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► **To cite this version:**

Erika Borrmann, Petra Möbius, Roland Diller, Heike Köhler. Divergent cytokine responses of macrophages to subsp. strains of Type II and III in a standardized model. *Veterinary Microbiology*, 2011, 152 (1-2), pp.101. 10.1016/j.vetmic.2011.04.002 . hal-00719075

**HAL Id: hal-00719075**

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Submitted on 19 Jul 2012

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## Accepted Manuscript

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

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



PII: S0378-1135(11)00213-6  
DOI: doi:10.1016/j.vetmic.2011.04.002  
Reference: VETMIC 5262

To appear in: *VETMIC*

Received date: 23-11-2010  
Revised date: 25-3-2011  
Accepted date: 5-4-2011

Please cite this article as: Borrmann, E., Möbius, P., Diller, R., Köhler, H., Divergent cytokine responses of macrophages to *Mycobacterium avium* subsp. *paratuberculosis* strains of Type II and III in a standardized *in vitro* model, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.04.002

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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](mailto: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- $\alpha$ , IL-1 $\beta$ , and IL-10 by quantitative RT-PCR.  
26 Secreted TNF- $\alpha$  protein was measured by a cytotoxicity test, IL-1 $\beta$  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- $\alpha$ , IL-1 $\beta$  and  
31 anti-inflammatory cytokine IL-10. Type III strain produced less IL-10 and IL-1 $\beta$  mRNA and  
32 protein but more TNF- $\alpha$  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- $\alpha$  and IL-1) as well as  
45 anti-inflammatory cytokines (IL-10 and TGF- $\beta$ ) (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 *Bst*EII and *Pst*I, 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  $3 \times 10^7$  cfu/ml were taken for inoculation. These  
110 suspensions were further diluted 1:2 and 1:4 corresponding to  $1.5 \times 10^7$  and  $7.5 \times 10^6$  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% CO<sub>2</sub>, and sub-cultivated 2 times per week  
139 at an approximate density of 2 x 10<sup>5</sup> 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 10<sup>5</sup>  
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 (7x10<sup>6</sup>; 1x10<sup>7</sup>, 2.6x10<sup>7</sup> and 1x10<sup>8</sup> 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 10<sup>7</sup> 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 $\beta$  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  $\mu$ g/ml to 100  $\mu$ 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  $\mu$ l final volume with  
199 Omniscript<sup>R</sup> reverse transcriptase (Qiagen, Germany) in the presence of RNase inhibitor  
200 (Promega, Germany) using random hexamer primer pd(N)<sub>6</sub> (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<sup>®</sup> 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<sup>®</sup> 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- $\alpha$ bioassay

221 TNF- $\alpha$  protein in the cell supernatants was determined using the bioassay described by  
222 Bertoni et al. (1993). Briefly, the amount of TNF- $\alpha$  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- $\alpha$  in U/ml was calculated in comparison to a dilution series  
227 of recombinant porcine TNF- $\alpha$  (MorphoSys AG, Germany) by the reader software. Negative  
228 control samples consisted of medium without TNF- $\alpha$ .

229

### 230 2.8.2. ELISA

231 The levels of IL-1 $\beta$  and IL-10 protein secreted into supernatants were determined using the  
232 ELISA kits human IL-1 $\beta$  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  $1 \times 10^7$  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  $3 \times 10^7$   
269 cfu/ml, further diluted 1:2 and 1:4 and an infection time of 8 h for the determination of IL-1 $\beta$  at  
270 24 h and IL-10 at all time points was chosen. The determination of TNF- $\alpha$  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- $\alpha$ 

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- $\alpha$  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- $\alpha$   
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  $1.5 \times 10^7$  and  $3 \times 10^7$  cfu/ml (Fig. 4b). At 4 h strain ATCC 19698 induced  
289 significantly more TNF- $\alpha$  protein using  $1.5 \times 10^7$  and  $3 \times 10^7$  cfu than both field strains and the  
290 TNF- $\alpha$  response was significantly dependent upon bacterial load.

291 Incubation times of 1 h and 8 h were also tested, but TNF- $\alpha$  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 $\beta$

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

298 Marked differences in IL-1 $\beta$  mRNA expression were determined between the three strains at  
299 4 h PI (Fig. 5a). The strain J1961 induced significantly more IL-1 $\beta$  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  $3 \times 10^7$  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- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 influence these processes. The pro-  
368 inflammatory cytokines TNF- $\alpha$ , and IL-1 have important T-cell stimulatory properties (Sohal  
369 et al., 2008). TNF- $\alpha$  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- $\alpha$   
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 $\alpha$  and  $\beta$ . IL-  
376 1 $\alpha$  remains cell-associated, whilst the bioactive IL-1 $\beta$  is released through a secretory  
377 pathway that avoids translocation into the endoplasmatic reticulum (Rubartelli 1993). IL-1 $\beta$  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 $\beta$  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- $\alpha$  /  
389 IL-10 balance is a major determinant of the macrophage activation status (Weiss et al.,  
390 2002). Since the cytokines TNF- $\alpha$ , IL-1 $\beta$  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- $\alpha$  incubation times of 2 and 4 h were chosen because TNF- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  transcript stability is  
406 responsible for lower TNF- $\alpha$  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 $\beta$  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 $\beta$  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 $\beta$  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 $\beta$  response in murine macrophages than  
419 pathogenic mycobacteria. Further investigations are necessary to clarify the kinetics of IL-1 $\beta$   
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- $\alpha$ , 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- $\alpha$  expression in comparison with  
437 non-pathogenic mycobacteria caused by insufficient activation of p38 MAPK. In our study,  
438 the TNF- $\alpha$  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- $\alpha$ , 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

## 453 5. Acknowledgements

454 We thank C. Muselmann, B. Burkert, A. Hinsching, D. Michel and U. Möller for the excellent  
455 technical assistance. C. Menge is acknowledged for critically reading the manuscript.

456 Part of the work was founded by the German Ministry of Research and Education (BMBF),  
457 grant-no. 01KI0755.

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- $\alpha$  mRNA in THP-1 cells by MAP strains after 2 h and 4 h. The

603 TNF- $\alpha$  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- $\alpha$  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 $\beta$  mRNA in THP-1 cells by MAP strains at time point 4 h and 24

613 h. The IL-1 $\beta$  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 $\beta$  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- $\alpha$  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 *BstEII*. 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

<b>Cytokine</b>	<b>Infection time [h]</b>	<b>Incubation with fresh medium [h]</b>	<b>Total incubation time [h]</b>
TNF- $\alpha$	2		2
	4		4
IL-1 $\beta$	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 $\beta$	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- $\alpha$	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 <sup>a</sup>	<i>Pst</i> I <sup>a</sup>	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 <sup>b</sup>	7 -12 - 3 - 4

3

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

6

Figure 1

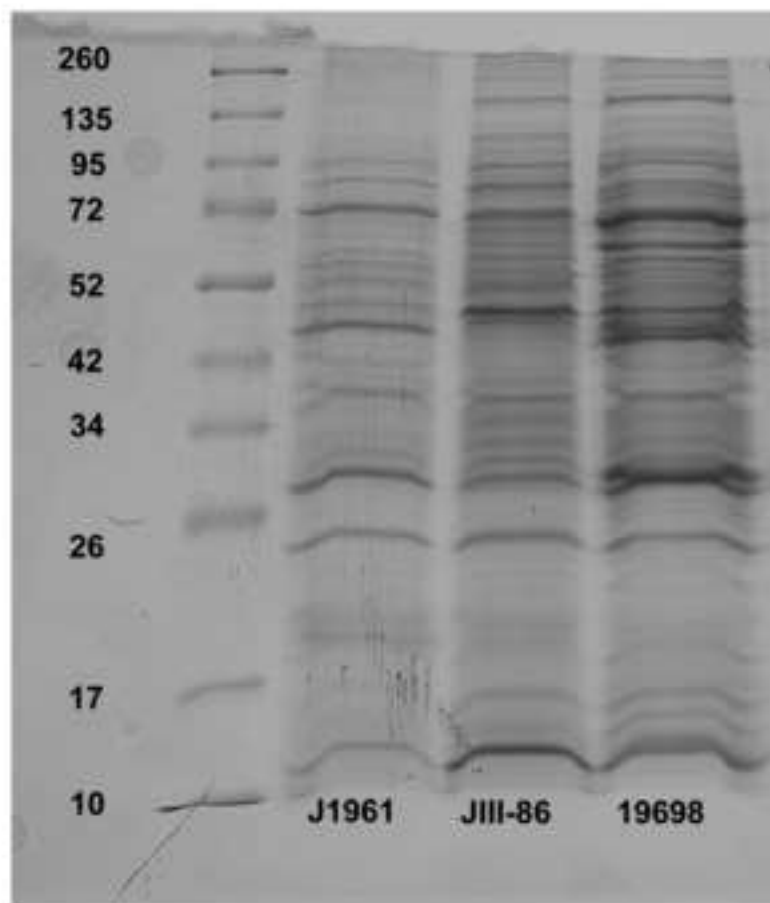


Figure 2

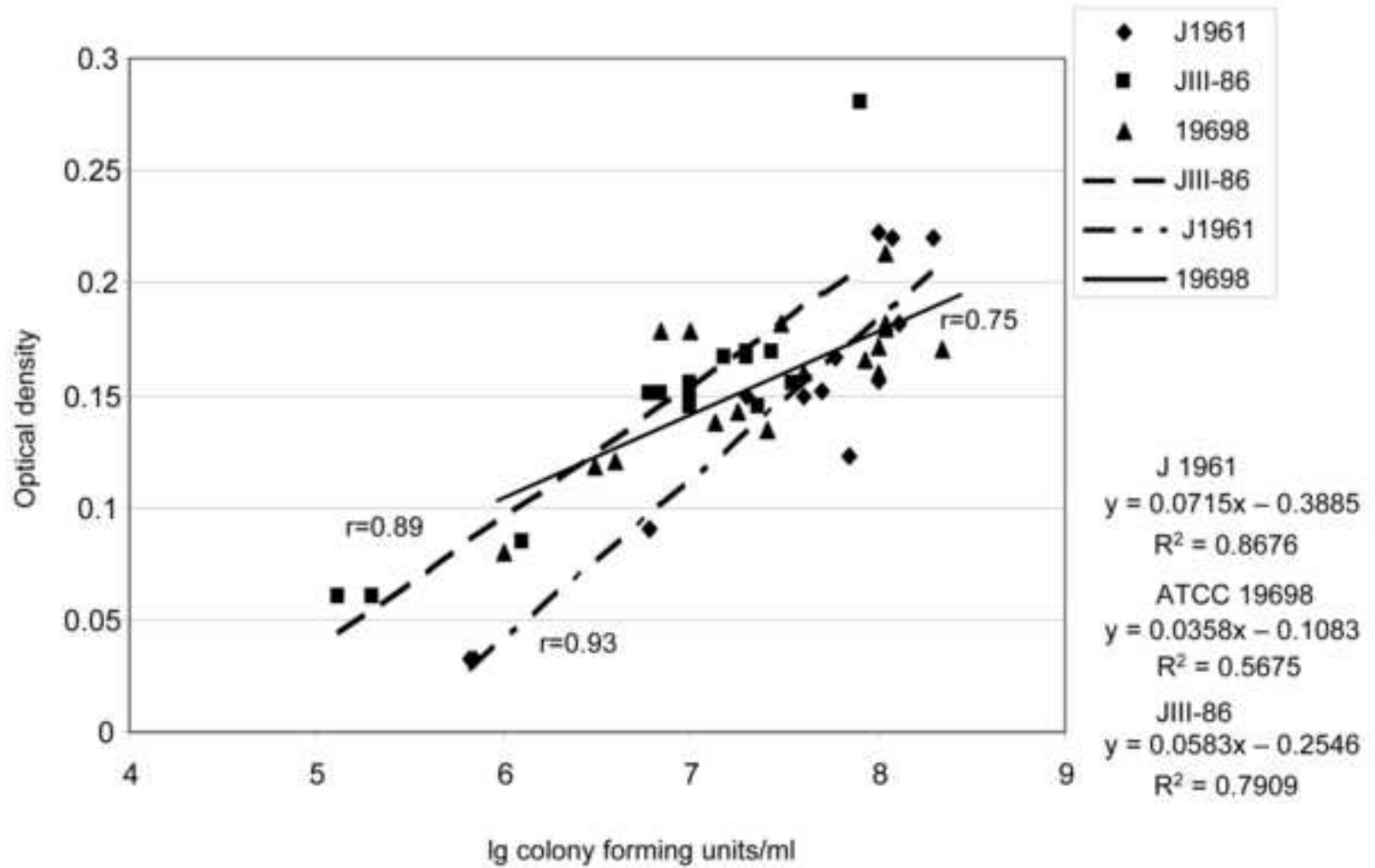


Figure 3

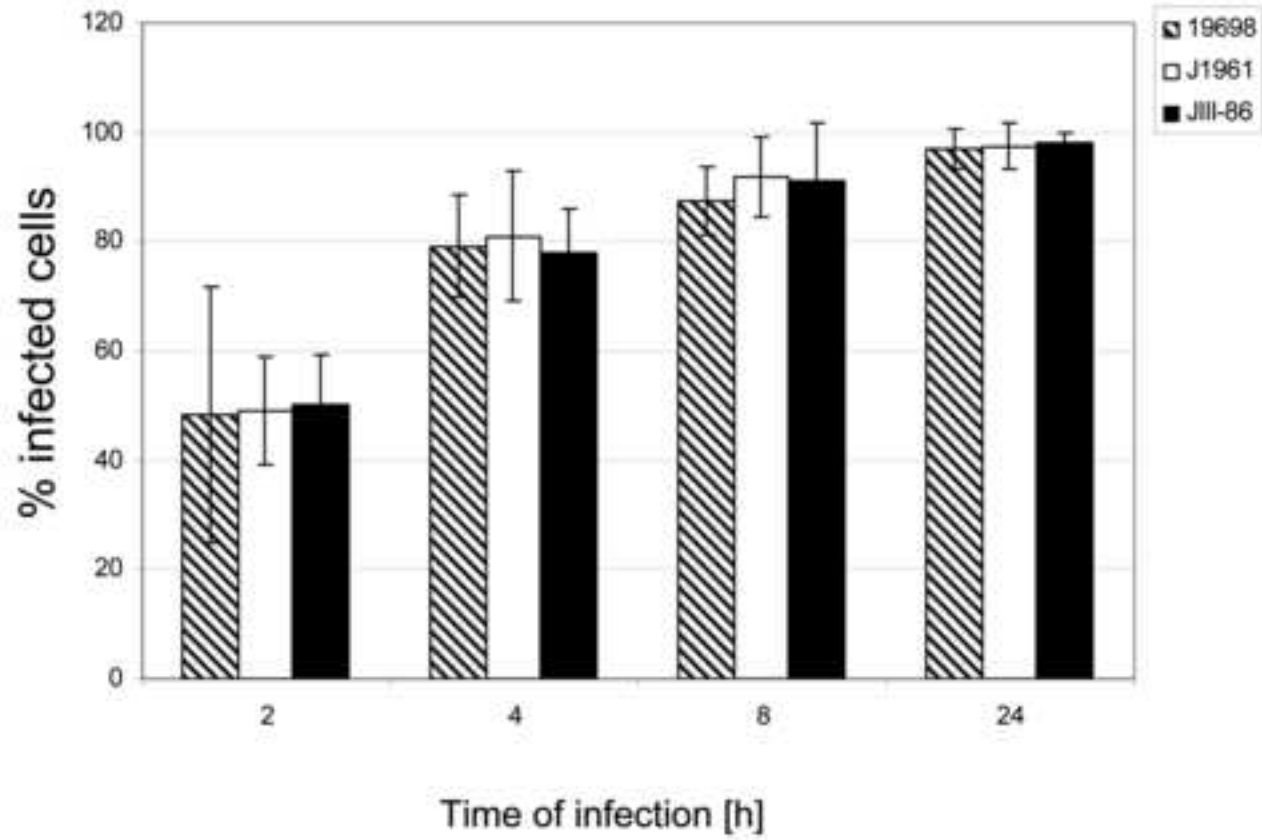




Figure 4a

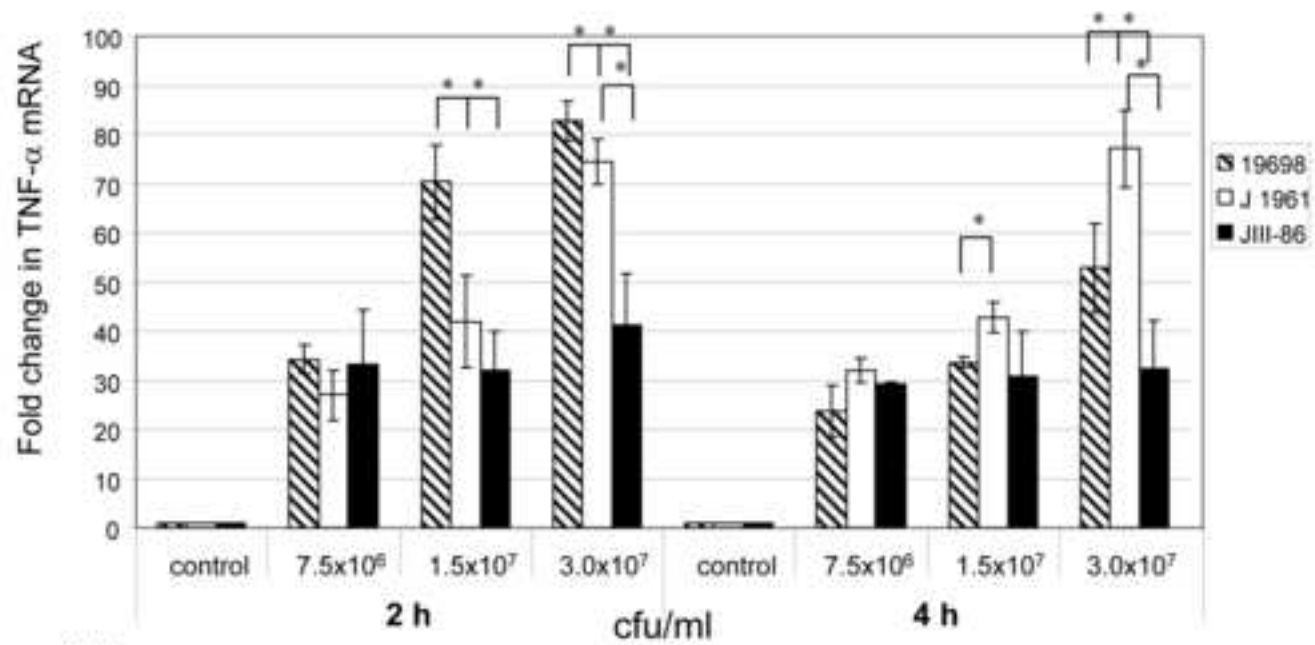
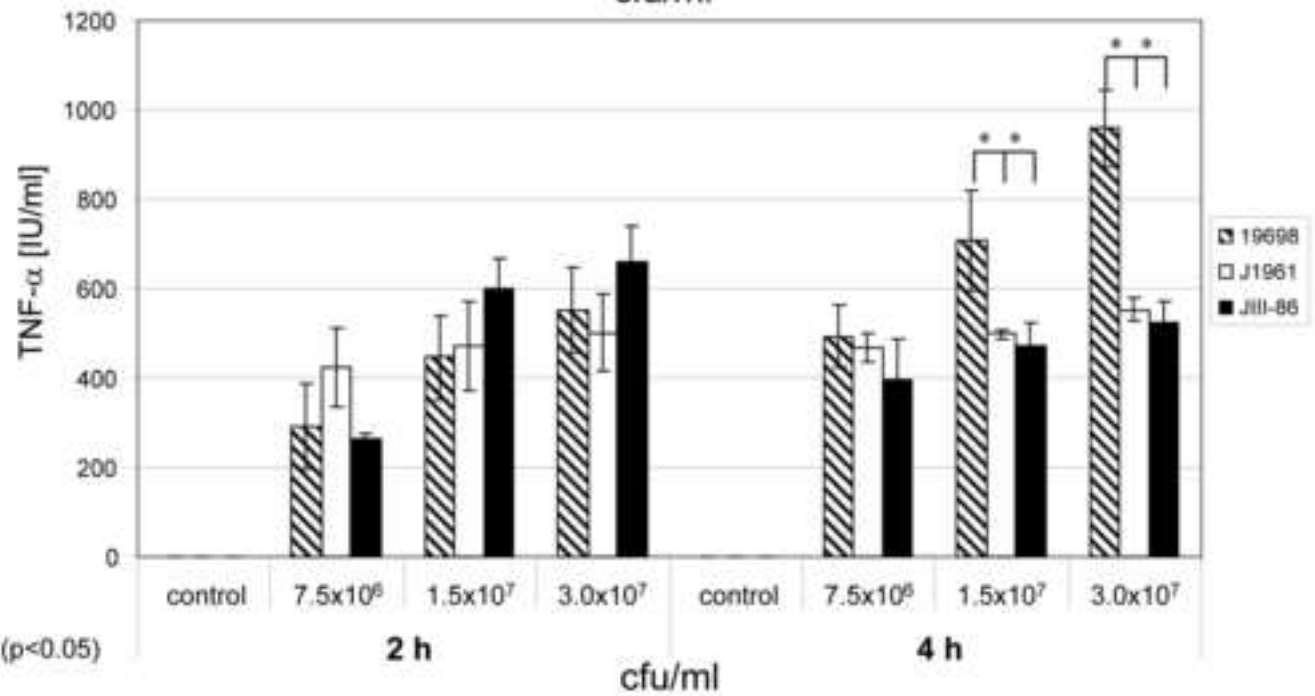


Figure 4b



\* significant difference (p<0.05)

Figure 5a

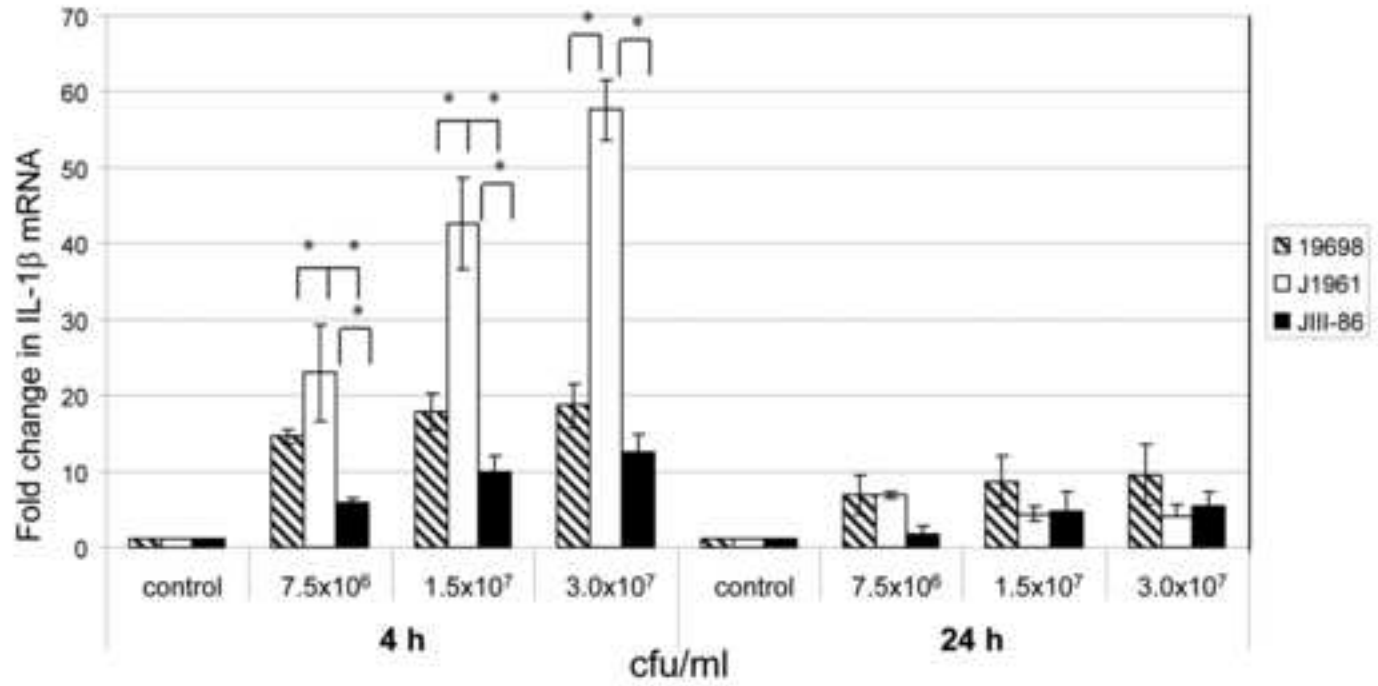
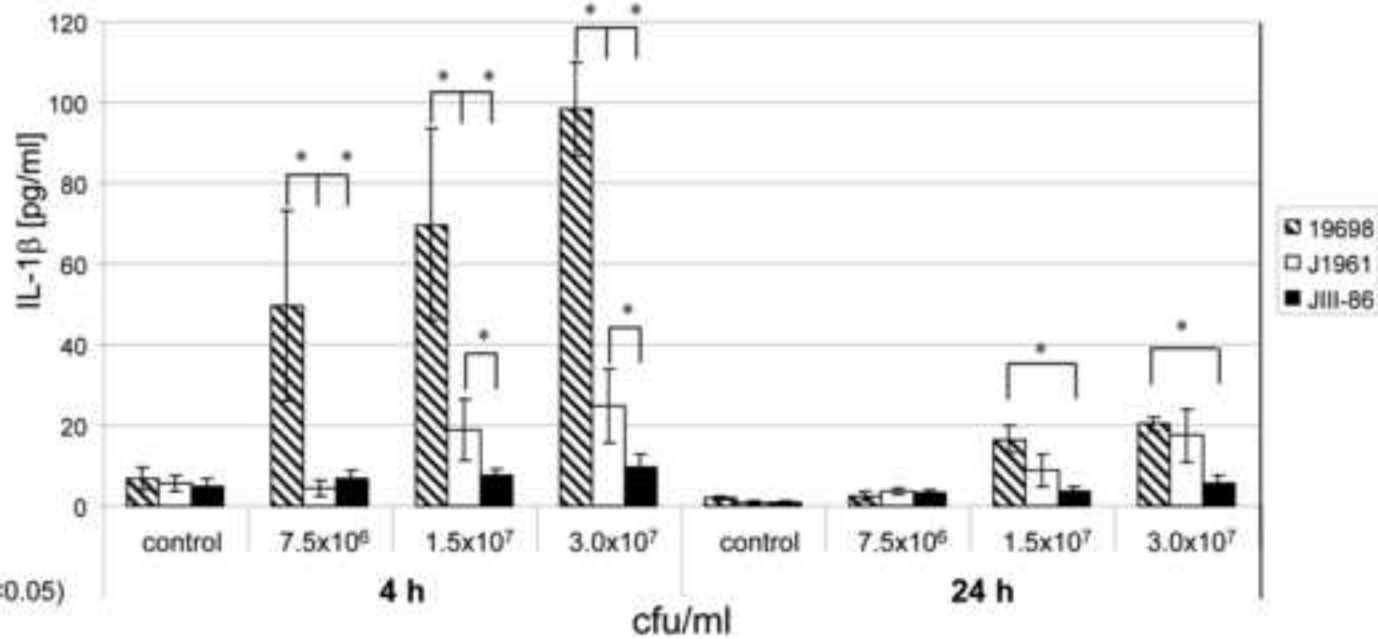


Figure 5b



\*significant difference (p < 0.05)

Figure 6a

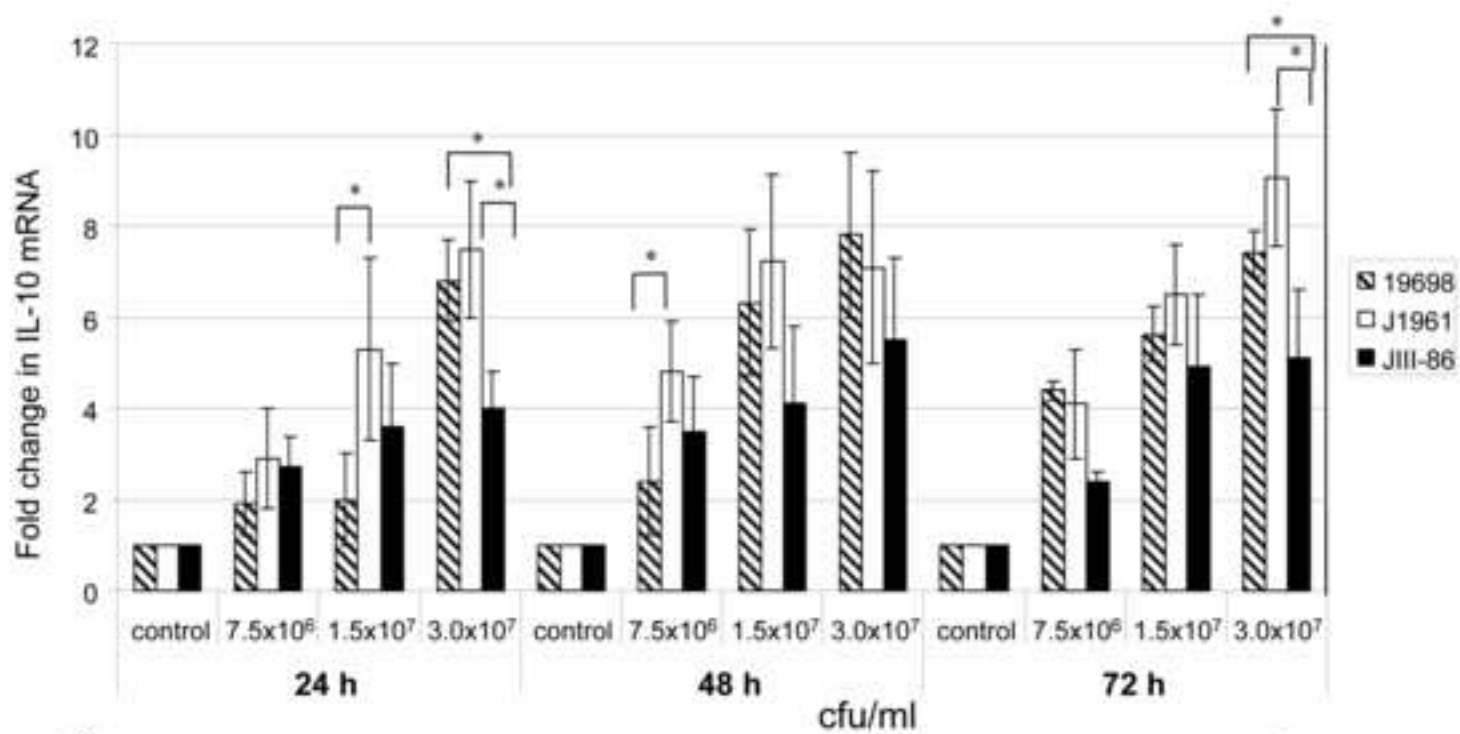
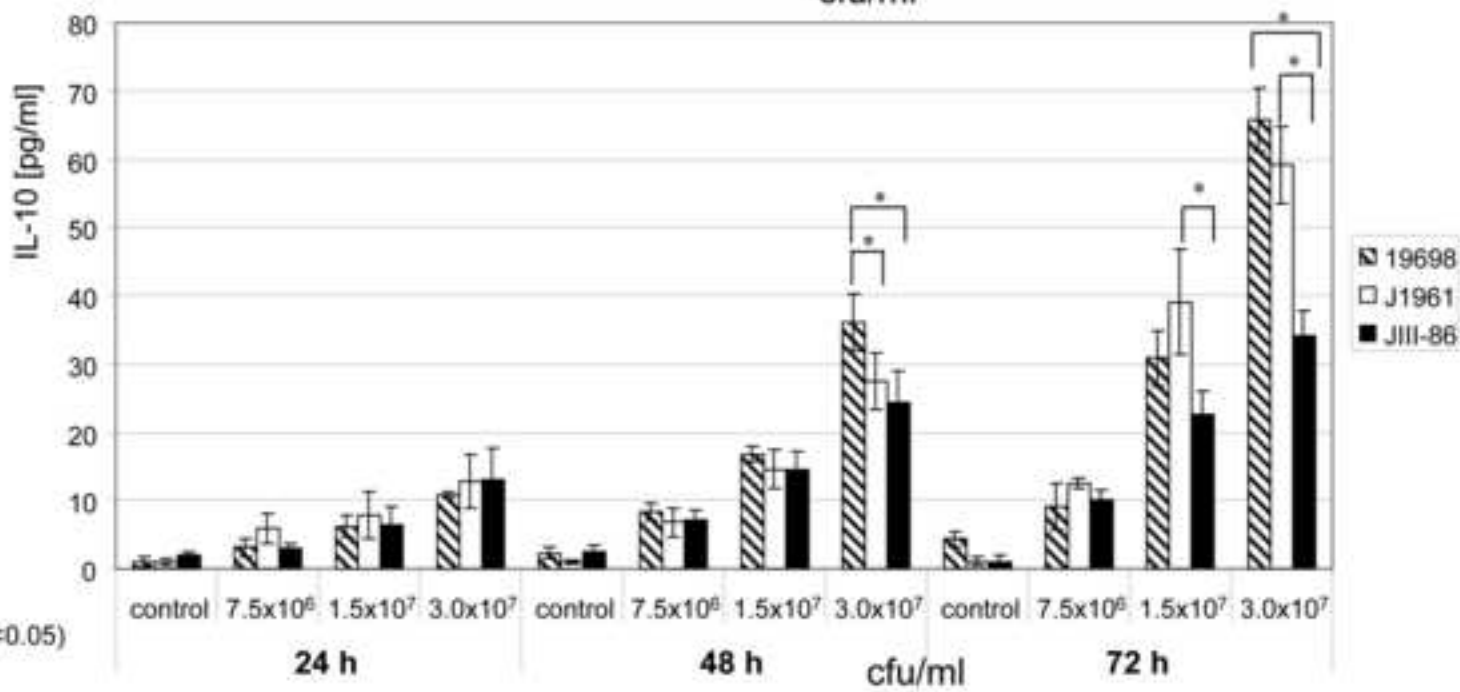


Figure 6b



\*significant difference (p < 0.05)