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Astrid de Greeff, Laurentiu Benga, Paul J. Wichgers Schreur, Peter Valentin-Weigand, Johanna M.J. Rebel, et al.. Involvement of NF- $\kappa$ B and MAP-kinases in the transcriptional response of alveolar macrophages to. *Veterinary Microbiology*, 2010, 141 (1-2), pp.59. 10.1016/j.vetmic.2009.07.031 . hal-00560842

**HAL Id: hal-00560842**

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Submitted on 31 Jan 2011

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## Accepted Manuscript

Title: Involvement of NF- $\kappa$ B and MAP-kinases in the transcriptional response of alveolar macrophages to *Streptococcus suis*

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PII: S0378-1135(09)00353-8  
DOI: doi:10.1016/j.vetmic.2009.07.031  
Reference: VETMIC 4523

To appear in: *VETMIC*

Received date: 2-4-2009  
Revised date: 2-7-2009  
Accepted date: 31-7-2009

Please cite this article as: de Greeff, A., Benga, L., Schreur, P.J.W., Valentin-Weigand, P., Rebel, J.M.J., Smith, H.E., Involvement of NF- $\kappa$ B and MAP-kinases in the transcriptional response of alveolar macrophages to *Streptococcus suis*, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.07.031

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1 **Involvement of NF- $\kappa$ B and MAP-kinases in the transcriptional response**  
2 **of alveolar macrophages to *Streptococcus suis***

3

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15

16 Keywords: *Streptococcus suis*, transcriptional response, macrophages

17

18 **Abstract**

19 Interaction of *Streptococcus suis* with primary porcine alveolar  
20 macrophages was studied using transcriptomics. Transcriptional  
21 response of macrophages to two different *S. suis* strains was studied:  
22 wild-type S10 that is resistant to phagocytosis, and a non-encapsulated  
23 mutant that is phagocytosed efficiently. The macrophages'  
24 transcriptional response was observed only after 60 minutes of  
25 incubation. Eleven genes were expressed significantly different  
26 between macrophages infected with streptococci and control mock-  
27 infected macrophages. These genes include IL-1- $\beta$ , MIP-2- $\alpha$  and TNF- $\alpha$ .  
28 When gene expression was studied as a function of time, transcriptional  
29 changes occurred in all macrophages independent of streptococci.  
30 The fold induction of induced genes however, was much stronger in  
31 macrophages incubated with the non-encapsulated *S. suis* strain that  
32 was phagocytosed. The genes that were higher induced due to *S. suis*  
33 suggest an innate immune response is induced in macrophages.  
34 Pathway analysis revealed that genes that are part of the putative  
35 MAP-kinase signal transduction system are overrepresented among the  
36 regulated genes. Using an immortalized alveolar macrophage cell line  
37 it was shown that macrophages respond to interaction with *S. suis* by  
38 translocation of NF- $\kappa$ B to the nucleus, independent of phagocytosis.  
39 This translocation subsequently induced expression of innate immune

40 genes. This strongly suggests besides the MAP-kinase signaling pathway,

41 NF- $\kappa$ B signaling is also induced upon interaction with *S. suis*.

42

Accepted Manuscript

## 43 Introduction

44

45 *Streptococcus suis* are gram positive cocci possessing a  
46 polysaccharide capsule that is responsible for the serotype specificity.  
47 Worldwide *S. suis* serotype 2 is most frequently isolated from diseased  
48 pigs (Staats et al., 1997). *S. suis* causes severe infections in piglets,  
49 typical symptoms of the disease include meningitis, arthritis and  
50 septicemia. Animals often do not survive an infection. Recently an  
51 outbreak of human *S. suis* infections in China was described in which  
52 200 people got infected, of whom 38 died (Yu et al., 2006).

53 The severity and outcome of infections caused by *S. suis* are likely to  
54 depend on the ability of host innate immune mechanisms to control  
55 bacterial growth and to limit spreading of the pathogen (Goldmann et  
56 al., 2007). Macrophages, together with the epithelial barrier, form the  
57 first line of defense against microorganisms. They identify pathogens  
58 using pattern recognition receptors (PRRs), and subsequently unfold a  
59 tightly regulated pathogen-specific immune response (Akira et al.,  
60 2006). Activation of PRRs leads to signal transduction via Myd88, and  
61 activates both NF- $\kappa$ B and MAP kinases, resulting in induction of genes  
62 involved in inflammatory responses. For *S. suis* it is known that after  
63 incubation with mononuclear cells, proinflammatory mediators are  
64 produced (Segura et al., 2006). Another study showed that *S. suis* can

65 induce an inflammatory response via the induction of prostaglandin E2  
66 and metalloproteinase 9 production in human macrophages (Jobin et  
67 al., 2006). These reactions are part of the general 'alarm signal' for  
68 infection that can lead to a cascade of immune responses (Jenner  
69 and Young, 2005).

70 In this paper we studied the 'alarm signal' of porcine alveolar  
71 macrophages in response to *S. suis* serotype 2 *in vitro*. This was  
72 achieved by comparing transcriptional profiles of alveolar  
73 macrophages after contact with either a *S. suis* strain that is resistant to  
74 phagocytosis or with an isogenic non-encapsulated mutant that is  
75 phagocytosed in this model (Smith et al., 1999). Our results showed that  
76 an innate immune response is induced in alveolar macrophages, that is  
77 probably induced via activation of both NF- $\kappa$ B and MAP-kinase  
78 pathways.

79

## 80 **Materials and Methods**

81

### 82 **Bacterial strains and growth conditions**

83 Streptococcal strains were grown on Columbia agar blood base plates  
84 (Oxoid Ltd., London, United Kingdom) containing 6% (vol/vol) horse  
85 blood. Cultures were grown in Todd-Hewitt broth (Oxoid). Pathogenic *S.*  
86 *suis* strain 10 (Smith et al., 1996; Vecht et al., 1991) and its avirulent non-  
87 encapsulated mutant strain S10cpsdeIEF were used (Smith et al., 1999).  
88 For phagocytosis experiments *S. suis* was grown for 16 h. One ml of  
89 culture was spun down for 2 min at 20,000 x g at 4°C. The pellet was  
90 washed once in cold Dulbecco's Modified Eagle's Medium (DMEM,  
91 Sigma-Aldrich, St. Louis, MO, USA) and resuspended in 1 ml of DMEM.  
92 The inoculum size was determined by plating serial dilutions on agar  
93 plates. For stimulation of 3D4 porcine alveolar macrophages cell line,  
94 bacteria were washed once with PBS, then adjusted in PBS to an OD<sub>600</sub>  
95 of 0.4, and subsequently diluted 1:10 in Iscove's Modified Dulbecco's  
96 Medium (IMDM). For NF-κB translocation studies, *S. suis* was grown for 16  
97 h in IMDM and 0.5 ml of the culture was directly used for stimulation of  
98 3D4 cells.

99

### 100 **Phagocytosis and stimulation of primary alveolar macrophages**

#### 101 Isolation of alveolar macrophages



102 Phagocytosis experiments were done as described before (Smith et al.,  
103 1999). Porcine lungs were collected from 6 individual specific pathogen  
104 free piglets, and used within 4 hours. As a control DMEM without  
105 bacteria was used. Animal experiments performed were approved by  
106 the ethical committee of the Central Veterinary Institute of  
107 Wageningen University and Research Center, in accordance with the  
108 Dutch law on animal experiments.

### 109 Phagocytosis

110 To each opsonized bacterial suspension 5 ml of macrophage  
111 suspension was added (MOI 1). The sample was mixed immediately  
112 after addition of bacteria and samples were taken at  $t = 0, 2, 5, 10, 15,$   
113 and 60 min. One part of the sample (1.5 ml) was spun down  
114 immediately for 3 min at max speed at 4°C. The supernatant was  
115 decanted and the pellet was snap frozen in liquid nitrogen for RNA  
116 isolation. The second part of the sample (0.4 ml) was added to 1,6 ml  
117 ice-cold DMEM. This sample was spun down for 10 min at 110 x g at  
118 4°C. Subsequently, the supernatant was plated in a serial dilution to  
119 determine the CFU/ml. Results of seven independent experiments were  
120 averaged. Results were analyzed using Mann Whitney testing, standard  
121 error of the mean is given for each timepoint.

122

### 123 **Phagocytosis and stimulation of 3D4 alveolar macrophages**

124 3D4 porcine alveolar macrophages derived from primary porcine  
125 alveolar macrophages as previously described (Weingartl et al., 2002)  
126 were used. Cells were maintained in Iscove's Modified Dulbecco's  
127 Medium (IMDM) containing 10% FCS, 5 mM glutamine, 100 units ml<sup>-1</sup>  
128 penicillin, and 100 µg ml<sup>-1</sup> streptomycin. For stimulation experiments,  
129 approximately 4 x 10<sup>5</sup> cells per well were seeded in antibiotic free  
130 media on 12 well tissue culture plates and incubated for 24 h. For NF-κB  
131 translocation, approximately 1 x 10<sup>5</sup> cells per well were seeded in  
132 antibiotic free media on 24 well tissue culture plates containing glass  
133 coverslips.

134 Phagocytosis of *S. suis* by adherent 3D4 alveolar macrophages cell line  
135 was determined during 90 min of co-cultivation by a gentamicin  
136 protection assay as previously described (Valenti-Weigand et al., 1996).  
137 3D4 cells in 12 well plates were stimulated with 1.5 ml *S. suis* suspension  
138 in IMDM for 6 h at 37°C to study the transcriptional activation.

139 For NF-κB translocation 3D4 cells were treated with *S. suis* wild-type for  
140 30 min at 37°C. Cells treated with 5 µg/ml LPS served as positive control,  
141 non-stimulated cells as negative control. After stimulation, cells were  
142 fixed with 3.7% formaldehyde for 10 min at room temperature, washed  
143 three times with PBS, and then permeabilized with 0.1% Triton-X 100 in  
144 PBS for 20 min at RT. Following, the cells were incubated for 30 min at RT  
145 with 10% FCS in PBS to block non-specific binding. Subsequently, cells

146 were double stained with anti-human NF- $\kappa$ B p65 antibodies (1:200, Cell  
147 Signalling, Frankfurt/Main, Germany) for 1h at room temperature. Cells  
148 were washed three times with PBS and then incubated with Alexa Fluor  
149 488 goat anti-rabbit antibodies (1:500, Invitrogen, Carlsbad, CA, USA)  
150 for 30 min at room temperature. After five washes, the cells were  
151 mounted with anti-fading solution and visualised under a fluorescence  
152 microscope.

153

#### 154 **RNA isolation**

155 The macrophage pellet was taken up in 1 ml of Trizol and 0.2 ml of  
156 chloroform was added. The suspension was shaken vigorously for 15  
157 sec, incubated for 3 min at RT and centrifuged for 15 min at 20,000 x g  
158 at 4°C. Two phenol-chloroform-isoamylethanol extractions were  
159 performed, and 1 chloroform extraction after which the RNA was  
160 precipitated using isopropanol, washed with 70% ethanol, and  
161 dissolved in water. RNA was further purified using the Nucleospin RNA II  
162 kit (Macherey Nagel, Düren, Germany) according to manufacturer's  
163 instruction with a prolonged DNase treatment (1hr at RT). RNA quality  
164 and quantity was checked using the NANOdrop (Thermo Fisher  
165 Scientific, Pittsburgh, PA, USA) and the BioAnalyzer (Agilent, Palo Alto,  
166 CA, USA). RNA samples were pooled per timepoint per strain.

167 In experiments with 3D4 cells, RNA was harvested 6h pi. For this, cells  
168 were washed three times with ice-cold PBS then harvested and lyzed in  
169 350  $\mu$ l lysis buffer (RLT-buffer, Qiagen, Hilden, Germany) containing 1%  
170  $\beta$ -mercaptoethanol. The RNA from the lysed cells was purified using the  
171 Qiagen RNeasy-Kit (Qiagen) according to the manufacturer's  
172 instructions.

173

#### 174 **Microarray analysis**

175 Biotin-labeled cRNA was synthesized using the one-cycle target  
176 labeling and control reagents (Affymetrix, Santa Clara, CA, USA)  
177 starting with 2  $\mu$ g of RNA. For hybridization to porcine whole genome  
178 Affymetrix chips, 12.5 – 20  $\mu$ g of cRNA was used for further  
179 fragmentation, 10  $\mu$ g of this fragmented cRNA was used for  
180 hybridization. Affymetrix protocols are followed for hybridization,  
181 washing, staining and scanning of the chips. Data analysis was done  
182 using GeneSpring software (Agilent). Pathway analysis was done using  
183 a Web Based Gene Set Analysis Toolkit (Webgestalt) (Zhang et al.,  
184 2005). All microarray data have been submitted MIAME complied to  
185 ArrayExpress under submission number E-MEXP-1756  
186 (<http://www.ebi.ac.uk/microarray-as/ae/>).

187

#### 188 **Quantitative PCR**

189 RT-PCR

190 200 ng RNA was used to synthesize cDNA in a reaction containing 25  
191 ng/ $\mu$ l random primers (Promega, Madison, WI, USA), 10 mM dNTPs  
192 (Promega), 10 mM DTT (Invitrogen), 40 U RNAsin (Promega) and  
193 SuperScriptII Reverse Transcriptase (Invitrogen) according to  
194 manufacturer's instructions.

195 qPCR

196 cDNA was diluted 20 times for qPCR analysis. Primers were designed  
197 using PrimerExpress software (Applied Biosystems, Foster City, CA, USA)  
198 (see Table 1). Each reaction contained 12.5 pmol forward primer, 12.5  
199 pmol reverse primer and POWR SYBR Green PCR Master Mix (Applied  
200 Biosystems) according to manufacturer's instructions. qPCR was  
201 performed using an ABI7500 (Applied Biosystems). The amount of  
202 *gapdh* was measured to control for variation in RNA-yield and RT-  
203 reaction conditions. In each run a standard curve was incorporated  
204 consisting of a vector (pGEMT-easy) containing the cloned gene. In this  
205 way both the gene and the external control gene could be related to  
206 a standard line. The efficiency of the PCR reaction was 90-100% for all  
207 reactions (slope standard line between -3.3 and -3.6). The standard line  
208 consisted of 7 10-fold dilutions of the control vector. For each reaction  
209 negative water controls were included. Analysis was performed using  
210 the ABI7500 Software (Applied Biosystems). Porcine IL-8 and MCP-1

211 transcripts of 3D4 cells were quantified by PCR using specific primers  
212 described previously (Segura *et al.*, 2006). Porcine ICAM-1 primers were  
213 used as described previously (Stocker *et al.*, 2000). *gapdh* gene was  
214 used for expression normalization, using the primers described  
215 previously (Segura *et al.*, 2006). The PCR mixture consisted 5  $\mu$ l of 10x  
216 Taq buffer, 1.5  $\mu$ l of 50 mM  $MgCl_2$ , 1  $\mu$ l of 10 mM dNTP, 0.5  $\mu$ l of each  
217 primer (50  $\mu$ M), 5  $\mu$ l cDNA, 0.5  $\mu$ l Taq DNA polymerase and water up to  
218 50  $\mu$ l. The PCR cycling consisted of denaturation at 94°C for 1 min,  
219 annealing at 56°C for IL-8, MCP-1 and at 58°C for ICAM-1 and E-  
220 Selectin, and elongation for 1 min at 72°C. The number of cycles used  
221 for amplification of chemokines (IL-8 and MCP-1) and ICAM-1 was 35  
222 and 30 respectively. The amplified DNA was separated in a 1.5 %  
223 agarose gel in Tris-borate EDTA buffer (TBE), and the intensity of the  
224 bands was quantified by densitometry using the software QuantityOne.  
225 Results were expressed as the ratio of the band intensities of the PCR  
226 products as compared to *gapdh* expression. Data were analyzed by  
227 analyses of variance followed by t test. A *P* value < 0.05 was  
228 considered significant.

229

230 **Results**

231

232 **Phagocytosis**

233 Freshly isolated porcine alveolar macrophages were either incubated  
234 with wild type strain 10, or with its non-encapsulated isogenic mutant  
235 strain S10cpsdeIEF, or with PBS (mock infection). Number of bacteria in  
236 the supernatant was measured by serial platings (Figure 1). The  
237 bacterial counts of wild-type bacteria remained constant in time, and  
238 even slightly increased during incubation, indicating that *S. suis* strain 10  
239 was able to survive, despite the presence of macrophages. The non-  
240 encapsulated mutant strain on the contrary, was phagocytosed very  
241 efficiently; after 5 minutes of incubation 70% of the bacteria were  
242 phagocytosed. The mock-infected macrophages remained sterile  
243 during the experiment. The difference in uptake of both strains was  
244 statistically significant ( $P < 0.05$ ) at 5 min pi and at later timepoints. These  
245 results confirmed previous findings (Smith et al., 1999).

246 Surprisingly, in contrast to the primary alveolar macrophages, the  
247 adherent alveolar macrophages cell line 3D4 was not able to  
248 significantly phagocytose the two *S. suis* strains used within 90 min of  
249 incubation as determined by a gentamycin protection assay (data not  
250 shown).

251

252 **Whole genome host response**

253 Microarray analysis

254 Gene expression profiles of macrophages were studied using  
255 microarrays. Transcription profiles of macrophages incubated with wild-  
256 type *S. suis*, non-encapsulated mutant, or PBS (mock) were studied as a  
257 function of time.

258 Gene expression of macrophages incubated with either the wild type  
259 strain 10 or with the non-encapsulated mutant was compared to gene  
260 expression of macrophages that were mock-infected with PBS at all  
261 timepoints. Significant differences were only found at timepoint 60  
262 minutes. After incubation with the wild type strain 10 that was not  
263 phagocytosed, one gene was expressed differentially compared to  
264 the mock infected macrophages (Table 2A). This gene showed  
265 homology to dynein. Ten genes were found to be differentially  
266 expressed in macrophages incubated with the non-encapsulated  
267 mutant compared to mock infected macrophages (Table 2A). Those  
268 genes include IL-1- $\beta$ , MIP-2a and TNF- $\alpha$  among others. The low numbers  
269 of genes that were found to be differentially regulated, suggested that  
270 either a limited response is induced in macrophages, or that mock-  
271 infected macrophages are activated as well.

272 To analyze this, gene expression of macrophages was studied in time  
273 by comparing expression at all timepoints to expression of timepoint  $t =$



274 0 of the same condition. This analysis showed that transcriptional  
275 changes occurred in all macrophages, independent of incubation with  
276 streptococci, and the highest number of regulated genes was found  
277 after 60 minutes. The process of phagocytosis of strain S10cpsdeIEF was  
278 nearly complete by then (95% was phagocytosed). In total 377 genes  
279 were found to be regulated in this experiment, 188 in the control  
280 macrophages, 244 after incubation with wild type strain 10, and 260  
281 genes after incubation with the non-encapsulated mutant. There is  
282 overlap among the regulated genes in the three conditions. Table 2B  
283 shows the toplist of genes that were found to be regulated. Ranking is  
284 based on macrophages incubated with S10cpsdeIEF. In general it was  
285 found that the toplists of all three conditions were very similar. However,  
286 the fold-induction of gene expression was strongest in macrophages  
287 that phagocytosed *S. suis* strain S10cpsdeIEF. This analysis indicates that  
288 the primary macrophages are activated in all three conditions. Despite  
289 this activation, genes could be identified that are differentially  
290 regulated due to interaction with *S. suis* (Table 2A), and *S. suis* could  
291 induced a stronger response in macrophage compared to the mock  
292 infected macrophages (Table 2B). A complete list of all regulated  
293 genes is given as supplementary material.

294 Pathway analysis

295 To gain insight into the biological processes of macrophage activation,  
296 a pathway analysis was performed on genes differentially expressed in  
297 time. To increase the power of pathway analyses, the cut-off for  
298 differential expression was lowered. Genes were included for analysis  
299 when gene expression was regulated at least 1.5 fold at timepoint  $t =$   
300 60 minutes compared to timepoint  $t = 0$  for (3,965 genes). Table 3 shows  
301 putative pathways found to be regulated. The MAPK-signaling  
302 pathway contained the highest number of regulated genes.  
303 Differentially expressed genes of the MAPK-signaling pathway all  
304 directed the macrophages towards proliferation/inflammation/anti-  
305 apoptosis. Expression of genes of the MAPK-signaling pathway was  
306 regulated under all three conditions, but similar to the other expression  
307 data the strongest induction/repression was found in macrophages  
308 incubated with the non-encapsulated strain s10cpsdeIEF.

309

### 310 **qPCR data**

311 To confirm the microarray data, qPCR analysis was performed on the  
312 same samples that were used in the microarray analysis. Expression of  
313 proinflammatory cytokines like TNF- $\alpha$ , IL-8, IL-1- $\beta$ , IL-6 and MIP-1- $\beta$  was  
314 studied. qPCR data confirmed the data found using microarray  
315 analysis (Figure 2). Strongest induction of proinflammatory cytokines  
316 was found in macrophages incubated with the non encapsulated *S.*

317 *suis* strain S10cpsdelEF, whereas much lower levels of induction were  
318 found in macrophages incubated with either S10 or PBS (mock).  
319 Expression of Toll like receptors was also studied using qPCR. No  
320 differences in expression were found, neither in time, nor between the  
321 different conditions (data not shown).

322

### 323 **Activation of porcine alveolar macrophages 3D4 cell line by *S. suis***

324 To study which signal transduction pathway is involved in the induction  
325 of proinflammatory cytokines, the 3D4 porcine alveolar macrophage  
326 cell line model was used (Weingartl et al., 2002). Although these cells  
327 did not phagocytose *S. suis* strains, this cell line can be used to study  
328 the effect of interaction between *S. suis* and alveolar macrophages  
329 independent of phagocytosis. NF- $\kappa$ B is an important transcriptional  
330 activator of pro-inflammatory cytokine gene expression. Therefore  
331 translocation of NF- $\kappa$ B to the nucleus of macrophages was studied after  
332 incubation with *S. suis* strain 10. Immunofluorescence microscopy  
333 revealed that the p65 subunit of NF- $\kappa$ B was translocated to the nucleus  
334 following infection with wild-type *S. suis*. Translocation of p65 was seen  
335 in approximately 10% of the macrophages (Figure 3). A similar picture  
336 was seen in LPS-treated cells (positive control), whereas in control cells,  
337 p65 remained in the cytoplasm. To confirm that NF- $\kappa$ B translocation  
338 indeed induces a proinflammatory cytokine response, *S. suis*-

339 dependent induction of IL-8, MCP-1, and ICAM-1 was studied by RT-  
340 PCR. Both *S. suis* strain 10 and the non-encapsulated mutant induced a  
341 strong expression of all three transcripts, whereas in non stimulated  
342 control cells no gene expression was induced (Figure 4). These data  
343 strongly suggest that after recognition of *S. suis* by alveolar  
344 macrophages, the signaling pathway of NF- $\kappa$ B is induced, as well as  
345 subsequent production of proinflammatory cytokines.

346

347 **Discussion**

348

349 In this article, gene expression of alveolar macrophages interacting  
350 with *S. suis* was studied using a phagocytosis model. Microarray analysis  
351 demonstrated that within the first 15 minutes of incubation of  
352 macrophages with bacteria, hardly any differences in gene expression  
353 occurred in the alveolar macrophages, whereas they did exert their  
354 biological function of clearing the bacteria within that time period.  
355 These data indicate that macrophages can phagocytose bacteria  
356 without adapting their gene regulation. Apparently, all reagents and  
357 mechanisms are ready-to-react in alveolar macrophages. It was  
358 previously shown that PMNs are capable of phagocytosing several  
359 pathogenic bacteria within 30 minutes of time, whereas regulation of  
360 gene expression started after 90 minutes (Kobayashi et al., 2003). Taken  
361 together, these data suggest that macrophages start signaling after  
362 their primary task of clearing bacteria from the host. The alveolar  
363 macrophage cell line was unable to phagocytose *S. suis*. Weingartl et  
364 al. described that the 3D4 cell line could phagocytose latex beads,  
365 although only a limited percentage of cells were able to exhibit this  
366 ability and only after very long incubations (3 hr – overnight) (Weingartl  
367 et al., 2002). Based on these results it seems likely, the 3D4 cells lost (part

368 of) their capacity to phagocytose efficiently during the process of  
369 immortalization.

370

371 We found that primary alveolar macrophages started regulating gene  
372 expression after 60 minutes of incubation with the two *S. suis* strains. At  
373 this timepoint, gene expression of macrophages that came into  
374 contact with the wild type strain or the non encapsulated mutant was  
375 compared to that of mock infected macrophages. Eleven genes were  
376 found to be expressed differentially due to incubation with *S. suis*. In  
377 macrophages that came into contact with the wild type strain, but did  
378 not phagocytose this strain, only dynein was found to be differentially  
379 expressed. Dynein is one of the microtubule associated motors.  
380 Overexpression of this gene suggests that despite the fact that *S. suis* is  
381 not phagocytosed, the macrophages are rearranging their  
382 membranes upon interaction with the wild type. Macrophages that  
383 came into contact with the non encapsulated mutant, that was  
384 phagocytosed, showed differential gene expression of ten genes.  
385 Genes involved in a proinflammatory immune response (TNF- $\alpha$ , MIP-2 $\alpha$ ,  
386 IL-1- $\beta$ ) were found to be regulated, but genes involved in cell cycling  
387 (G0/G1 switch protein, BTG2 protein) were regulated as well. In  
388 conclusion, after contact with the wild type strain, macrophages seem  
389 to change morphologically. After interaction with the non

390 encapsulated mutant macrophages raised an innate immune  
391 response and changed their cell cycle. This might potentially lead to  
392 phagocytosis or proliferation of macrophages.

393 The relatively low number of regulated genes due to incubation with *S.*  
394 *suvis* that was found can be explained by the activation of  
395 macrophages due to the isolation procedure or the incubation time.

396 When transcriptional responses were studied in time, 377 genes were  
397 differentially expressed compared to timepoint zero. This response was

398 observed in macrophages incubated with S10cpsdeIEF as well as in  
399 macrophages that came into contact with S10, and in control

400 macrophages. This general transcriptional response of macrophages  
401 was described previously as the common macrophage activation

402 program by Nau et al. (Nau et al., 2002). These authors describe that  
403 macrophages respond to a broad range of bacteria with a robust,

404 shared pattern of gene expression. The alveolar macrophages in our  
405 study were activated after contact with *S. suvis* independent of

406 phagocytosis. Genes that were described to be part of this 'common  
407 activation program' share a lot of homology with our gene list of

408 differentially regulated genes at timepoint 60 min (compared to  
409 timepoint 0). These data suggest that the alveolar macrophages in our

410 study were all activated, independent of bacterial interaction.  
411 However, not all macrophages were activated to the same extent.

412 Both in our microarray results and in the conformational qPCR analysis,  
413 differences in the level of induction are observed between the  
414 incubation conditions. The strongest induction of gene expression was  
415 observed in alveolar macrophages incubated with S10cpsdeIEF.  
416 Macrophages that came into contact with wild type strain S10 induced  
417 the same genes, but a much weaker induction was observed, whereas  
418 control macrophages showed an even weaker induction of gene  
419 expression. The strong transcriptional response observed in  
420 macrophages after phagocytosing the capsular mutant, will probably  
421 result in a strong local innate immune response *in vivo*. This will create a  
422 hostile environment for the bacteria to prevent them from  
423 multiplication and spreading through the host. Since the transcriptional  
424 response of macrophages to the wild type strain 10 is much weaker, it is  
425 expected that a weaker immune response is induced locally. In this  
426 way capsule not only protects *S. suis* from phagocytosis, but indirectly  
427 suppresses the immune response.

428

429 When the list of regulated genes at timepoint 60 compared to  
430 timepoint 0 is studied in more detail, it is clear that the strongest  
431 regulated genes encode proteins involved in the proinflammatory host  
432 defense like IL-1- $\beta$ , TNF- $\alpha$ , MIP-2- $\alpha$ , IL-1- $\alpha$  and I $\kappa$ B being the signal  
433 transducer. The regulation of a selected number of genes encoding



434 pro-inflammatory proteins was confirmed using qPCR. Our data confirm  
435 previous publications that describe induction of a proinflammatory  
436 immune response after incubation of human monocytes with *S. suis*  
437 (Dominguez-Punaro et al., 2007; Graveline et al., 2007; Segura et al.,  
438 1999; Segura et al., 2002; Segura et al., 2006). CD163 is one of the  
439 genes most strongly downregulated in alveolar macrophages. CD163 is  
440 a macrophage marker that is known to be regulated by pro- and anti-  
441 inflammatory stimuli (Buechler et al., 2000). The low expression level  
442 observed in alveolar macrophages correlates with the strong  
443 proinflammatory response that is also observed. This indicates that our  
444 data fit the known regulation of innate immune response in  
445 macrophages, thereby strengthening our findings. Activation of the  
446 innate immune response is controlled in large part by the Toll-like  
447 receptor (TLR) family of pattern-recognition receptors. TLRs are  
448 transmembrane proteins that recognize specific pathogen-associated  
449 molecular patterns (Akira et al., 2006). It was shown that *S. suis* can  
450 indeed bind to TLR2, both on human macrophages, as well as *in vivo* in  
451 murine brain (Dominguez-Punaro et al., 2007; Graveline et al., 2007). In  
452 our primary alveolar macrophages no increased expression of either  
453 one of the TLRs was observed upon stimulation with *S. suis*. However,  
454 expression of all TLRs included in our analysis (TLR 1, 2, 3, 4, 6, 8, 9, 10)  
455 was detected on macrophages at all timepoints. This expression

456 correlates very well with the ready-to-react status described previously.  
457 It seems that alveolar macrophages are constantly armed with sensors  
458 to detect invading bacteria. Therefore, it seems very likely that *S. suis*  
459 binds to one or more of the TLRs, resulting in the observed  
460 proinflammatory response.

461

462 When the induced genes were clustered to functional groups, some  
463 putative pathways were strongly regulated upon contact with *S. suis*  
464 such as the MAPK-signaling pathway, cytokine-cytokine receptor  
465 interaction, and cell cycle. The highest number of regulated genes  
466 were involved in the MAPK-signaling pathway . MAP-kinases are key  
467 regulators for the synthesis of numerous cytokines, chemokines, and  
468 other inflammatory mediators (Dong et al., 2002). ERK, one of the MAP-  
469 kinase subfamilies is strongly upregulated, thereby inducing the  
470 inflammatory cascade. However, ERK has not yet induced its self-  
471 limiting mechanism of MKP1, since MKP1 is downregulated at 60  
472 minutes (Wang and Liu, 2007). This means that the process that  
473 terminates cytokine production is completely off after 60 minutes,  
474 allowing the inflammatory cascade to amplify

475

476 NF- $\kappa$ B represents another important transcriptional regulator, which also  
477 induces expression of proinflammatory cytokines (Pahl, 1999). The

478 inactive form of NF- $\kappa$ B is located in the cytoplasm, whereas stimulation  
479 of the NF- $\kappa$ B pathway leads to a degradation of the inhibitory proteins  
480 I $\kappa$ B, resulting in NF- $\kappa$ B translocation into the cell nucleus. To study if NF- $\kappa$ B  
481 is also involved in the induction of expression of proinflammatory  
482 cytokines we identified with the microarray, an alveolar macrophages  
483 cell line was used (3D4) (Weingartl et al., 2002). Despite the fact that  
484 this cell line was unable to phagocytose either wild type *S. suis* or the  
485 non encapsulated mutant, the cell line offers a good model to study  
486 the transcriptional effects of interaction with *S. suis*. An advantage o  
487 using a cell line is that the conditions are more standardized. When  
488 using primary cells, there is always the risk the cells get activated upon  
489 isolation. When using a cell line, this 'background' effect is lacking.  
490 Immunofluorescent staining of the p65 subunit of NF- $\kappa$ B allowed us  
491 to demonstrate nuclear translocation of the transcriptional regulator in  
492 response to *S. suis* infection indicating activation of NF- $\kappa$ B. This  
493 demonstrates for the first time that *S. suis* is able to induce NF- $\kappa$ B. NF- $\kappa$ B  
494 activation plays an established central role in the inflammatory process  
495 (Pahl, 1999). The *S. suis* induced translocation of NF- $\kappa$ B suggest that  
496 signal transduction upon interaction with *S. suis* not only acts via MAP-  
497 kinase, but also via NF- $\kappa$ B. RT-PCR analyses showed that the  
498 translocation of NF- $\kappa$ B indeed resulted in an upregulation of innate  
499 immune genes such as IL-8, MCP, and ICAM. This regulation of

500 expression was not different between stimulation with encapsulated  
501 and non-encapsulated *S. suis* strains.

502 Taken together, we show that after interaction of *S. suis* with alveolar  
503 macrophages a strong proinflammatory response is induced. Although  
504 this effect occurs independent of phagocytosis, the induction of gene  
505 expression was stronger in a population of macrophages that were  
506 incubated with the non-encapsulated *S. suis* that was phagocytosed.  
507 Pathway analysis and NF- $\kappa$ B translocation studies showed that the  
508 differential gene expression is probably induced by two major signal  
509 transduction pathways, MAP-kinase and NF- $\kappa$ B. This is the first time *S. suis*  
510 induced translocation of NF- $\kappa$ B is described.

511 **Acknowledgements**

512 This project was financially supported by the Ministry of Agriculture,  
513 Nature and Food Quality (KB-08) and results were obtained through the  
514 EC funded EADgene network (contract no. FOOD-CT-2004-506416). The  
515 text represents the authors' views and does not necessarily represent a  
516 position of the Commission who will not be liable for the use made of  
517 such information.

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608 **Figure Legends**

609

610 **Figure 1.** Bacteria present in supernatant of culture medium containing  
611 porcine alveolar macrophages. (*S. suis* strain 10, open circles; non-  
612 encapsulated mutant strain S10cpsdelEF, closed circles). Each  
613 datapoint depicts the mean of 7 independent experiments. Error bars  
614 show standard error of the mean. \*  $p < 0.05$

615

616 **Figure 2.** Gene expression of alveolar macrophages using qPCR. Gene  
617 expression is depicted as relative expression compared to *gapdh* gene  
618 expression. Macrophages were either incubated with *S. suis* S10 (black  
619 bars), *S. suis* S10cpsdelEF (hatched bars) or with PBS (mock) (white  
620 bars). Each sample consists of a pool of 7 individual samples.

621

622 **Figure 3.** Immunofluorescence detection of p65 translocation into the  
623 nuclei of porcine cells. 3D4 porcine alveolar macrophages - were  
624 treated with *S. suis* wild-type strain, LPS or cell culture medium as  
625 described in the Material and Methods. After 30 min of treatment, the  
626 cells were fixed, permeabilized and stained for p65.

627

628 **Figure 4.** Contribution of *S. suis* to transcriptional activation of IL-8, MCP-1  
629 and ICAM-1 by 3D4 porcine alveolar macrophages. Cells were



630 stimulated with wild type *S. suis* S10 (black bars) and non-encapsulated  
631 *S. suis* strain S10cpsdeIEF (hatched bars) or were left unstimulated  
632 (white bars). Gene expression was measured by RT-PCR analysis. Data  
633 are expressed as the mean ratio of target molecule over *gapdh*  
634 expression. Means and SD of three independent experiments are  
635 shown. \*, *P* values < 0.05.

636 -

637

638 **Table 1.** Primer sequences.

<b>Gene name</b>	<b>Forward primer</b>	<b>Reverse primer</b>
TLR1	TGCTGGATGCTAACGGATGTC	AAGTGGTTTCAATGTTGTTCAAAGTC
TLR2	TCACTGTCTAACTTATCATCCTCTTG	TCAGCGAAGGTGTCATTATTGC
TLR3	AGTAAATGAATCACCCCTGCCTAGCA	GCCGTTGACAAAACACATAAGGACT
TLR4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG
TLR6	AACCTACTGTCATAAGCCTTCATTC	GTCTACCACAAATTCACITTTCTTCAG
TLR8	AAGACCACCACCAACTTAGCC	GACCCTCAGATTCTCATCCATCC
TLR9	CACGACAGCCGAATAGCAC	GGGAACAGGGAGCAGAGC
TLR10	CCTGTCCAACCTGCCTCATTTG	CTAAGTGTCTAAGGATGTGTTTCTG
IL-1 $\beta$	GGCCGCCAAGATATAACTGA	GGACCTCTGGGTATGGCTTTC
IL-12	GGAGTATAAGAAGTACAGAGTGG	GATGTCCCTGATGAAGAAGC
IL-8	TTCGATGCCAGTGCATAAATA	CTGTACAACCTTCTGCACCCA
MIP-1 $\beta$	CAGCACCAATGGGCTCAGA	TTCCGCACGGTGTATGTGA
TNF- $\alpha$	CGCCCACGTTGTAGCCAATGT	CAGATAGTCGGGCAGGTTGATCTC
IL-6	GACAAAGCCACCACCCCTAA	CTCGTTCTGTGACTGCAGCTTATC
GAPDH	TGCCAACGTGTCGGTTGT	TGTCATCATATTTGGCAGGTTTCT

639

640

641 **Table 2A.** Genelist of regulated genes in alveolar macrophages after  
 642 incubation with *S. suis* strain 10 (S10), or its non-encapsulated mutant  
 643 (S10cpsdeIEF). Fold induction is expressed as expression level after  
 644 incubation with S10 or S10cpsdeIEF at timepoint 60 min compared to  
 645 cells that were mock infected at timepoint 60 min. (-) Gene expression  
 646 below cut-off of 2 fold induction

Gene name	Fold induction	
	S10	S10delcpsEF
Dynein	2.9	-
Tumor necrosis factor (TNF- $\alpha$ )	-	3.9
Chemokine ligand 4 (CCL4)	-	3.8
Interleukin 1 beta (IL-1- $\beta$ )	-	3.6
Amphiregulin	-	3.0
Put. Lymphocyte G0/G1 switch protein 2	-	2.9
BTG2 protein	-	2.2
Macrophage inflammatory protein 2 alpha (MIP-2- $\alpha$ )	-	2.2
Jumonji domain containing 3	-	2.1
Serine/threonine proteins kinase PLK2	-	2.0
Pyruvate dehydrogenase kinase isozyme 4	-	-2.1

647

648

649 **Table 2B.** Genelist of regulated genes in alveolar macrophages after  
 650 incubation with *S. suis* strain 10 (S10), its non-encapsulated mutant  
 651 (S10cpsdeIEF) or PBS (mock). Fold induction is expressed as expression  
 652 level at timepoint 60 min compared to timepoint 0 min of the same  
 653 strain. (-) Gene expression below cut-off of 2 fold induction.

Gene Name	Fold Induction		
	PBS (mock)	S10	S10 cpsdeIEF
Interleukin-1- $\beta$	16.1	16.6	47.2
TNF- $\alpha$	9.6	13.1	43.4
Macrophage inflammatory protein-2- $\alpha$	16.0	25.5	34.3
Mitogen-activated kinase (MAP3K8)	13.9	16.9	23.5
Interleukin-1- $\alpha$	12.6	16.0	22.1
I $\kappa$ B	14.6	19.8	21.9
Adrenomedullin	6.2	6.9	18.0
Immediate early protein	4.3	5.9	15.8
Activating transcription factor 3 (ATF-3)	12.8	12.4	14.8
FosB	10.9	13.7	12.2
Interleukin-8	8.1	10.3	10.8
Mitogen-inducible gene 6	6.6	5.5	9.9
Cytochrome P450	7.1	10.1	9.5
Small inducible cytokine like 3 A1	-	-	9.2
Chemokine Ligand 4 like (CCL4)	2.0	2.3	8.5
Vascular endothelial growth factor A	8.1	8.9	8.4
Phosphatidylcholine:ceramide cholinephosphotransferase 1	-2.8	-2.7	-3.0
Nuclear factor 1A	-	-	-2.8
Muscleblind-like protein	-3.2	-3.0	-3.0
P2Y purinoreceptor 5 (P2Y5)	-3.0	-2.9	-3.0

Map3K12 binding inhibitory protein	-	-2.4	-3.1
No homology	-2.3	-2.5	-3.2
No homology	-2.8	-2.4	-3.4
No homology	-3.3	-2.5	-3.9
Cardiomyopathy associated 4	-	-	-4.1
No homology	-2.7	-2.9	-4.3
Dual specificity protein phosphatase 6	-3.8	-3.8	-4.3
CD163 antigen	-4.5	-4.3	-5.3
Ras-related protein Rab-3B	-5.0	-5.3	-5.8
Adapter-related protein complex 3	-	-1.7	-8.3
Neuronal protein 3.1	-6.9	-7.2	-9.0

654

655

656 **Table 3.** Pathways that contain an overrepresentation of regulated  
 657 genes in alveolar macrophages after incubation with *S. suis* strain  
 658 S10cpsdeIEF at timepoint 60. Genes were considered regulated when  
 659 fold induction at  $t = 60$  min. compared to  $t = 0$  min. was  $>1.5$ .

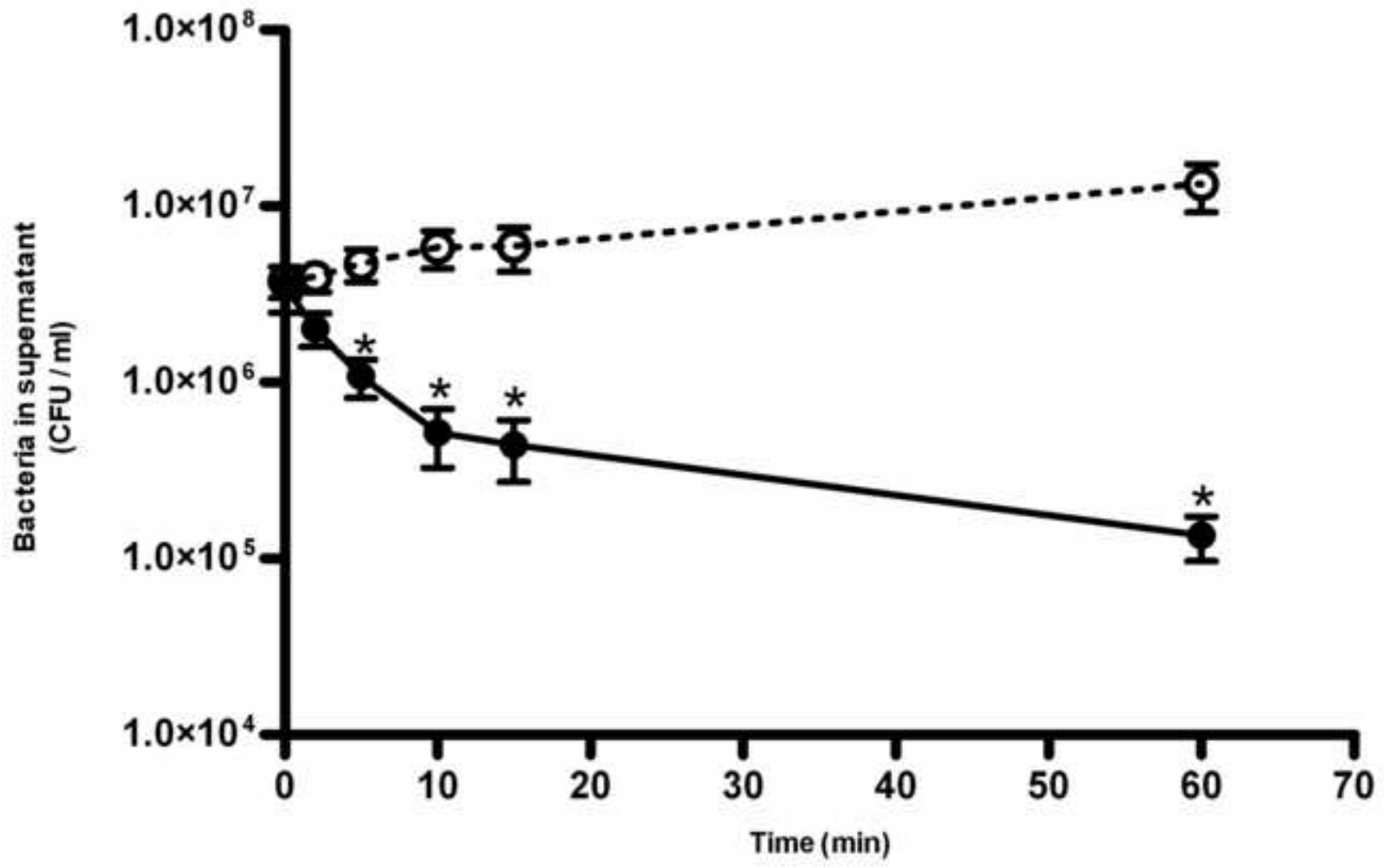
<b>KEGG pathway</b>	<b>Gene number</b>
MAPK signaling pathway	31
Focal adhesion	27
Regulation of actin cytoskeleton	21
Cytokine – cytokine receptor interaction	18
Cell cycle	17
Axon guidance	17
Insulin signaling pathway	16
ECM-receptor interaction	14
Tight junction	13
Colorectal cancer	12
T Cell receptor signaling pathway	11
Cell communication	11
Purine metabolism	11
Calcium signaling pathway	10

Apoptosis	10
Wnt signaling pathway	10

660

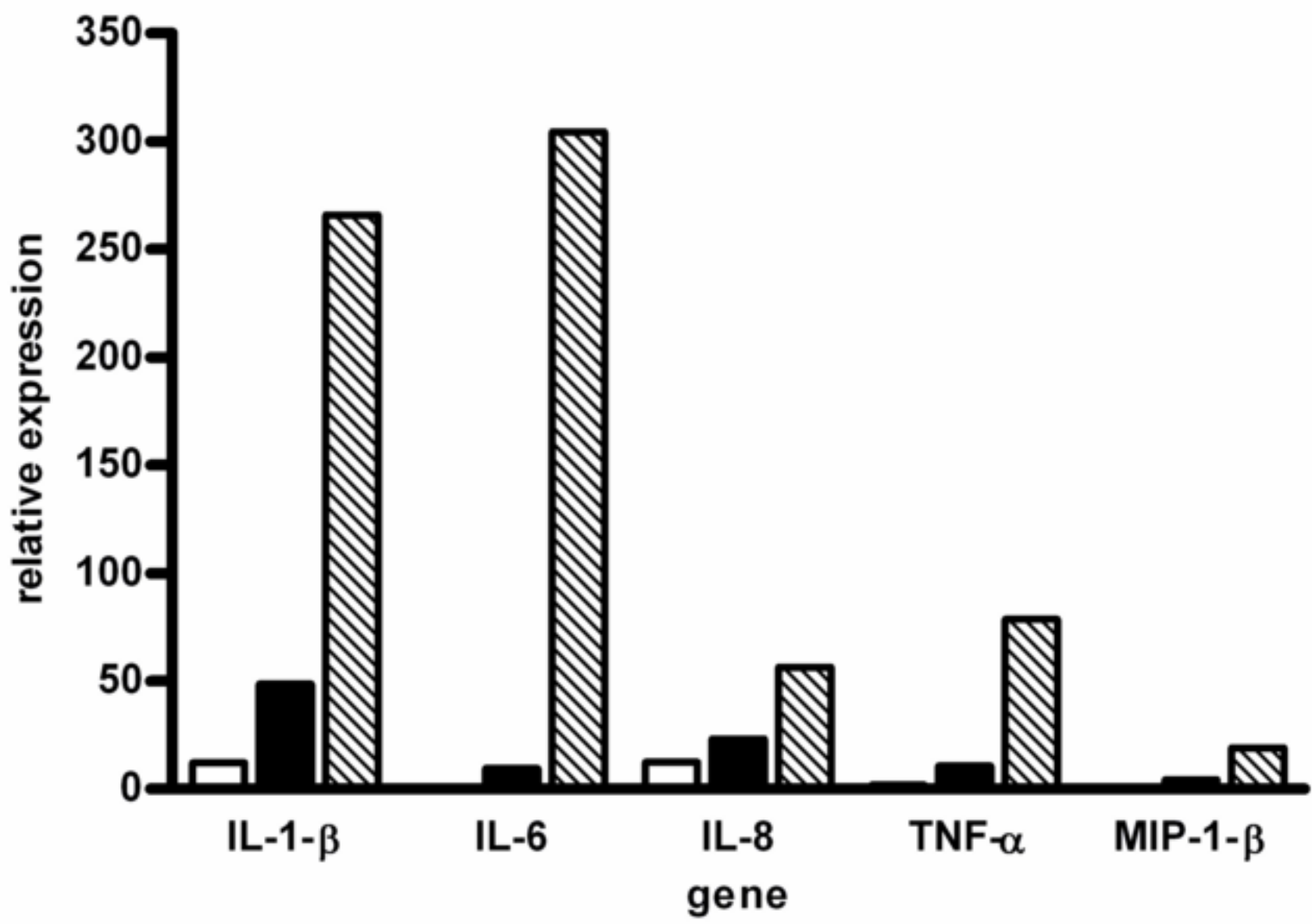
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Figure 1





Criip



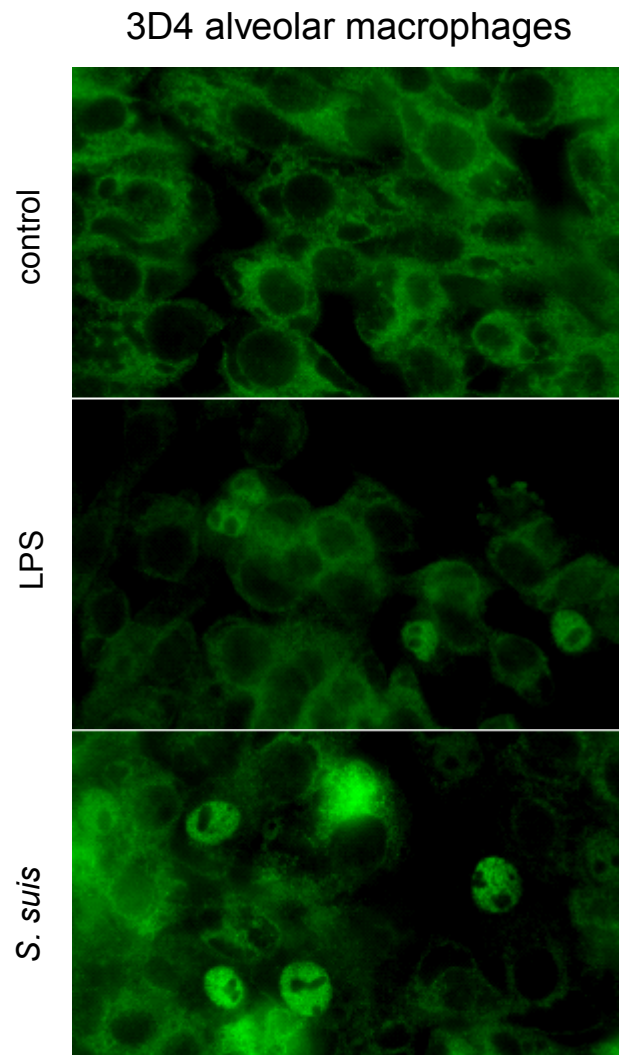


Figure 1

## 3D4 alveolar macrophages

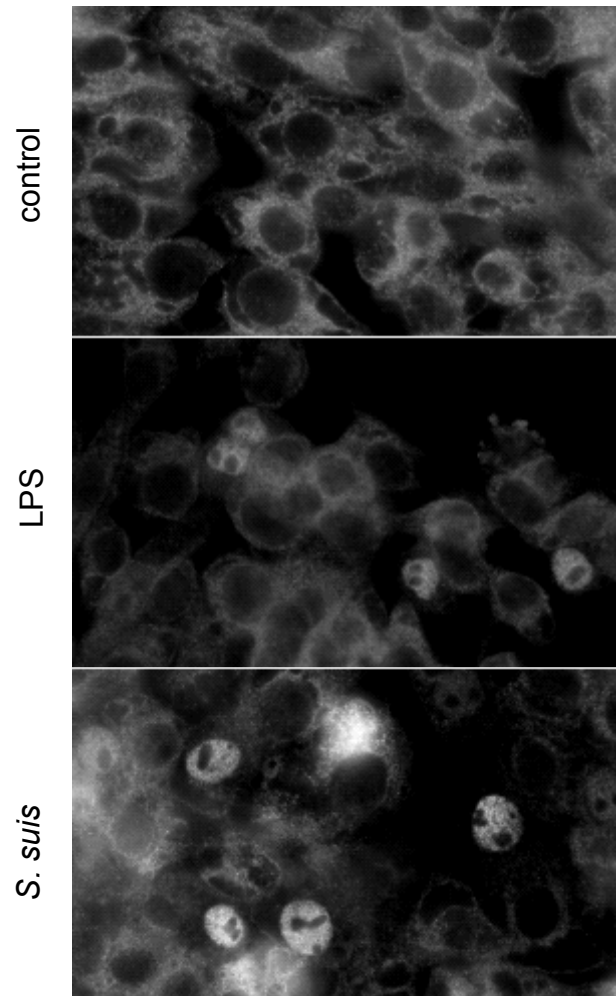


Figure 4

