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Authors: Erika Borrmann, Petra Möbius, Roland Diller, Heike Köhler



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1 Divergent cytokine responses of macrophages to *Mycobacterium avium* subsp.
2 *paratuberculosis* strains of Type II and III in a standardized *in vitro* model

3

4 Erika Borrmann*, Petra Möbius, Roland Diller, Heike Köhler

5

6 Friedrich-Loeffler-Institut, Institute of Molecular Pathogenesis,

7 D-07743 Jena, Naumburger Str. 96 a, Germany

8

9 *Corresponding author: Erika Borrmann, D-07743 Jena, Naumburger Str. 96 a

10 Phone: +49 3641 804 2427

11 Fax: +49 3641 804 2228

12 E-mail: erika.borrmann@fli.bund.de

13

14 Abstract

15 Based on epidemiological and clinical observations, different strains of *Mycobacterium avium*
16 subsp. *paratuberculosis* (MAP) are suspected to significantly differ in their virulence for
17 ruminants. In the pathogenesis of paratuberculosis, macrophages represent the principal
18 target cell for MAP. In order to judge the ability of different MAP-genotypes to modulate
19 macrophage responses, the cytokine responses of the monocyte cell line THP-1 were
20 studied after challenge with three different MAP strains under standardized conditions. The
21 bovine field isolate J1961 (major Type II) and the ovine field isolate JIII-86 (Type III) were
22 compared with the laboratory adapted reference strain ATCC 19698 (Type II). Strains were
23 shown by three different typing methods (IS900-RFLP-, MIRU-VNTR-, and SSR-analysis) to
24 substantially differ in several genotypic features. Macrophage function was assessed by
25 quantifying mRNA of the cytokines TNF- α , IL-1 β , and IL-10 by quantitative RT-PCR.
26 Secreted TNF- α protein was measured by a cytotoxicity test, IL-1 β and IL-10 using ELISA
27 tests.

28 The three MAP strains of various genotypes differ in their effect on human macrophages
29 depending on challenge dose and infection time. These differences concerned both the
30 mRNA level and secreted protein amounts of proinflammatory cytokines TNF- α , IL-1 β and
31 anti-inflammatory cytokine IL-10. Type III strain produced less IL-10 and IL-1 β mRNA and
32 protein but more TNF- α protein at 2 h than the Type II strains.

33 In summary, our results support the hypothesis that strain characteristics might have
34 relevance for the host response towards MAP and, consequently, for the pathogenesis of
35 paratuberculosis.

36

37 Key words: *Mycobacterium avium* subsp. *paratuberculosis*, MAP-genotypes, cytokines, THP-
38 1, quantitative real-time PCR

39 1. Introduction

40 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of
41 paratuberculosis (Johne's disease), a chronic, incurable granulomatous enteritis of
42 ruminants. In the pathogenesis of paratuberculosis, macrophages represent the principal
43 target cell for MAP. Upon contact of macrophages with MAP, a variety of cellular reactions
44 are triggered, including the release of proinflammatory cytokines (TNF- α and IL-1) as well as
45 anti-inflammatory cytokines (IL-10 and TGF- β) (Weiss et al., 2002; Weiss and Souza, 2008).
46 These cytokines play an important role in the pathogenesis of paratuberculosis, because
47 increased pro-inflammatory cytokine expression in the intestine coincide with failed or
48 misdirected immune responses and contribute to the course of this disease (Rossi et al.,
49 2009). Herd management (Hayton, 2007), resistance/susceptibility of cattle to MAP infection
50 (Bhide et al., 2009) and the different genotypes of implicated MAP strains (Castellanos et al.,
51 2009) are considered risk factors for the clinical manifestation of paratuberculosis. Based on
52 IS900 restriction fragment length polymorphism analysis (IS900-RFLP) and phenotypic
53 properties like growth characteristics and pigmentation, MAP strains have been subdivided
54 into three major types: sheep, cattle and intermediate type, whereas sheep type corresponds
55 to Type I, cattle type to Type II and the intermediate type to Type III using pulse-field gel-
56 electrophoresis (PFGE) (de Juan et al., 2005, Sohal et al, 2010). Several studies addressed
57 the question if specific genotypes of mycobacteria in general can cause differential immune
58 responses. Significant differences in both cytokine profiles and global gene expression
59 profiles in established cell lines (THP-1 or U937) as well as in peripheral blood mononuclear
60 cells (PBMCs) in response to diverse pathogenic and non-pathogenic mycobacterial species
61 have been demonstrated recently (Theus et al., 2005, McGarvey et al., 2004).
62 The aim of this study was to develop a standardized protocol for the characterization of MAP-
63 strains of different genotype regarding their property to modulate cytokines involved in the
64 pathogenesis of paratuberculosis. The effects of one well characterized Type II and one
65 Type III field strain of MAP were compared to an ATCC-reference strain (Type II) depending
66 on challenge dose and incubation time.

67 2. Material and Methods

68 2.1. Bacterial strains and growth conditions

69 *Mycobacterium avium* subsp. *paratuberculosis* strain DSM 44133 (ATCC 19698) was
70 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ,
71 Braunschweig, Germany). The bovine field isolate J1961 was recovered from intestinal
72 tissue, cultivated and characterized at the Friedrich-Loeffler-Institut, Jena. This isolate
73 originated from a naturally MAP infected cow from Germany. Strain JIII-86 isolated from the
74 ileal mucosa of one sheep has been described recently (Möbius et al., 2009).
75 The field strains had been isolated from tissues by cultivation on Herrold's Egg Yolk medium
76 (HEYM, BD Biosciences, Germany) and modified Loewenstein-Jensen Medium (Bioservice
77 Waldenburg, Germany) both containing mycobactin J. The isolates were further maintained
78 on HEYM. Several loopfuls of colonies were taken from solid medium and inoculated into
79 Middlebrook 7H9 broth (BD Biosciences, Germany) supplemented with 10% (vol/vol) oleic
80 acid-albumin-dextrose-catalase (OADC; BD Biosciences, Germany), 0.2% (vol/vol) glycerol,
81 2 µg/ml mycobactin J (Allied Monitor, USA) and 0.05% Tween 80 (Serva, Germany). After
82 about 8 weeks starter cultures were transferred into larger batches and these broth cultures
83 were cultivated at 37°C under constant shaking to allow uniform growth. The final cultures
84 were harvested after 12 to 16 weeks depending on the strain.
85 Characterization of the strains was carried out by phenotype (pigmentation, growth kinetics)
86 and genotype: IS1311-PCR/REA according to Whittington et al. (2001) to differentiate
87 between Type II and Type I / III strains, *gyrB*-PCR/REA as described by Castellanos et al.
88 (2007) to differentiate between the Type I, Type II and Type III groups. Furthermore, strains
89 were extensively sub-typed by three diverse typing techniques: IS900-RFLP using the
90 digestion enzymes *BstEII* and *PstI*, mycobacterial interspersed repetitive unit and variable-
91 number of tandem repeat (MIRU-VNTR) analysis using ten specific markers (MIRU loci 1-4;
92 VNTR loci 292, 25, 47, 3, 7, 32) and by multi-locus short sequence repeat (MLSSR)
93 sequencing (loci 1, 2, 8, 9) as described in detail previously (Möbius et al., 2008; 2009).

94

95 2.2 Preparation of bacterial suspensions for inoculation

96 For infection of macrophages, mycobacteria were harvested from broth cultures by
97 centrifugation (4000 U/min for 10 min), washed 3 times with phosphate-buffered saline (PBS)
98 including 0.05% Tween 80 and resuspended in cell culture medium with 1% foetal calf serum
99 (FCS). For homogenization the bacterial suspensions were vortexed in the presence of glass
100 beads for 5 min and centrifuged for 5 min at 50 x g (Biofuge, Hereaus, Germany). Ziehl-
101 Neelsen staining and microscopy confirmed that the resulting supernatant contained mainly
102 single bacteria. These bacterial suspensions were adjusted to an optical density (OD) of
103 approximately 0.15 at 580 nm with cell culture medium containing 1% FCS. The mean
104 numbers of colony forming units (cfu) per ml were quantified by performing standard plate
105 counts.

106 Correlation coefficients between OD at 580 nm and cfu for each of the strains were
107 calculated to obtain an OD which is the equivalent of cfu.

108 In order to standardize the performance of challenge tests for detection of cytokines, only
109 bacterial suspensions with approximately 3×10^7 cfu/ml were taken for inoculation. These
110 suspensions were further diluted 1:2 and 1:4 corresponding to 1.5×10^7 and 7.5×10^6 cfu/ml.
111 Viability of bacteria was examined by the BacLight system (Invitrogen, US).

112

113 2.3. SDS-PAGE

114 Sample preparation for the SDS-PAGE was carried out as described by Gumber et al.
115 (2007) with modifications. Briefly, mycobacterial suspensions of each strain were centrifuged
116 at 10.000 x g (Biofuge, Hereaus, Germany) and washed with PBS (pH 7.2). The
117 supernatants were discarded and cell pellets were washed with PBS (pH 7.2) including 1%
118 Tween 80. The weights of cell pellets were recorded and lysis buffer (PBS pH 7.0 with 0.4%
119 (vol/vol) Tween 20 and 0.2% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich, Germany)
120 were added. 250 µl lysis buffer corresponded to 10 mg cell pellet (wet weight). The mixture
121 (cell pellet and lysis buffer) was given to Lysing matrix tubes B including glass beads (MP
122 Biomedicals, Germany). Cells were disrupted in a cell homogenizer (Fast Prep®-24, MP

123 Biomedicals, Germany) using 7 x 20 s pulses at speed 4.0 m/s with 2 min rest on ice
124 between pulses. The beads were allowed to settle; the supernatants were removed and
125 centrifuged at 14.000 x g (Centrifuge 5415 R, Eppendorf, Germany). The protein
126 concentration of the supernatant was determined using the BCA Protein Assay Kit (Thermo
127 Scientific, Germany).
128 The mycobacterial proteins were solubilised in SDS reducing buffer (Bio-Rad Laboratories,
129 US) and boiled for 5 min. Afterwards, the proteins were analysed under discontinuous
130 electrophoresis buffer conditions using a 12% separating gel. Electrophoresis was performed
131 in a Mini Protean® II Dual Slab Cell (Bio-Rad Laboratories, US) at a constant current of 30
132 mA.

133

134 2.4. Cells

135 The human monocytic cell line THP-1 was obtained from the DSMZ (Braunschweig,
136 Germany). Cells were maintained in RPMI 1640 medium with L-glutamine (PAA,
137 Laboratories GmbH, Germany), 5% FCS, 0.05 mM mercaptoethanol, referred to as cell
138 culture medium in the manuscript, at 37 °C and 5% CO₂, and sub-cultivated 2 times per week
139 at an approximate density of 2 x 10⁵ cells/ml.

140

141 2.5. Determination of the infection rate of THP-1 cells

142 Preliminary infection assays were performed with each isolate in order to determine the
143 bacterial concentration and time needed to obtain approximately 85% of the macrophages
144 infected. THP-1-cells were seeded into wells of 24-well-flat bottom plates (Costar Corning,
145 The Netherlands) containing 12-mm-diameter glass cover slips at a cell number of 5 x 10⁵
146 cells per well. Differentiation into macrophages was triggered by addition of 2.5 ng/ml PMA
147 overnight followed by replacement with fresh cell culture medium. The cells were allowed to
148 rest 24 h following activation to ensure that they converted to adherent cells before infection.
149 The differentiated, adherent cells were infected with the MAP suspension produced as
150 described above (2.2.) at different bacterial numbers (7x10⁶; 1x10⁷, 2.6x10⁷ and 1x10⁸ cfu/ml)

151 and for a different time period (2, 4, 8, and 24 h). Subsequently, cells were washed with
152 warm PBS (pH 7.2) three times, then fixed with 4% paraformaldehyd for 15 min,
153 permeabilized with ice-cold methanol/acetone (1:1) for 5 min and stained with
154 auramin/rhodamin using the TB Fluorostain™ Kit (Polyscience Europe GmbH, Germany).
155 The coverslips were removed from the wells, mounted on glass slides with mounting medium
156 and sealed with nail polish. The stained coverslips were observed by fluorescence
157 microscopy (Leitz Labor Lux S, Leica, Germany) and at least 100 cells were counted per
158 coverslip (Woo et al., 2006).

159 Viable intracellular bacteria were assessed after 2, 4, 8 and 24 h of incubation using the
160 standard colony-counting technique (Weiss et al. 2002). Cells were washed three times with
161 PBS, incubated with PBS containing gentamycin (150 µg/ml gentamycin, Sigma, Germany)
162 for 2 h and lysed using Triton-X100 (Sigma, Germany) in sterile water for 10 min. After the
163 addition of Middlebrook 7H9 broth, samples were disrupted by vigorous pipetting.
164 Suspensions were serially diluted and plated for cfu determination onto Middlebrook 7H11
165 agar containing mycobactin J (BD Biosciences, Germany). The determination of cfu occurred
166 after 5-10 weeks.

167

168 2.6. Sample preparation for detection of cytokine induction

169 THP-1 cells were differentiated with PMA and inoculated with MAP strains as described
170 above (2.5.). The highest bacterial numbers for inoculation amounted to approximately 3 x
171 10⁷ cfu/ml. They were further diluted 1:2 and 1:4 as described in 2.2. As determined during
172 preliminary infection assays, the bacteria were removed after 8 h interaction by washing
173 twice with warm PBS (pH 7.2), (Table 1). Then fresh cell culture medium was added.
174 Supernatants were harvested after different incubation times, 4 samples with the same
175 challenge dose were pooled, filtered through 0.22-µm pore-size syringe filters and stored at -
176 80°C until analyzed. This procedure was used for the determination of IL-10 and IL-1β at 24
177 h. For determination of TNF-α in the bioassay, supernatants were recovered after 2 and 4 h
178 infection time respectively, filtered and stored at -80°C until analyzed. For the determination

179 of IL-1 β supernatants were harvested 4 h PI and 24 h PI (8 h incubation with bacteria + 16 h
180 incubation with cell culture medium with 1% FCS). For the determination of IL-10
181 supernatants were harvested after total incubation times of 24 h (8 h incubation with bacteria
182 +16 h with fresh medium with 1%FCS), 48 h (8 h + 40 h), and 72 h (8 h + 64 h) as described
183 above and stored at -80°C until analyzed for IL-10. An overview of infection and total
184 incubation times is also given in Table 1. Corresponding cell pellets were washed three times
185 with warm PBS (37°C) and mRNA was isolated using the RNeasy Mini kit (Qiagen,
186 Germany).

187 In preliminary experiments the ability of activated THP-1 cells to release cytokines was
188 tested using lipopolysaccharide (LPS) from *E. coli* 026:B6 (Sigma-Aldrich, Germany) in the
189 concentration range from 0.1 μ g/ml to 100 μ g/ml as an inductor. The isolated mRNA from
190 these cell pellets was used as positive control in further investigations.

191 Negative control samples were cells activated by PMA without addition of bacteria and with
192 cell culture medium including 1% FCS.

193

194 2.7. Determination of cytokine mRNA expression by real-time RT-PCR

195 Quantity and purity of the isolated mRNA from cell pellets (2.6.) were determined
196 spectrophotometrically by measuring the absorbance at 260 nm and the 260/280 nm
197 absorbance ratio (Biophotometer, Eppendorf, Germany).

198 Reverse transcription (RT) of the mRNA was performed in 20 μ l final volume with
199 Omniscript^R reverse transcriptase (Qiagen, Germany) in the presence of RNase inhibitor
200 (Promega, Germany) using random hexamer primer pd(N)₆ (Roche Diagnostics, Germany).

201 The quantification of cDNA and consequently of mRNA was carried out by the Taqman real
202 time PCR system (ABI Prism 7000 Sequence Detection System, Applied Biosystems,
203 Germany) in a 96-well microtiter format using SYBR[®] Green PCR Mastermix according to the
204 instructions of the manufacturer (Applied Biosystem, Germany). Description of primers is
205 given in Table 2. All primers used were synthesized by Jena Bioscience (Germany).

206 The target genes and the endogenous control gene GAPDH were amplified in separate wells

207 of 96-well plates. The PCR reactions contained 300 nM of the gene specific primers and the
208 SYBR[®] Green PCR Mastermix, which includes SYBR Green I as a fluorescent reporter and
209 Rox as internal reference for normalization of the fluorescence signal. The concentration of
210 cDNA was equivalent to 7.5 ng RNA. In every run, three no-template controls (DEPC-water)
211 were included. Amplification conditions were 2 min at 50°C, 10 min at 95°C, followed by 40
212 cycles of 15 s at 95°C and 1 min at 60°C. Subsequently, melting curve analysis was
213 performed to measure the specificity of amplification. The threshold cycle number (C_t) of
214 triplicate reactions was calculated using the ABI Prism software 1.23 (relative quantification).
215 Final quantification was done using the comparative C_t -method and is reported as relative
216 transcription or the n-fold difference relative to a calibrator cDNA, here non-infected cells
217 (Borrmann et. al., 2007).

218

219 2.8. Determination of cytokine secretion

220 2.8.1. TNF- α bioassay

221 TNF- α protein in the cell supernatants was determined using the bioassay described by
222 Bertoni et al. (1993). Briefly, the amount of TNF- α was determined by its cytotoxic effect on
223 the porcine kidney cell line PK 15 clone 15 in 96-well plates. The viability of the cells was
224 assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-
225 Aldrich, Germany). The OD was measured at 590 nm using an ELISA-reader (TECAN,
226 Germany) and the amount of TNF- α in U/ml was calculated in comparison to a dilution series
227 of recombinant porcine TNF- α (MorphoSys AG, Germany) by the reader software. Negative
228 control samples consisted of medium without TNF- α .

229

230 2.8.2. ELISA

231 The levels of IL-1 β and IL-10 protein secreted into supernatants were determined using the
232 ELISA kits human IL-1 β Cytoset and human IL-10 Cytoset following the manufacturer's
233 instructions (Invitrogen, US). Amounts of cytokines were calculated in comparison to the
234 corresponding standards by the reader software (Tecan, Germany).

235

236 2.9. Statistical Analysis

237 Results from independent cell tests generated by bioassay, ELISA and real-time RT-PCR are
238 presented as mean values \pm standard deviation. Statistical analyses of the comparison
239 between control and samples, among the three strains and dependency of time and infection
240 dose for every strain were performed by the Mann-Whitney-test. A p value of < 0.05 was
241 considered statistically significant.

242

243 3. Results

244 3.1. Characterization of bacterial strains

245 The type strain ATCC 19698 and the bovine field strain J1961 were classified as Type II and
246 strain JIII-86 as Type III by phenotype and genotype. All three strains were non pigmented.

247 In contrast to the sheep strain JIII-86 characterized by a very long growth period up to seven
248 months, both other strains showed visible colonies after 6 to 10 weeks of cultivation. Typing
249 by IS900-RFLP and *Bst*EII digestion revealed the intermediate pattern I6 for JIII-86,

250 supporting its classification as Type III. Both other strains exhibited C-patterns typical for
251 Type II strains. Results of the *gyrB*-gene-PCR/REA and the IS1311-PCR/REA confirmed this
252 classification. Sub-typing results of these strains using IS900-RFLP, MIRU-VNTR- and
253 MLSSR- analysis are shown in Table 3 and Fig. 1 (suppl.). Additionally, protein samples of
254 the three strains showed clearly different protein banding patterns on coomassie-stained
255 SDS-PAGE (Fig. 1).

256

257 3.2. Correlation between optical density and colony forming units

258 The comparison between OD and cfu resulted in different slopes of the regression lines of
259 the three strains (Fig. 2). The regression lines of both field strains run almost parallel, whilst
260 the regression line of the laboratory-adapted strain ATCC 19698 had a lower slope than the
261 lines of both field strains and differed markedly from both field strains. Consequently, inocula
262 for challenge studies were adjusted based on cfu counts.

263

264 3.3. Infection kinetics of THP-1 cells by MAP

265 When the THP-1 cells were incubated with 1×10^7 MAP organisms for 8 h $87.5 \pm 6.3\%$ (strain
266 ATCC 19698), $91 \pm 5\%$ (strain JIII-86) and $91 \pm 10.6\%$ (strain J 1961) of macrophages
267 contained one or more organisms (Fig. 3). Furthermore, approximately 80% of the cells were
268 infected already after 4 h. Based on these results, a challenge dose of approximately 3×10^7
269 cfu/ml, further diluted 1:2 and 1:4 and an infection time of 8 h for the determination of IL-1 β at
270 24 h and IL-10 at all time points was chosen. The determination of TNF- α was carried out at
271 an infection time of 2 and 4 h (Tab.1).

272

273 3.4. Cytokine release

274 3.4.1. TNF- α

275 All three MAP strains investigated induced significantly higher amounts of mRNA and protein
276 in comparison to cell controls (THP-1 cells activated by PMA and cell culture medium only)
277 (Fig. 4a).

278 The amount of TNF- α mRNA induced by the three strains differed in its extent of dependency
279 from infection time and challenge dose. The mRNA level induced by the Type II strains
280 (ATCC 19698 and J1961) increased significantly with rising challenge dose at both time
281 points (2 and 4 h), whilst the mRNA level induced by JIII-86 did not change significantly.
282 Strain ATCC 19698 showed a down-regulation of the mRNA expression from 2 h to 4 h
283 incubation. The field strains J1961 and JIII-86 did not differ in their time dependency referring
284 to mRNA expression (Fig. 4a).

285 The laboratory-adapted strain ATCC 19698 differed from both field strains concerning TNF- α
286 protein secretion. It was significantly up-regulated from 2 to 4 h after stimulation with strain
287 ATCC 19698 for all bacterial concentrations, but down-regulated at 4 h after challenge with
288 strain JIII-86 at 1.5×10^7 and 3×10^7 cfu/ml (Fig. 4b). At 4 h strain ATCC 19698 induced
289 significantly more TNF- α protein using 1.5×10^7 and 3×10^7 cfu than both field strains and the
290 TNF- α response was significantly dependent upon bacterial load.

291 Incubation times of 1 h and 8 h were also tested, but TNF- α could not be determined at 1 h
292 post infection (PI) and the protein amounts decreased strongly at 8 h PI (data not shown).

293

294 3.4.2. Interleukin-1 β

295 At 4 h MAP infected macrophages showed significantly higher expression of IL-1 β mRNA
296 and production of IL-1 β protein compared to the cell controls, whereas at 24 h the effect was
297 minimal (Fig. 5).

298 Marked differences in IL-1 β mRNA expression were determined between the three strains at
299 4 h PI (Fig. 5a). The strain J1961 induced significantly more IL-1 β mRNA at time point 4 h
300 than strain ATCC 19698 and JIII-86. Significant differences were also detected in protein
301 secretion among the strains. Strain ATCC 19698 induced the highest amount of protein in
302 comparison to the field strains. Strain ATCC 19698 and J1961 showed significant differences
303 in protein secretion between the lowest and the highest challenge dose whereas Type III
304 strain JIII-86 triggered the lowest protein secretion throughout.

305

306 3.4.3. Interleukin-10

307 MAP infected macrophages harboured significantly more IL-10 mRNA and protein than the
308 cell controls (Fig. 6).

309 IL-10 mRNA expression was measured after 24, 48 and 72 h without significant differences
310 among the time points for all three strains, but the mRNA levels were influenced by the
311 challenge dose (Fig. 6a). At the highest challenge dose the intermediate strain JIII-86 at all
312 time points induced IL-10 mRNA to a lesser extent than the bovine strains.

313 IL-10 protein secretion rose steadily until 72 h (Fig. 6b) for all strains. The amounts of the
314 secreted protein were significantly different at a challenge dose of 3×10^7 between 24, 48
315 and 72 h and were significantly dependent on the challenge dose at 48 h and 72 h. These
316 results proved to be valid for all three strains.

317 Using the highest challenge dose at 48 h the IL-10 protein level induced by strain ATCC
318 19698 was significantly higher than those induced by the field strains. However, at 72 h PI

319 both bovine strains induced a significantly higher IL-10 protein secretion than the
320 intermediate strain.

321

322 4. Discussion

323 Paratuberculosis in cattle caused by MAP is a widely distributed, debilitating chronic
324 granulomatous enteritis of domestic ruminants such as cattle, sheep and goats as well as
325 wildlife (Sohal et al., 2010). MAP is also widespread within the environment in most
326 developed countries, and this may include exposure of man through the food chain (Eltholth
327 et al. 2009).

328 A key step in the pathogenesis of mycobacterial infections is phagocytosis of the bacteria by
329 macrophages. Inhibition of phagosomal maturation into functional phagolysosomes caused
330 by numerous mechanisms allows mycobacteria to survive inside of macrophages and
331 thereby to evade the defence mechanisms of the host (Sohal et al., 2008). The hypothesis is
332 discussed that these processes are dependent on the genotype of strains (Motiwala et al.,
333 2006). Gollnick et al. (2007) observed that bovine strains of MAP (SSR genotyped), isolated
334 from a faecal sample of a cow, are more successful for survival in bovine MDMs than ovine
335 strains (SSR genotyped), isolated from an intestinal tissue sample of a sheep.

336 On this reason we determined the effect of genetically different MAP strains, bovine field
337 strain J1961 (Type II) and sheep strain JIII-86 (Type III) in comparison to the reference strain
338 ATCC 19698 (Type II) on macrophages.

339 Macrophage-like cell lines of human origin have been shown to be a suitable model for
340 studying the interaction of macrophages with mycobacteria (Theus et al., 2004; Motiwala et
341 al., 2006). Preliminary experiments using the bovine macrophage cell line BoMac obtained
342 from the collection of cell lines in veterinary medicine (CCLV, Insel Riems, Germany) showed
343 that this cell line was not able to produce any cytokine response (data not shown),
344 corroborating results of Janagama et al. (2006).

345 The three MAP strains included differed in their genotypes as well as in their protein patterns
346 produced by SDS-PAGE. The combination of bead beating and usage of Tween 20 as

347 solubilisation buffer was a suitable method for protein analysis, confirming data in the
348 literature (Gumper et al., 2007). This method revealed unique protein patterns for each strain
349 indicating differences in phenotype or metabolism which could be pathogenetically important.
350 In order to standardize the mycobacteria-to-macrophage ratios for all experiments we
351 determined the relation between optical density of the bacterial suspension and colony
352 forming units. The regression lines of the three strains differed in their course and slope (Fig.
353 2), possibly due to variation in clumping from strain to strain and from batch to batch.
354 Therefore we strongly recommend preparing the inocula for *in vitro* challenge studies based
355 on colony forming unit counts rather than on the optical density.

356 Intracellular survival of the three MAP strains in THP-1 cells was compared according to the
357 method described by Weiss et al. (2002). Uptake of the MAP strains increased in a time
358 dependent manner from 2 h to 24 h. At 8 h PI more than 85% of the cells were infected by
359 MAP. Longer incubation times led to a higher number of dead and lysed THP-1-cells. Hence,
360 an incubation time of 8 h was chosen. The three strains were internalized with similar
361 efficiency providing the basis for a comparison of the cytokine results without the need to
362 normalize the data to the infection rate of the strains in the current study. Nevertheless, when
363 using the model developed herein to assess the virulence of other strains, investigators will
364 have to confirm that all strains considered display a comparable infection efficacy.

365 Macrophages have a central role in mycobacterial killing, antigen processing and
366 presentation and directing inflammatory and immune processes. Cytokines produced by
367 macrophages such as TNF- α , IL-1 β , IL-6 and IL-10 influence these processes. The pro-
368 inflammatory cytokines TNF- α , and IL-1 have important T-cell stimulatory properties (Sohal
369 et al., 2008). TNF- α is produced by activated macrophages and is associated with
370 granulomatous inflammation and cachexia. It is an activator of both inflammatory and
371 immune responses and is implicated in the initiation of a protective immune response to
372 mycobacterial infections (Sano et al., 1999; Beltan et al., 2000; Weiss et al., 2002). TNF- α
373 also plays an important role in the inhibition of mycobacterial growth *in vitro* (Basler et al.
374 2010). IL-1 is a major macrophage product with a fundamental role in immunoregulation and

375 inflammation (Dinarello, 1994). Two distinct genes encode two forms of IL-1, IL-1 α and β . IL-
376 1 α remains cell-associated, whilst the bioactive IL-1 β is released through a secretory
377 pathway that avoids translocation into the endoplasmatic reticulum (Rubartelli 1993). IL-1 β is
378 converted into its active form by the interleukin-converting-enzyme (ICE) which is also known
379 as capase-1. Studies have shown IL-1 β to play a protective role for the host during
380 mycobacterial infections but drastic over-expression of IL-1 by MAP-infected intestinal
381 macrophages could result in same late effects of paratuberculosis to the host. (Roach and
382 Schorey, 2002, Kabara et al. 2010).

383 IL-10 is a major anti-inflammatory cytokine that suppresses the activation of macrophages,
384 cytokine secretion and microbicidal activity (Souza et al., 2008). It is described as a mediator
385 of MAP survival within bovine mononuclear phagocytes. This cytokine enhances the Th2-
386 type and suppresses the Th1-type immune responses (Weiss and Souza, 2008). At late
387 stages of paratuberculosis the production of IL-10 is associated with increased intracellular
388 replication of mycobacteria (Rossi et al., 2009). Studies have documented that the TNF- α /
389 IL-10 balance is a major determinant of the macrophage activation status (Weiss et al.,
390 2002). Since the cytokines TNF- α , IL-1 β and IL-10 exhibit an important role in
391 paratuberculosis, we have focused our studies on the determination of these cytokines after
392 interaction between MAP and macrophages.

393 Extent and time course of the cytokine response of the THP-1 cells was dependent on the
394 MAP strain and the challenge dose. For all three strains induced mRNA levels as well as
395 amounts of proteins secreted strongly depended on the challenge dose (Fig 4-6).

396 For the determination of TNF- α incubation times of 2 and 4 h were chosen because TNF- α is
397 identified as one of the early mediators involved in cellular cross talk (Haller et al. 2000).

398 Determinations of this cytokine at 1 h and 8 h were also performed, but TNF- α could not be
399 determined at 1 h post infection (PI) and the protein amounts decreased strongly at 8 h. The
400 highest amount of TNF- α protein was determined at 2 h when cells were stimulated with the
401 intermediate strain JIII-86. Afterwards the amount decreased. Hence, the strain JIII-86
402 caused a faster reaction of the THP-1 cells than both bovine strains which could be a hint

403 that the sheep strain is less virulent than both bovine strains. Our results concerning the
404 kinetic of TNF- α secretion are in agreement with those published by Adams et al. (1995) and
405 Reiling et al. (2001). Basler et al. (2010) could show that reduced TNF- α transcript stability is
406 responsible for lower TNF- α expression in MAP-infected macrophages and that this correlate
407 with lower p38MAPK activation. It remains to investigate if different p38MAPK activation is
408 also one of the causes for the differences between the three strains.

409 The three MAP strains induced a fast IL-1 β response on the mRNA as well as on the
410 secreted protein level. The response was high at 4 h and decreased rapidly at 24 h. The
411 reason for that could be the synthesis of a transcriptional repressor as well as a short mRNA
412 half-life (Dinarelo, 1994). Our results correspond to those reported by Fenton et al. (1988).

413 They could show that IL-1 β mRNA accumulates for 4 h followed by a rapid decrease.

414 Motiwala et al. (2006) described that cattle strains entered the human THP-1 cells without
415 triggering any major pro-inflammatory cascades. In contrast, in our study both Type II strains
416 from cattle and the Type III strain from sheep up-regulated the IL-1 β genes in a dose-
417 dependent manner. Roach and Schorey (2002) showed that non-pathogenic mycobacteria
418 are capable of inducing a much faster IL-1 β response in murine macrophages than
419 pathogenic mycobacteria. Further investigations are necessary to clarify the kinetics of IL-1 β
420 expression induced by other MAP genotypes and by mycobacteria with accepted
421 pathogenicity for man.

422 IL-10 mRNA was expressed from 24 to 72 h without significant differences among the time
423 points, but the amounts of secreted protein increased steadily. This is confirmed by findings
424 of Janagama et al. (2006). They described that bovine monocyte-derived macrophages
425 (MDM) stimulated with different MAP isolates gradually up regulated IL-10 secretion from 2
426 to 96 h. In their study, the time course of mRNA induction by a sheep strain was similar to
427 that of the sheep strain JIII-86 (Type III) in our study. At 72 h the Type II strains from cattle
428 induced significantly more IL-10 protein than strain JIII-86. Since IL-10 has a short half-life, it
429 has to be secreted continuously (Tanaka et al. 2008). The key role of IL-10 in intracellular

430 survival of MAP is supported by a study of Weiss et al. (2005) in which treatment of MDM
431 with anti-IL-10 antibodies enabled the macrophages to kill MAP organisms.

432 Non-pathogenic species of mycobacteria induce higher levels of pro-inflammatory cytokines
433 such as TNF- α , GC-CSF and IL-6 (Beltan et al. 2000). This is considered one of the
434 mechanisms leading to fast elimination of non-pathogenic mycobacterial species, whereas
435 pathogenic species persist since the levels of these cytokines are lower. Basler et al. (2010)
436 showed that pathogenic mycobacteria induce lower TNF- α expression in comparison with
437 non-pathogenic mycobacteria caused by insufficient activation of p38 MAPK. In our study,
438 the TNF- α response after stimulation of THP-1 cells with strain JIII-86 was faster than after
439 stimulation with bovine strains, but at 4h, laboratory-adapted strain ATCC 19698 produced
440 the highest amount of TNF- α , which could be a hint that the reference strain is less virulent
441 than both field strains. Additionally, the effect of the strain JIII-86 (Type III) on THP-1 cells
442 was less pronounced than that of both Type II strains in conformity with results of Janagama
443 et al. (2006). They have shown genetic differences between MAP strains isolated from cattle
444 when compared to those from sheep. Sheep strains tend to be less virulent than cattle
445 strains (Kabara 2010).

446 In conclusion, the *in vitro* model allowed the reliable quantitation of macrophage responses
447 that depend on MAP-genotype, challenge dose, and infection time. Each of the strains
448 showed characteristic patterns of pro- and anti-inflammatory cytokine induction. The model
449 will help to increase our understanding as to whether the genotype of the infecting strain
450 plays a role in the modulation of the cellular response and, eventually, for the pathogenesis
451 of paratuberculosis.

452

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458

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589

590

591 7. Legends of Figures

592

593 Figure 1: SDS-PAGE analysis of protein samples extracted from different MAP strains. Lane
594 1-Standard; Lane 2-strain J1961; Lane 3-strain JIII-86; Lane 4-strain ATCC 19698.

595

596 Figure 2: Correlation between OD of the broth culture from MAP strains measured by 580 nm
597 and colony forming units assessed by the standard colony-counting technique.

598

599 Figure 3: Ingestion of MAP strains by THP-1 cells depending on the challenge time.

600 Percentages of infected cells were determined by three independent experiments per strain

601

602 Figure 4: (a) Induction of TNF- α mRNA in THP-1 cells by MAP strains after 2 h and 4 h. The

603 TNF- α mRNA production was determined by real-time RT-PCR. The mRNA expression as

604 shown in the histogram was calculated as fold change relative to non-infected cells

605 (control) and normalized against GAPDH (house keeping gene) expression.

606 (b) Secretion of TNF- α protein following 2 h and 4 h incubation of THP-1 cells with MAP

607 strains. Protein amounts were determined using a bioassay with the porcine kidney cell line

608 PK cell clone15.

609 The results of four independent tests are presented as mean values \pm standard deviation

610 (SD).

611

612 Figure 5: (a) Induction of IL-1 β mRNA in THP-1 cells by MAP strains at time point 4 h and 24

613 h. The IL-1 β mRNA production was determined by real-time RT-PCR. The mRNA expression

614 as shown in the histogram was calculated as fold change relative to non-infected cells

615 (control) and normalized against GAPDH (house keeping gene) expression.

616 (b) Secretion of IL-1 β protein following 4 h and 24 h (8 h incubation of the cells with bacteria

617 + 16 h incubation with fresh cell culture medium) incubation of THP-1 cells with MAP strains.

618 Protein amounts were determined using a commercially available ELISA kit following the

619 manufacturer's instructions.

620 The results of five independent tests are presented as mean values \pm standard deviation
621 (SD).

622 Figure 6: (a) Induction of IL-10 mRNA in THP-1 cells by MAP strains at 24 h, 48 h and 72 h
623 incubation time. The TNF- α mRNA expression was determined by real-time RT-PCR. The
624 mRNA expression as shown in the histogram was calculated as fold change relative to
625 non-infected cells (control) and normalized against GAPDH (house keeping gene)
626 expression. (b) Secretion of IL-10 protein after 24 h, 48 h and 72 h total incubation time of
627 THP-1 cells with MAP strains. Protein amounts were determined using a commercially
628 available ELISA kit following the manufacturer's instructions.

629 The results of four independent tests are presented as mean values \pm standard deviation
630 (SD).

631
632 Figure 1 (supplemented): IS900 RFLP profiles of *M. avium* subsp. *paratuberculosis* isolates,
633 after digestion with *BstE*I. The numbers above the lanes are the IS900 RFLP type
634 designations, description see Table 3. Lane M, molecular weight (Mw) marker. The numbers
635 below the lanes show the strain designations: (J1961) field isolate from cattle of Type II in
636 comparison to (JIII-86) field isolate from sheep of Type III and reference strain ATCC 19698
637 of Type II.

638

639

640 8. Legends of Tables

641

642 Table 1: Standardized conditions for the performance of the cytokine release

643

644 Table 2: Primer sequences for real-time RT-PCR

645

646 Table 3: IS900-RFLP-, MIRU-VNTR-, and SSR-profiles of MAP strains included in this study

1 **Table 1**

2

Cytokine	Infection time [h]	Incubation with fresh medium [h]	Total incubation time [h]
TNF- α	2		2
	4		4
IL-1 β	4		4
	8	16	24
IL-10	8	16	24
	8	40	48
	8	64	72

3

4

1 **Table 2**

2

Target	Sequence (5'-3')	Length (bp)	Accession no.	Reference
IL-1 β	Forward: TGA TGG CTT ATT ACA GTG GCA ATG	87 bp	NM000576	Naldini et al. 2006
	Reverse: GTA GTG GTG GTG GGA GAT TCG			
IL-10	Forward: GTG ATG CCC CAA GCT GAG A	138 bp	AF043333	Giulietti et al., 2001
	Reverse: CAC GGC CTT GCT GTT GTT TT			
TNF- α	Forward: ATC TCT CGA ACC CCG AGT GA	149	M10988	
	Reverse: CCT CTG ATG GCA CCA			
GAPDH	Forward: TGG GTG TGA ACC ATG AGA AG	76	BC029340	Brink et al., 2000
	Reverse : GCT AAG CAG TTG GTG GTG C			

3

4

1 **Table 3**

2

	IS900-RFLP		MIRU	VNTR	MLSSR
	<i>Bst</i> EII ^a	<i>Pst</i> I ^a	loci 1, 2, 3, 4	loci 292-25-47-3-7-32	loci 1(g), 2(g), 8(ggt), 9(tgc)
J1961	C17	9	3-9-5-1	4-3-3-2-1 -8	7 - 9 - 4 - 4
ATCC19698	C5	7	3-7-5-1	3-3-3-2-2 -8	7 -10 - 5 - 5
JIII-86	I6	13	1-9-5-1	4-1-3-1-(1*)-8 ^b	7 -12 - 3 - 4

3

4 ^a IS900-RFLP-*Bst*EII and *Pst*I designations as described by Möbius et al.(2008; 2009)5 ^b (1*), some additional nucleotides were identified at this tandem repeat

6

Figure 1

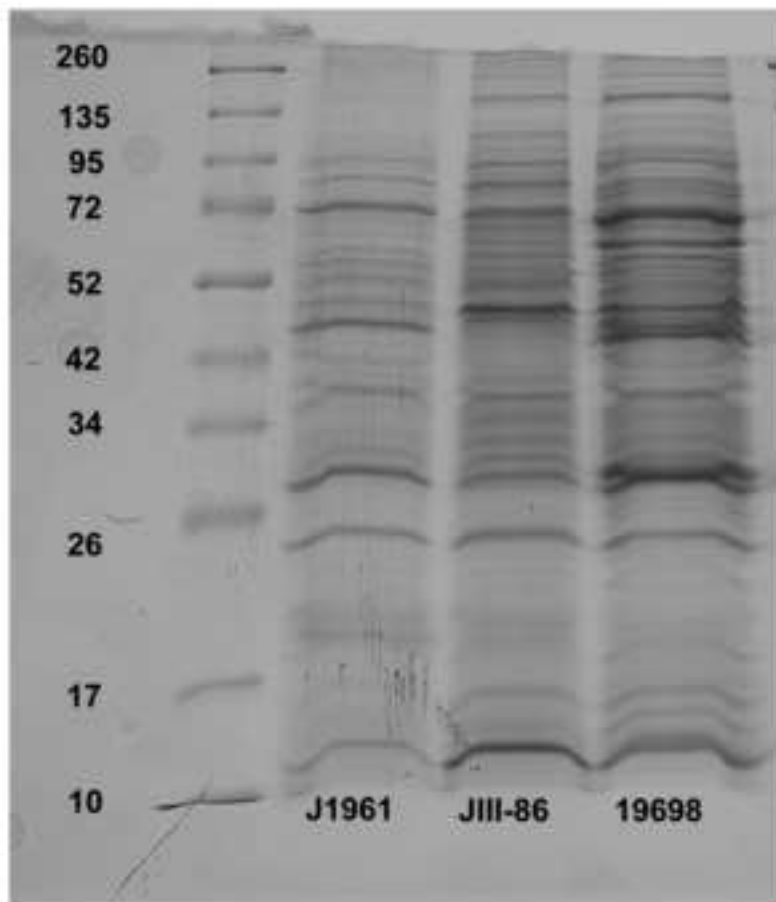


Figure 2

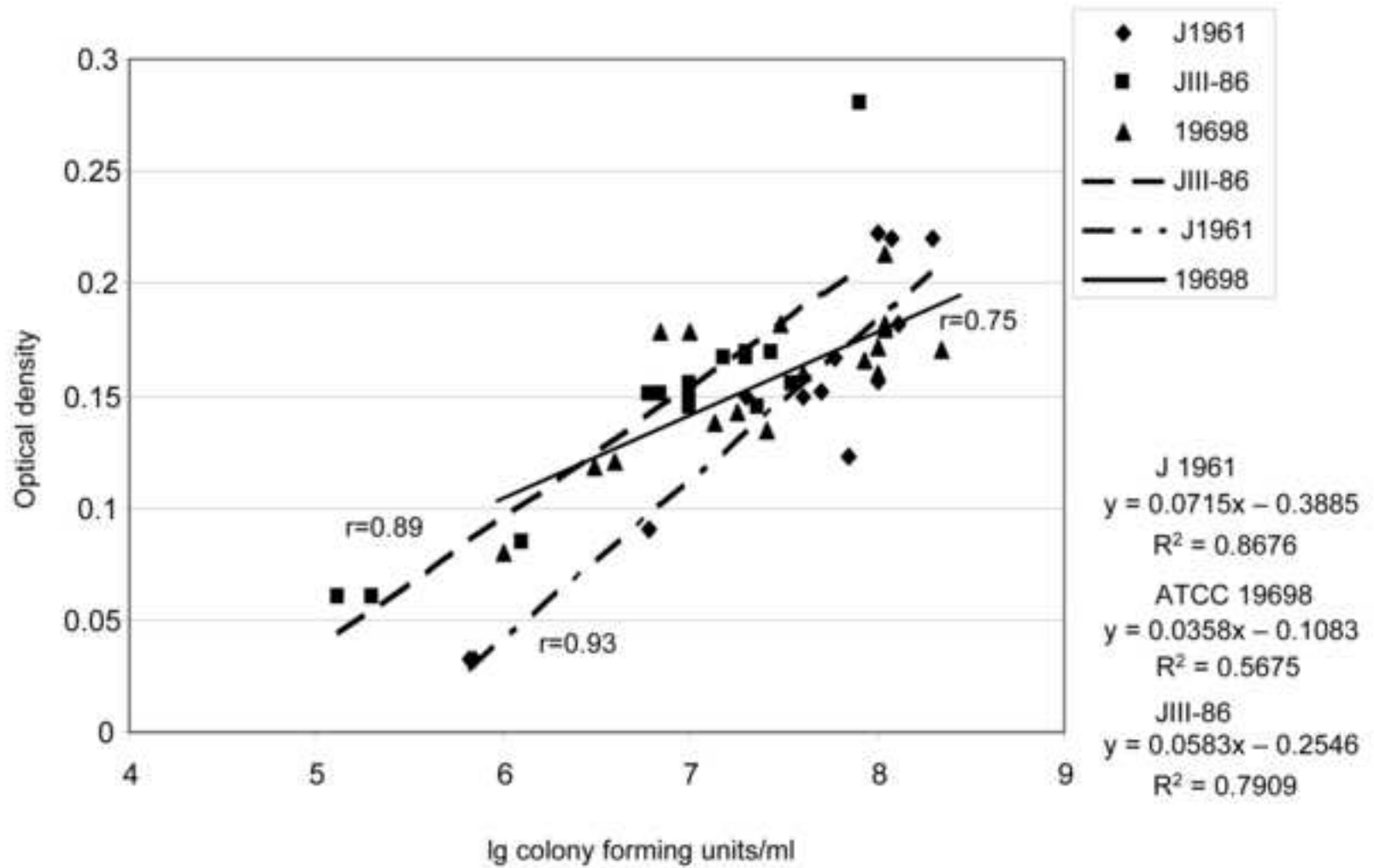


Figure 3

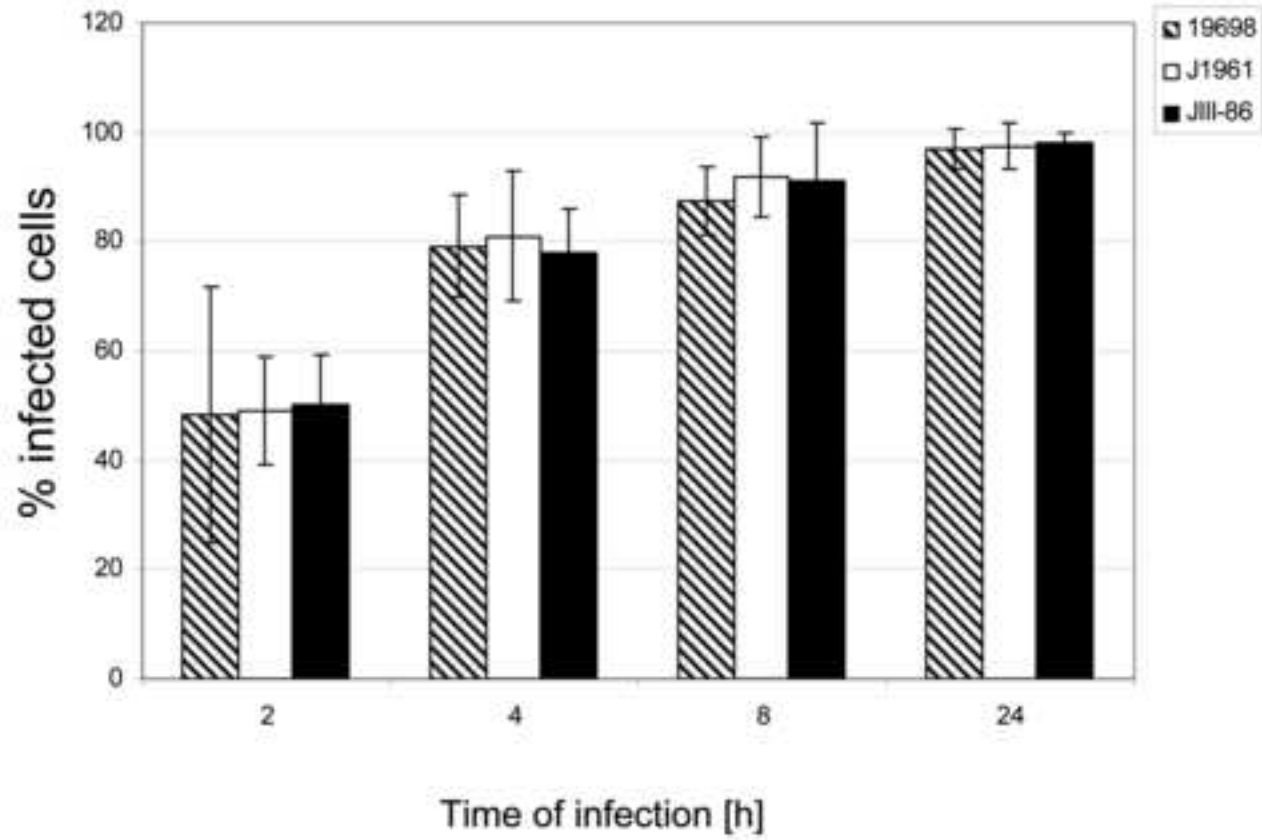


Figure 4a

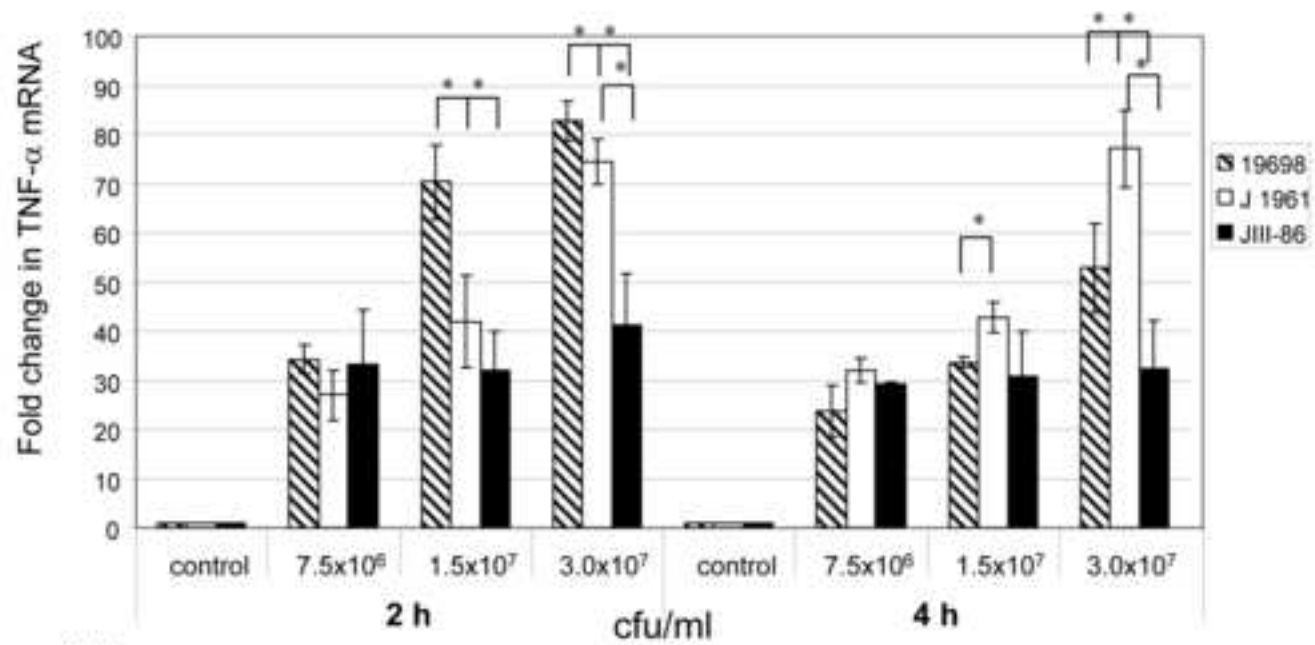
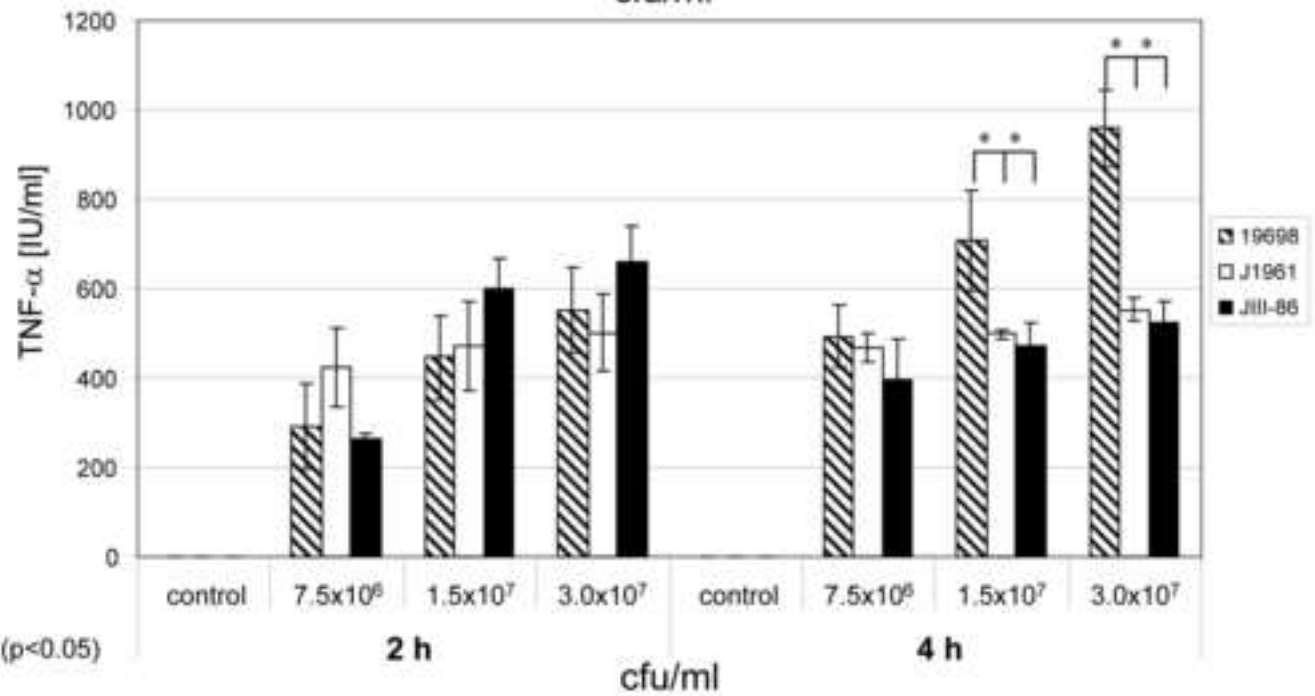


Figure 4b



* significant difference (p < 0.05)

Figure 5a

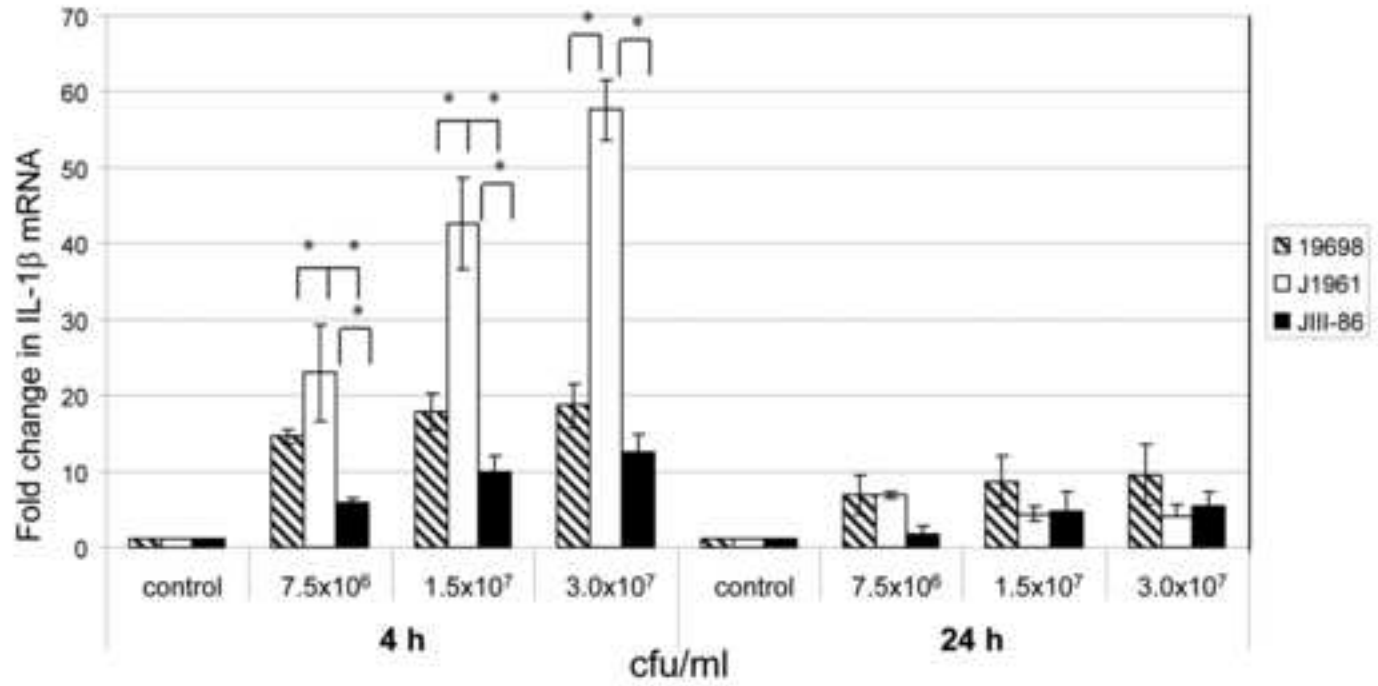
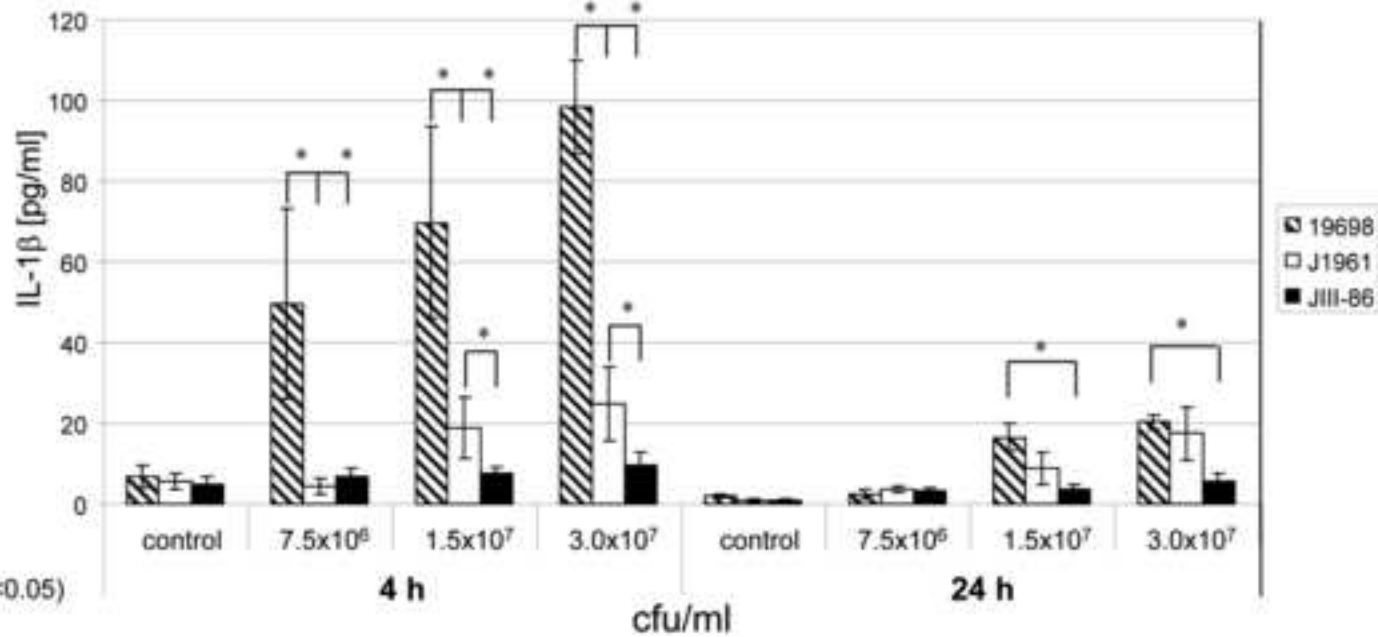


Figure 5b



*significant difference (p < 0.05)

Figure 6a

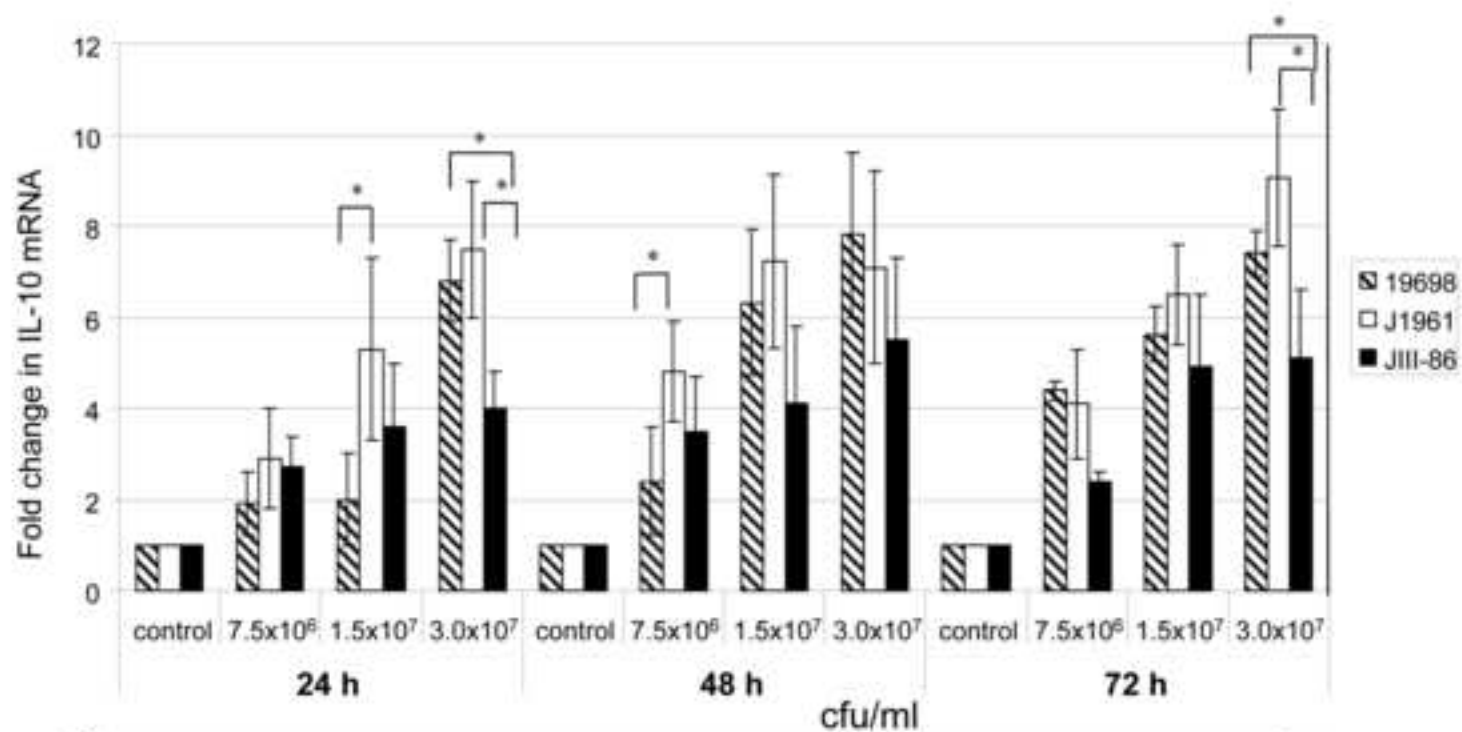
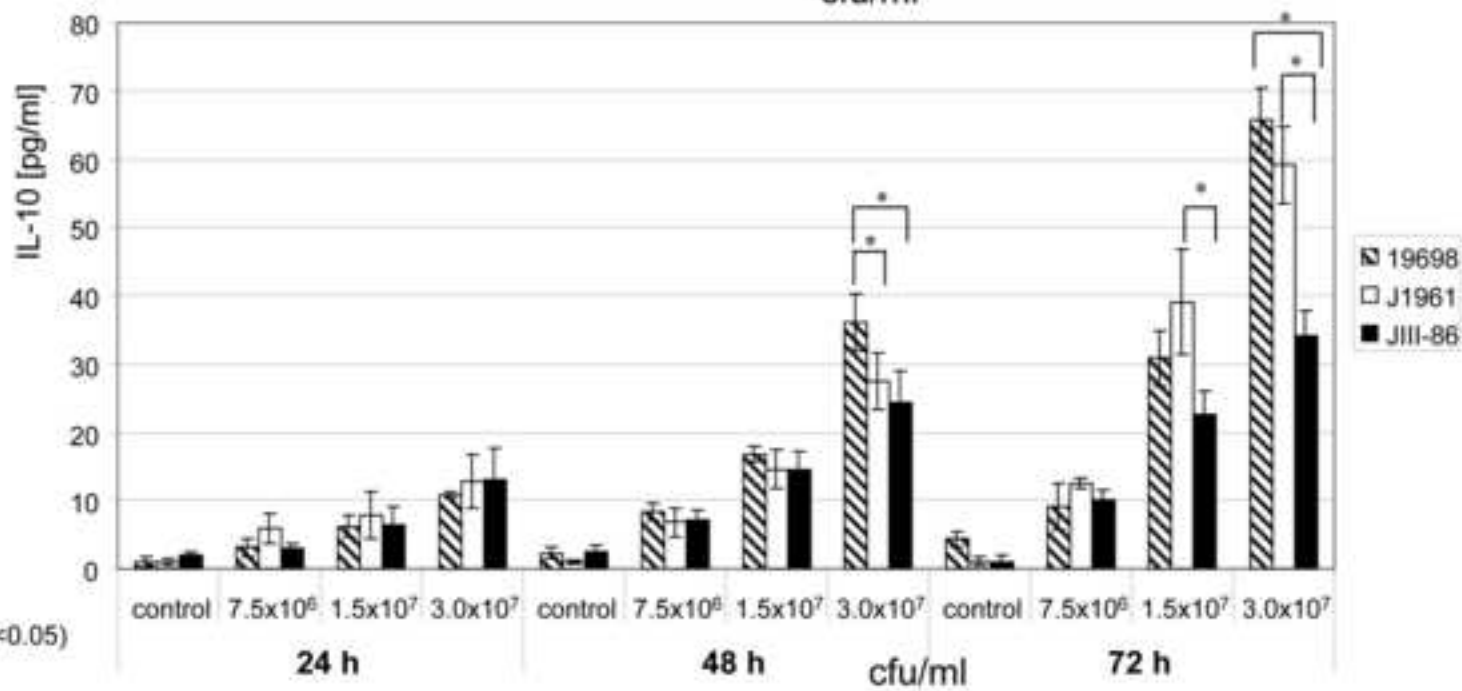


Figure 6b



*significant difference (p < 0.05)