member RAS oncogene family	BG066967	15.96
Cd3e	141803351	CD3 antigen, epsilon polypeptide	NM_007648	15.92
Socs3	31982458	suppressor of cytokine signaling 3	NM_007707	13.27
Ralgds	6677734	ral guanine nucleotide dissociation stimulator	NM_009058	12.49
Rgs1	7657511	regulator of G-protein signaling 1	NM_015811	11.85
Cmkor1	15929639	chemokine orphan receptor 1	BC015254	9.13
Cish	118129844	cytokine inducible SH2-containing proteincytokine	NM_009895	3.99

		inducible SH2-containing protein		
Gpr35	142356082	G protein-coupled receptor 35	NM_022320	3.99
Plaur	53277	urokinase plasminogen activator receptor	X62701	2.41
Csk	12576639	c-src tyrosine kinase	BG094076	-2.85
Il16	20070724	interleukin 16	BC026894	-4.86
Apoptosis				
Gadd45b	12845848	growth arrest and DNA-damage-inducible 45 beta	AK010420	188.84
Pmaip1	118130467	phorbol-12-myristate-13-acetate-induced protein 1	NM_021451	38.91
Tnfaip3	31543879	tumor necrosis factor, alpha-induced protein 3	NM_009397	33.59
Gadd45g	12840945	growth arrest and DNA-damage-inducible 45 gamma	AK007410	22.50
Casp4	6671681	caspase 4, apoptosis-related cysteine protease	NM_007609	5.10
Birc2	141803312	baculoviral IAP repeat-containing 2	NM_007464	4.68
Bcl2a1a	293273	B-cell leukemia/lymphoma 2 related protein A1a	L16462	4.66
Bcl2l11	16399030	BCL2-like 11 (apoptosis facilitator)	BB667581	3.05
Cflar	131889125	CASP8 and FADD-like apoptosis regulator	NM_009805	2.92
Cell cycle				
Gadd45a	6681148	growth arrest and DNA-damage-inducible 45 alpha	NM_007836	10.54
Cdkn1a	12841291	cyclin-dependent kinase inhibitor 1A (P21)	AK007630	8.88
Fosb	110350004	FBJ osteosarcoma oncogene B	NM_008036	8.86
Mapk6	19353313	mitogen-activated protein kinase 6	BC024684	4.73
Map3k8	118131172	mitogen activated protein kinase kinase kinase 8	NM_007746	4.26
Junb	6680511	Jun-B oncogene	NM_008416	3.81
Ccnl;	31505036	cyclin L	BM250672	3.24
Dusp1	145301574	dual specificity phosphatase 1	NM_013642	2.80
Ccng2	2149913	cyclin G2	U95826	2.53
Electronic transport				
Mt2	2859721	metallothionein 2	AA796766	32.65
Arg2	4779068	arginase type II	AV002218	12.76
Slc20a1	7657578	solute carrier family 20, member 1	NM_015747	11.73
Txnrd1	110224443	thioredoxin reductase 1	NM_015762	5.21
Sod2	76253932	superoxide dismutase 2, mitochondrial	NM_013671	4.22
Fabp4	14149634	fatty acid binding protein 4, adipocyte	NM_024406	3.72
Hbb-b1	20071755	hemoglobin, beta adult major chain	BC027434	2.72
Abca1	15411280	ATP-binding cassette, sub-family A (ABC1), member 1	BB305534	2.62
Lcn2	49710	lipocalin 2	X14607	2.58
Clic4	16987473	chloride intracellular channel 4 (mitochondrial)	BB814844	2.17
Por	6679420	P450 (cytochrome) oxidoreductase	NM_008898	2.03
Cell adhesion				
Icam1	14250386	intercellular adhesion molecule	BC008626	28.72
Cd44	53677	CD44 antigen	X66083	2.89
Thbs1	4803484	thrombospondin 1	AV026492	2.51

1



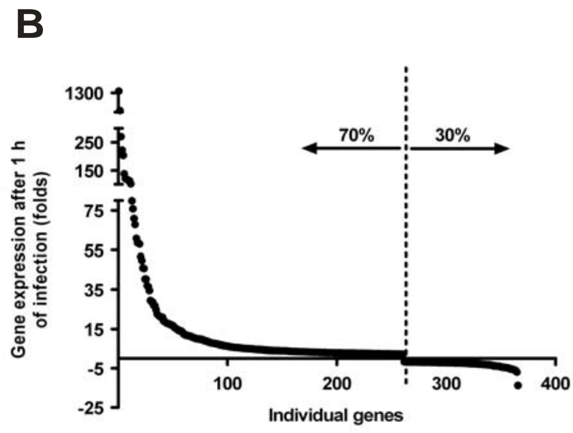


Figure 1

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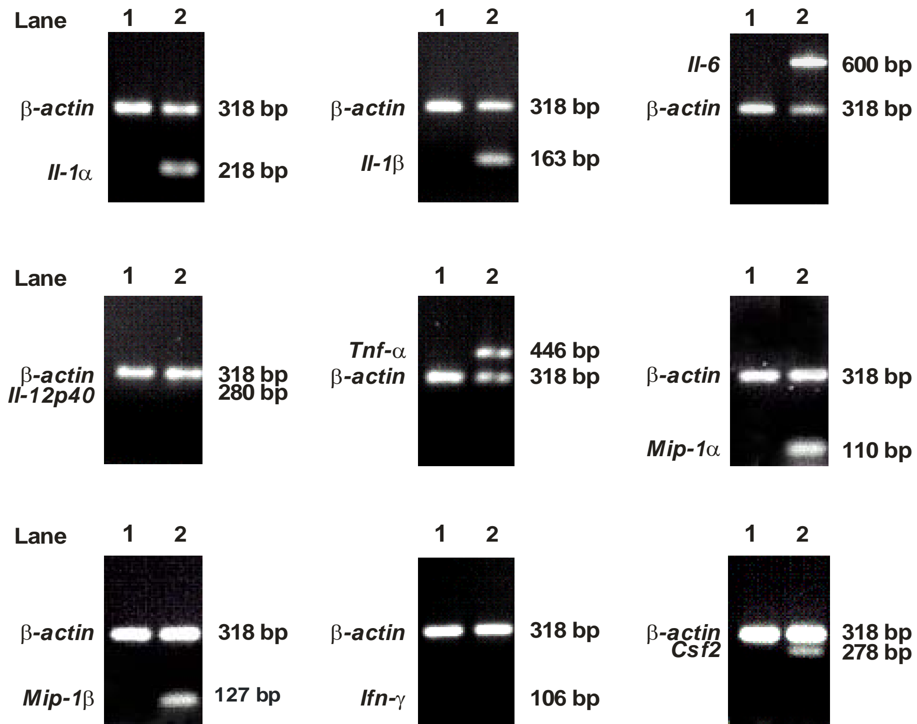

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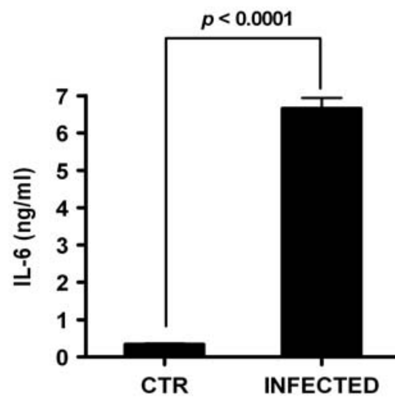


Figure 2

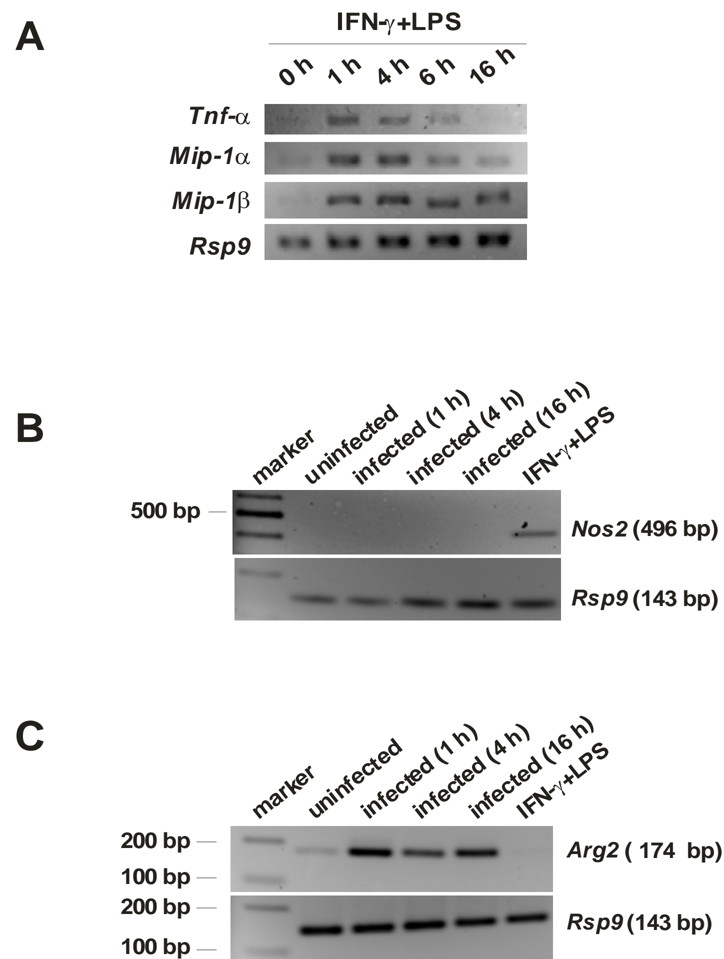


Figure 3

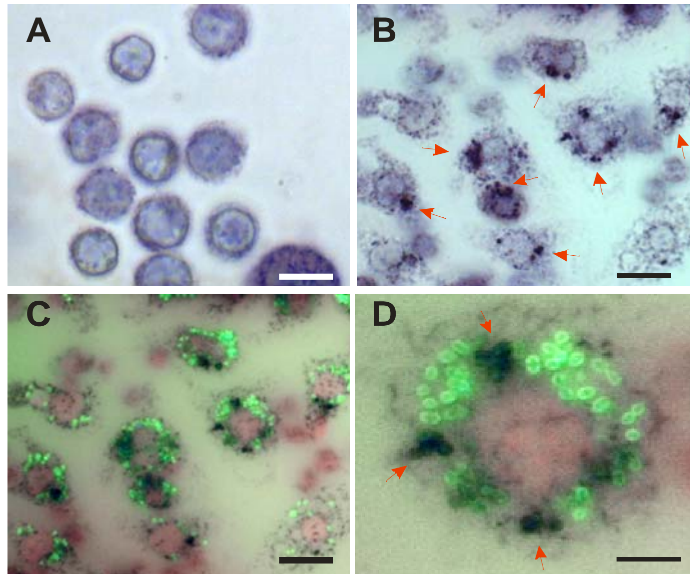
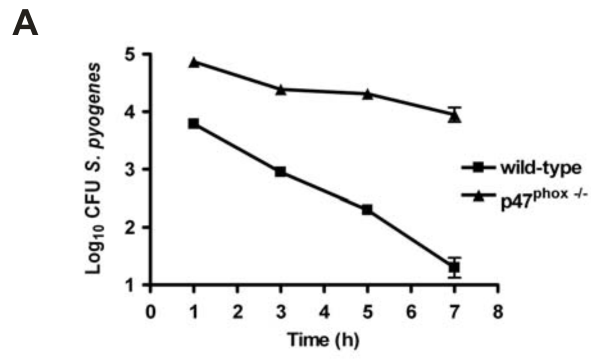


Figure 4

A



B

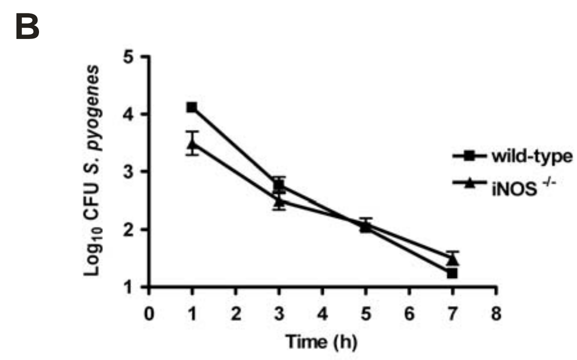


Figure 5

1

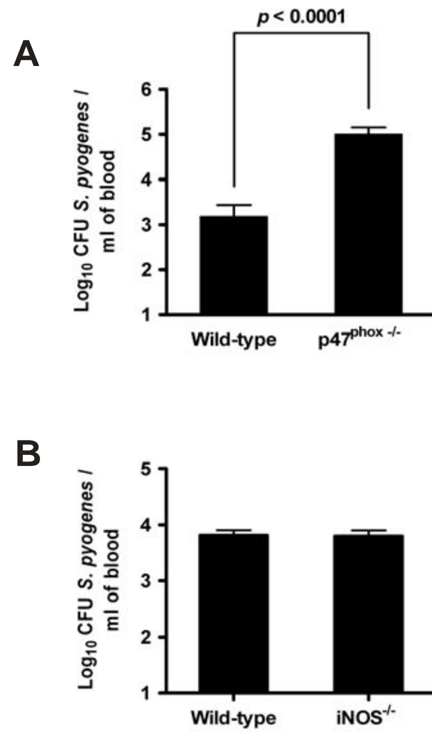


Figure 6