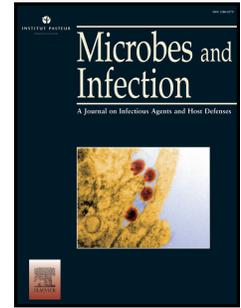# Accepted Manuscript

Endocrine modulation of *Brucella abortus*-infected osteocytes function and osteoclastogenesis via modulation of RANKL/OPG

Ayelén Ivana Pesce Viglietti, Guillermo Hernán Giambartolomei, María Victoria Delpino

Please cite this article as: A.I. Pesce Viglietti, G.H. Giambartolomei, M.V. Delpino, Endocrine modulation of *Brucella abortus*-infected osteocytes function and osteoclastogenesis via modulation of RANKL/OPG, *Microbes and Infection*, https://doi.org/10.1016/j.micinf.2019.01.004.

1   **Endocrine modulation of *Brucella abortus*-infected osteocytes function and**

2   **osteoclastogenesis via modulation of RANKL/OPG**

3   Ayelén Ivana Pesce Viglietti[1], Guillermo Hernán Giambartolomei[1], María Victoria Delpino[1*].

4

5   Instituto de Inmunología, Genética y Metabolismo (INIGEM). Universidad de Buenos Aires

6   (UBA). Consejo Nacional de Investigaciones Científicas y técnicas (CONICET). Buenos

7   Aires, Argentina[1].

8

9

10   *Corresponding author:

11   M. Victoria Delpino, PhD.

12   INIGEM, Instituto de Inmunología, Genética y Metabolismo. Hospital de Clínicas "José de

13   San Martín". Universidad de Buenos Aires, Córdoba 2351 piso 3 sala 4, 1120. Buenos Aires,

14   Argentina.

15   Phone: 54-11-5950-8755.

16   Fax: 54-11-5950-8758.

17   E-mail: mdelpino@ffyb.uba.ar

18

19 **Abstract**

20   Osteoarticular brucellosis is the most frequent complication of active disease. A large

21 amount of cells in bone are osteocytes. Since bone remodeling process is regulated by

22 hormones we sought to study the effect of cortisol and DHEA in *B. abortus*-infected

23 osteocytes. Cortisol treatment inhibited the expression of IL-6, TNF-α, MMP-2 and RANKL

24 in *B. abortus*-infected osteocytes. DHEA could reverse the inhibitory effect of cortisol on

25 MMP-2 production. *B. abortus* infection inhibited connexin 43 (Cx43) expression in

26 osteocytes. This expression was increased when cortisol was incorporated during the infection

27 and DHEA treatment partially reversed the effect of cortisol. Osteocytes-infected with *B.*

28 *abortus* induced osteoclast´s differentiation. Yet, the presence of cortisol, but not DHEA,

29 during osteocyte infection inhibited osteoclastogenesis. Glucocorticoid receptor (GR) is

30 implicated in the signaling of cortisol. Infection with *B. abortus* was able to increase $GR\alpha/\beta$

31 ratio. Levels of intracellular cortisol are not only dependent on GR expression but also a result

32 of the activity of the isoenzymes 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 (cortisone

33 to cortisol conversion), 11β-HSD2 (cortisol to cortisone conversion). *B. abortus* infection

34 increased 11β-HSD 1/ 2 ratio and cortisone mimicked the effect of cortisol. Our results

35 indicated that cortisol and DHEA could modulate osteocyte responses during *B. abortus*

36 infection.

37

38

39

41

42   **1. Introduction**

43   *B. abortus* infection frequently induces osteoarticular damage during the active form

44   of the disease [1]. However, during many years the mechanisms implicated in bone

45   injurycaused by *B. abortus* infection remained unknown. Regardless of the fact that *Brucella*

46   infections in animal models have limitations because they do not reproduce all the signs and

47   symptoms of the disease, studies performed using murine models described some aspects of

48   *Brucella* infection in bone[2-6]. Additionally, results obtained in our laboratory revealed the

49   important role of macrophages, osteoblasts, osteocytes, B lymphocytes and T lymphocytes in

50   the induction of osteoclast differentiation during *B. abortus* infection [7-11]. Osteocytes are

51   encrusted in the mineralized matrix of bone and constitute the final differentiated form of

52   osteoblasts. They are up to 95% of all types of bone cells in the adult skeleton, and then

53   constitute the most numerous cells of bone. Osteocytes constitute the main regulators of

54   activity and differentiation of both osteoclast (cells involved in bone resorption) and

55   osteoblast (cells involved in organic and mineral matrix deposition) during bone remodeling

56   [12].Given that, osteocytes are responsible for the control of bone remodeling, the modulation

57   of their activity by *B. abortus* infection could contribute to the bone loss observed during the

58   osteoarticular form of the disease.In addition, osteocytes are the main mechanosensors in the

59   repair of bone microdamage, and then it can be postulated that loss of these cells is important

60   in bone stability.

61   We have previously demonstrated that *B. abortus* invades and replicates in murine

62   osteocytes with concomitant induction of the expression of proinflammatory cytokines,

63   RANKL and MMP-2 [13]. Recently, it has been established a cross-regulation connecting

64   adrenal steroids (glucocorticoids and dehydroepiandrosterone [DHEA]) and osteocyte

65   function [14-18]. Glucocorticoid reduces cell to cell communication by inducing the

66   degradation of connexin 43 (Cx43) with concomitant osteocyte dead by apoptosis. The effects

67   of cortisol are frequently opposed by other adrenal steroids. The cortisol /DHEA ratio is

68   altered in most pathological conditions. Accordingly, it has been found significantly elevated

69   amounts of cortisol in serum from patients with acute brucellosis with respect to healthy

70   controls [19, 20]. Then, we hypothesized that the responses during *Brucella* infection could be

71   modulated by the neuroendocrine system. Therefore, to investigate the relevance of the

72   adrenal axis on osteocyte function during *B. abortus* infection deserves to be studied.

73

74   **2.   Materials and Methods**

75   **2.1. Bacterial culture**

76   *Brucella abortus* S2308 was grown overnight with constant agitation in 10 ml of

77   tryptic soy broth (Merck, Buenos Aires, Argentina) at 37°C. Bacteria were harvested by

78   centrifugation for 15 min at 6,000 x g at 4°C and washed twice with 10 ml of phosphate-

79   buffered saline (PBS). Comparison or optical densities (OD) with a standard curve obtained in

80   our laboratory was used to determine the number of bacteria in cultures. Cultures were diluted

81   in sterile PBS to the desired bacterial concentration on the basis of the optical density

82   readings, and the number was scored by plating cells onto tryptic soy agar (Britania, Buenos

83   Aires, Argentina). *Brucella* manipulations were performed in biosafety level 3 facilities

84   located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS).

85

86   **2.2. Cells and Media**

87   MLO-Y4 cell line was kindly provided by Professor Lynda Bonewald (University of

88   Missouri-Kansas City). All experiments were performed at 37°C in a 5% CO_2 atmosphere

89   unless specified. MLO-Y4 cells were cultured in standard tissue culture flasks containing

90   alpha minimum essential medium (α-MEM), 10% fetal bovine serum (FBS), 100 U/ml of

91   penicillin, and 100 g/ml of streptomycin (complete medium). Medium was replaced every 3-4

92   days, and after confluence, cells were harvested using trypsin and resuspended in complete

93   medium.

94

95   **2.3. Cellular infection**

96   Cells were seeded at a concentration of $1x10^5$ cells per well in 24 well plates and

97   infected at different multiplicities of infection (MOI) in the presence or absence of cortisol (1

98   x 10^{-6} M) and/or dehydroepiandrosterone, DHEA (1 x 10^{-8} M) and incubated for 1 h at 37ºC in

99 a 5% CO_2 atmosphere. Then, cells were washed with α-MEM to remove extracellular bacteria

100 and were incubated in medium supplemented with 100 µg/ml of gentamicin and 50 µg/ml of

101 streptomycin to kill extracellular bacteria. All experiments were achieved in the presence or

102 absence of the indicated concentrations of cortisol and DHEA. To determine intracellular

103 replication, cytokine production, MMP secretion, 11β-HSD1, 11β-HSD2, GRα, GRβ

104 expression and to obtain culture supernatants to perform osteoclastogenesis assay, MLO-Y4

105 cells and culture supernatants were harvested at 24 or 48 hours.

106   Cells were lysed with 0.1% (vol/vol) Triton X-100 in H_2O to monitor *Brucella*

107 intracellular survival. Then, serial dilutions were plated on tryptic soy agar plates to

108 enumerate CFU.

109

110   **2.4. Zymography**

111   Zymography were conducted by the method of Hibbs et al. [21] with modifications, as

112 described [22, 23].

113

114   **2.5.Measurement of cytokine concentrations**

115   IL-6, TNF-α, (BD Biosciences) and RANKL (R&D systems) were quantified by

116 ELISA from in culture supernatants.

117

118   **2.6. Immunoflourescence**

119   *B. abortus*-infected MLO-Y4 cells were fixed in 4% paraformaldehyde for 60 min at

120 room temperature and then permeabilized with 0,3% TritonX-100 for 10 min. Cells were first

121 incubated for 18 h at 4°C with mouse anti-Cx43 (Invitrogen) diluted in phosphate-buffered

122 saline (PBS)- 5% SFB and then with Alexa 488 anti-mouse (Invitrogen) in PBS-5%SFB for 1

123 h at room temperature, nuclear staining was performed with DAPI. After cells were washed in

124 PBS, they were mounted and analyzed by fluorescence microscopy, using an Olympus

125 microscope and counted using NIH ImageJ software.

126

2.7. Osteoclast formation assay

128 Bone marrow-derived monocytes (BMM) were induced to undergo osteoclastogenesis

129 as was previously described[11, 24]. Briefly, BMM cells from Balb/c mice were cultured in

130 complete medium containing 5 ng/ml murine recombinant M-CSF (R&D Systems) in 24-well

131 plates for 12 h. Non adherent cells were collected and subsequently cultured with 30 ng/ml

132 M-CSF in 24-well plates for an additional 24 h. Adherent cells were used as BMM which

133 were seeded at a concentration of $5x10^4$ cells per well onto 24-well plates and cultured in 0.3

134 ml of complete medium in the presence of 0.2 ml of culture supernatants from MLO-Y4

135 osteocytes infected with *B. abortus* and 30 ng/ml M-CSF. The culture was maintained for 7

136 days. 50 ng/ml murine RANKL was used as positive controls. 1 µg/ml of anti-OPG antibody

137 (R&D Systems) was used in neutralization experiments. Osteoclasts were identified by TRAP

138 staining (Sigma-Aldrich). TRAP-positive, multinucleated cells (more than three nuclei) were

139 defined as osteoclasts, and the number was determined by count in microscopic.

140

2.8. mRNA preparation and quantitative RT-PCR

142 RNA extraction was performed by using the Quick-RNA MiniPrepKit (Zymo

143 Research) and 1 µg of RNA was subjected to reverse transcription using Improm-II Reverse

144 Transcriptase (Promega). PCR analysis was achieved with StepOne™ Real-Time PCR

145 System (Applied Biosystems) using SYBR Green as fluorescent DNA binding dye. The

146 primer sets used for amplification were: β-actin sense: 5'-AACAGTCCGCCTAGAAGCAC-

147 3', β-actin antisense: 5'-CGTTGACATCCGTAAAGACC-3'; 11β-HSD1 sense 5'-

148 GTCCTTGGCCTCATAGACACAG-3' antisense 5'-GGAGTCAAAGGCGATTTGTCAT.

149   11β-HSD2   sense   5´-GTTAACAACGCTGGCCTCAATATC-3´antisense   5´-

150   CAACGGTCACAATACGTCCCCTC-3´.GRα   sense   5´-

151   AAAGAGCTAGGAAAAGCCATTGTC-3´   antisense   5´-

152   TCAGCTAACATCTCTGGGAATTCA-3´.   GRβ   sense   5´-

153   AAAGAGCTAGGAAAAGCCATTGTC-3´antisense   5´-

154   CTGTCTTTGGGCTTTTGAGATAGG-3´

155   The amplification cycle for GRβ and 11β-HSD2 were 95°C for 15 s, 55°C for 30 s and

156   72°C for 60 s for GRα and11β-HSD1 were 95°C for 15 s, 58°C for 30 s and 72°C for 60 s. All

157   primer sets yielded a single product of the correct size. Relative expression levels were

158   normalized against β-actin.

159

160   **2.9. Statistical analysis**

161   Statistical analysis was performed with one-way ANOVA, followed by Post Hoc

162   Tukey Test. Analysis was made by using GraphPad Prism 5.0 software. Data were

163   represented as mean ±SD.

164

165

166 **3. Results**

167 **3.1. Intracellular replication of *B. abortus* in osteocytes is modulated by adrenal steroids**

168   Adrenal steroids do not only modulate the function of host cells but can also modulate

169 bacterial intracellular replication, including *B. abortus* replication in monocytes/macrophages

170 and osteoblast [19, 25-27]. In previous studies performed in our laboratory, we demonstrated

171 that osteocytes support *B. abortus* replication [13]. Then, experiments were performed to

172 determine if cortisol and DHEA treatment could vary the ability of *B. abortus* to replicate in

173 osteocytes. Our results indicated that the ability of *B. abortus* to replicate in osteocytes was

174 increased when experiments were performed in the presence of cortisol with respect to

175 untreated cells at 24 and 48 h postinfection. By contrast, intracellular replication of *B. abortus*

176 was not affected by DHEA respect to untreated cells (Fig. 1A). Infection experiments

177 conducted in the presence of cortisol and DHEA administrated together revealed not

178 significant differences in intracellular bacterial survival with respect to untreated cells.

179 Together, these results indicate that intracellular replication of *B. abortus* is increased in the

180 presence of cortisol and that DHEA treatment is able to reverse this effect.

181

182 **3.2. Cortisol inhibits IL-6, TNF-α, RANKL and MMP-2 expression and DHEA partially**

183 **reverses the effect of cortisol on MMP-2 expression in *B. abortus*-infected osteocytes**

184   Most of functions of different cell types including osteocytes (the most abundant bone

185 cells) are modulated by adrenal hormones [28].Thus, we hypothesized that osteocyte function

186 would be modified by cortisol and DHEA during *B. abortus* infection. Osteocytes infected

187 with *B. abortus* in the presence of cortisol, secreted significantly lower amounts of TNF-α,

188 IL-6, RANKL and MMP-2 with respect to untreated infected cells (Fig. 1 B, C, D and E). In

189 contrast, DHEA had no significant effect on the levels of TNF-α, IL-6, RANKL and MMP-2

190 respect to untreated infected cells. When both cortisol and DHEA were administrated

191   together, DHEA could only reverse the inhibitory effect of cortisol on MMP-2 expression.

192   These results indicate that cortisol reduces the proinflammatory microenvironment induced by

193   *B. abortus* infection of osteocytes; and DHEA partially reverses this effect on MMP-2

194   expression.

195

196   **3.3. DHEA reverses the effect of cortisol on Cx43 expression in *B. abortus*-infected**

197   **osteocytes**

198   Cx43 is the most abundant gap junction in bone cells. It is required for cell to cell

199   communication and to maintain the viability of osteocytes [29]. Previous studies performed in

200   our laboratory revealed that *B. abortus* infection inhibits Cx43 expression in osteocytes. Then,

201   experiments were conducted to determine the role of cortisol and DHEA in the modulation of

202   Cx43 expression during *B. abortus* infection. Using specific antibodies, Cx43 expression was

203   evaluated by immunofluorescence. *B. abortus* infection reduced the expression of Cx43

204   demonstrating that infection could modify gap junction in osteocytesas was previously

205   reported [13]. This phenomenon was reversed when infection experiments were performed in

206   the presence of DHEA (Fig. 1F and G). Cortisol inhibited Cx43 expression in uninfected

207   osteocytes, as it was previously described [14] and also inhibited Cx43 expression in *B.*

208   *abortus*-infected osteocytes. In addition, when both cortisol and DHEA were administrated in

209   conjunction, DHEA could reverse the inhibitory effect of cortisol on Cx43 expression in

210   infected and non-infected cells. These results indicate that DHEA reverses the effect of *B.*

211   *abortus* infection on Cx43 expression even in the presence of cortisol.

212

213   **3.4. Osteoclastogenesis induced by *B. abortus*-infected osteocytes was inhibited by**

214   **cortisol**

215 Osteocytes are the main bone cells that modulate osteoclast differentiation. In

216 pathological conditions osteocyte misbalance could cause excessive osteoclastogenesis and

217 pathological bone loss. Then, experiments were conducted to determine the role of adrenal

218 steroids on osteocytes and in their ability to induce osteoclastogenesis during *B. abortus*

219 infection. To this end, osteoclast precursors were stimulated in the presence of M-CSF with

220 supernatants from osteocytes that were previously infected with *B. abortus* in the presence or

221 not of cortisol, DHEA, both cortisol and DHEA, or with supernatants from uninfected cells as

222 control. Our results indicated that supernatants from *B. abortus*-infected osteocytes induced

223 osteoclastogenesis (Fig. 2). When we studied the effect ofconditioned medium from *B.*

224 *abortus*-infected osteocytes in the presence of adrenal steroids, our results indicated that

225 supernatants form *B. abortus*-infected osteocytes cultured in the presence of cortisol inhibited

226 osteoclastogenesis, and supernatants from *B. abortus*-infected osteocytes cultured in the

227 presence of DHEA had no effect on osteoclastogenesis. When osteoclast differentiation was

228 performed with supernatants from *B. abortus*-infected osteocytes in the presence of both

229 adrenal steroids (cortisol and DHEA), cortisol was also able to inhibit osteoclastogenesis

230 induced by *B. abortus* infection (Fig. 2 A and B).

231 To determine if the modulation of osteoclast differentiation induced by supernatants

232 from *B. abortus*-infected osteocytes in the presence of cortisol and DHEA was due to a direct

233 effect of these hormones on osteoclast precursors or as a result of the modulation of adrenal

234 steroids on the cytokine production by *B. abortus*-infected osteocytes; experiments were

235 performed with supernatants from *B. abortus*-infected osteocytes that were added on

236 osteoclast precursors together with exogenously added adrenal steroids. Our results indicated

237 that, adrenal steroids were unable to modulate osteoclast differentiation induced by culture

238 supernatants from *B. abortus*-infected osteocytes (Fig. 2 C and D).

239   Taken together, these results indicate that adrenal steroids can modulate the secretion

240   of cytokines by osteocyte during *B. abortus* infection resulting in a different ability to induce

241   osteoclast differentiation.

242

243   **3.5. Cortisol inhibits osteoclast differentiation through a mechanism that depends on**

244   **osteoprotegerin (OPG)**

245   Cortisol could not only inhibitosteoclastogenesis through the inhibition of RANKL,

246   but it could also induce the expression of OPG, the natural antagonist of RANKL. Since by

247   ELISA assay it was measured only the free form of RANKL, experiments were performed to

248   determine the contribution of OPG in the inhibition of osteoclastogenesis induced by

249   supernatants from *B. abortus*-infected osteocytes in the presence of cortisol respect to

250   supernatants from *B. abortus*-infected but untreated osteocytes. To this end, osteoclast

251   differentiation experiments were performed with supernatants from *B. abortus*-infected

252   osteocytes in the presence or not of anti-OPG neutralizing antibodies. Our results indicated

253   that in the presence of anti-OPG antibodies, supernatants from *B. abortus*-infected osteocytes

254   in the presence of cortisol induced significantly higher levels of osteoclast differentiation

255   respect to supernatants not treated with anti-OPG antibodies. When anti-OPG neutralizing

256   antibodies were added in conjunction with supernatants from *B. abortus*-infected osteocytes

257   in the presence of DHEA, this treatment had no significant effect on osteoclast differentiation.

258   The neutralizing antibodies anti-OPG were able to partially reverse the inhibitory effect on

259   osteoclastogenesis induced bysupernatants from *B. abortus*-infected osteocytes in the

260   presence of both cortisol and DHEA. Isotype control antibodies had no effect on any of the

261   described phenomena (Fig. 3A). Taken together these results indicate that cortisol inhibits

262   osteoclastogenesis induced by *B. abortus*-infected osteocytes at least in part through the

263   increase of OPG expression.

3.6. Cortisol inhibits the induction of functional osteoclasts

Our hypothesis is that *B. abortus* infection creates a microenvironment that promotes osteoclastogenesis, leading to bone loss. Thus, osteoclast precursors were treated with culture supernatants from *B. abortus*-infected osteocytes in the presence of M-CSF and in the presence or not of cortisol, DHEA and both cortisol and DHEA. Our results indicated that supernatants from *B. abortus*-infected osteocytes induced functional osteoclasts formation that were able to resorb dentine. Additionally, supernatants from *B. abortus*-infected osteocytes in the presence of cortisol were unable to induce dentine resorption; and this effect was reversed by anti-OPG treatment (Fig. 3 B and C). Taken together, these results indicate that culture supernatants from *B. abortus*-infected osteocytes promote functional osteoclast formation, and this can be inhibited by the presence of cortisol.

3.7. *B. abortus* infection inhibits GRα and GRβ gene transcription

Cortisol exerts effects bybinding to GR, which is expressed in different cell types including osteocytes [15]. Thus, the cellular sensitivity to cortisol is not only dependent on serum concentration but also is dependent on the ratio between different GR isoforms. It is know that GRβ does not have the capacity to bind glucocorticoids and, in addition, it can induce GRα/GRβ heterodimers formation that inhibits GRα-mediated transcriptional activation [30]. Then, experiments were conducted to assess the effect of *B. abortus* infection in the transcriptional levels of GRα and GRβ. Our results indicated that *B. abortus* infection reduced the transcription of both GRα and GRβ genes, but increase the GRα/β transcriptional ratio (Fig. 4 A, B and C). These results suggest that *B. abortus* infection could favor cortisol action on osteocytes through the increase in GRα/β transcriptional ratio.

288   **3.8. Transcription of 11β-HSD1 and 11β-HSD2 genes in osteocytes is modulated by**

289   ***B. abortus* infection**

290   GR expression is important in the recognition of cortisol by cells. However, cell

291   response to cortisol is also dependent on its intracellular presence and bioavailability [16].

292   Intracellular levels of cortisol are dependent on the action of the isoenzymes 11β-

293   hydroxysteroid- dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) which convert

294   cortisone to cortisol and vice versa, respectively. Then, experiments were performed to

295   determine if 11β-HSD1/2 mRNA levels could be modulated by *B. abortus* infection. 11β-

296   HSD1 transcription was increased by *B. abortus* infection (Fig. 4D). By contrast, *B. abortus*

297   infection inhibits 11β-HSD2 transcription (Fig. 4E). In concordance, when we analyzed 11β-

298   HSD1/2 ratio, our results indicated that *B. abortus* infection induces an increase in 11β-

299   HSD1/2 ratio (Fig. 4F). Taken together, these results suggest that *B. abortus* infection

300   increase intracellular bioavailability of cortisol, thought an increase of the 11β-HSD1/2 ratio.

301

302   **3.9. Cortisone mimics the effect of cortisol on *B. abortus*-infected osteocytes**

303   Experiments were then conducted to determine the relevance of the increase of 11β-

304   HSD1/2 ratio induced by *B. abortus* infection. To this end, we evaluated the ability of

305   cortisone to mimic the effect of cortisol to stimulate *B. abortus* intracellular replication in

306   osteocytes and to modulate TNF-α, IL-6 and RANKL secretion; Cx43 and MMP-2

307   expression. Our results indicated that when osteocytes were infected with *B. abortus* in the

308   presence of cortisone, the levels of intracellular bacteria were similar to that of cells infected

309   in the presence of cortisol (Fig. 5 A). In addition, *B. abortus*-infected cells in the presence of

310   cortisone secreted significantly lower quantities of the cytokines TNF-α, IL-6, RANKL and

311   MMP-2 and express lower quantities of Cx43 than untreated infected-cells. The levels of

312   cytokines, MMP-2 and Cx43 in *B. abortus*-infected osteocytes in the presence of cortisone

313   were similar to the ones produced by osteocytes infected with *B. abortus* in the presence of

314   cortisol (Fig. 5 B, C, D, E and F). These results indicate that both cortisol and cortisone are

315   able to reduce proinflammatory microenvironment induced by *B. abortus* infection on

316   osteocytes.

317

318   4. **Discussion**

319   In brucellosis, we and others have demonstrated that cortisol/ DHEA ratio is higher in

320   infected patients with acute manifestations of the disease than in healthy controls and in

321   patients with disease remission [19, 20]. This increase could modulate bone responses and

322   affect the control of the disease during osteoarticular localization of *Brucella* infection. In

323   particular, adrenal steroids could modulate osteocyte responses during *B. abortus* infection.

324   The importance of osteocytes is because they are the most abundant bone cells and they are

325   involved in bone homeostasis. We have previously demonstrated that *B. abortus* invades and

326   replicates in osteocytes and this infection affects osteocyte function through the induction of

327   proinflammatory cytokines, MMP-2 and RANKL with a concomitant induction of

328   osteoclastogenesis. In addition, the most abundant gap junction in osteocytes that facility

329   intercellular communication to maintain osteocytes viability, Cx43, was inhibited by *B.*

330   *abortus* infection. It has been previously demonstrated that glucocorticoids have adverse

331   effects on osteocytes, and these effects included the decrease in Cx43 protein expression [14].

332   In contrast, DHEA was reported to have beneficial effects in osteoporosis by increasing bone

333   mineral density [17, 18]. However, besides the beneficial effects of DHEA in bone, its role in

334   modulating osteocytes function has not been studied yet. Our results indicate that DHEA has

335   beneficial effect on *B. abortus*-infected osteocytes. DHEA treatment was able to reverse the

336   inhibitory effect of *B. abortus* infection on Cx43 expression. Moreover, DHEA reversed the

337 effect of *B. abortus* infection on Cx43 expression when infection experiments were performed

338 in the presence of cortisol.

339   Adrenal steroids could not only modulate cell responses during *B. abortus* infection,

340 but also could modulate bacterial intracellular replication. Our results indicated that cortisol

341 treatment significantly increases *B. abortus* intracellular proliferation in osteocytes and this

342 phenomenon was reversed when both cortisol and DHEA were administrated in conjunction.

343 This increase in intracellular replication due to glucocorticoids treatment has been described

344 by us and others in macrophages infected with *B. abortus*, *Salmonella typhimurium* and

345 *Mycobacterium tuberculosis* and in *B. abortus*-infected osteoblast [19, 25-27]. The reversion

346 of the effect of cortisol mediated by DHEA on *B. abortus* intracellular replication added

347 support to the beneficial effect of DHEA as it was previously demonstrated in non-infectious

348 bone disease [28].

349   The effect of cortisol, is dependent on its availability, on the ratio between GRα and

350 GRβ isoforms [30] and on its intracellular presence dependent on 11β-HSD1 and 11β-HSD2

351 expression [16]. In osteocytes, *B. abortus* increased GRα/β and 11β-HSD1/2 ratio suggesting

352 an increase in intracellular concentration of cortisol. The increase observed in GRα/β and

353 11β-HSD1/2 ratio was not exclusive of osteocytes infected with *B. abortus*, since it was also

354 observed in mononuclear cells from patients with *M. tuberculosis* infection and also in

355 osteoblast infected with *B. abortus* [25, 31]. Our results demonstrated the importance of the

356 increase of 11β-HSD1/2 ratio induced by *B. abortus* infection in osteocytes, since in

357 experiments performed with cortisone instead of cortisol; we demonstrated that cortisone

358 could mimic the effect of cortisol on osteocytes during *B. abortus* infection.

359   This study constitutes the first analysis on the adrenal steroids modulation of

360 osteocytes response in a context of a bacterial infection. Although our previous studies reveals

361 the importance of DHEA in reducing the damage induced by *B. abortus* infection [19, 25],

362 DHEA was not able to modulate most of the responses induced by *B. abortus* in osteocytes.In

363 addition, DHEA was also unable to reverse most of the effect of cortisol on *B. abortus*-

364 infected osteocytes. However, the reversion in the inhibition of Cx43 expression during *B.*

365 *abortus* infection still when infection experiments were performed in the presence of cortisol,

366 added an important role of DHEA. In this context a supplementation with DHEA could be

367 considered to reduce the bone damage induced by *B. abortus*-infected osteocytes. Then

368 considering the effect of DHEA on the modulation of immune response in *B. abortus*-infected

369 monocytes [19], cells that infiltrated the osteoarticular localization and the benefic effect of

370 DHEA on osteoblast [25] and the effect of DHEA in osteocytes during osteoarticular

371 brucellosis; antibiotic therapy with the addition of DHEA or its derivatives could be consider

372 as a new possible treatment for brucellosis with the aim to reduce bone lesion and sequelae.

373 The results of this study were summarized in Fig. 6.

374

375

376

377

378

379

380

381

382

383

384

385

386

387

399

400 **Conflict of interest**

401 The authors declare no conflict of interest.

402

403

404 **References**

405

406 [1] Madkour MM. Osteoarticular brucellosis. Edited by Madkour MM, Madkour's brucellosis,

407 2nd ed. Springer-Verlag, Berlin, Germany, 2001.

408 [2] Skyberg JA, Thornburg T, Kochetkova I, Layton W, Callis G, Rollins MF, et al. IFN-

409 gamma-deficient mice develop IL-1-dependent cutaneous and musculoskeletal inflammation

410 during experimental brucellosis. J Leukoc Biol 2012;92:375-87.

411 [3] Rajashekara G, Glover DA, Krepps M, Splitter GA. Temporal analysis of pathogenic

412 events in virulent and avirulent *Brucella melitensis* infections. Cell Microbiol 2005;7:1459-

413 73.

414 [4] Adarichev VA, Vegvari A, Szabo Z, Kis-Toth K, Mikecz K, Glant TT. Congenic strains

415 displaying similar clinical phenotype of arthritis represent different immunologic models of

416 inflammation. Genes Immun 2008;9:591-601.

417 [5] Farkas B, Boldizsar F, Tarjanyi O, Laszlo A, Lin SM, Hutas G, et al. BALB/c mice

418 genetically susceptible to proteoglycan-induced arthritis and spondylitis show colony-

419 dependent differences in disease penetrance. Arthritis Res Ther 2009;11:R21.

420 [6] Lacey CA, Keleher LL, Mitchell WJ, Brown CR, Skyberg JA. CXCR2 Mediates Brucella-

421 Induced Arthritis in Interferon gamma-Deficient Mice. J Infect Dis 2016;214:151-60.

422 [7] Pesce Viglietti AI, Arriola Benitez PC, Giambartolomei GH, Delpino MV. *Brucella*

423 *abortus*-infected B cells induce osteoclastogenesis. Microbes Infect 2016;18:529-35.

424 [8] Delpino MV, Fossati CA, Baldi PC. Proinflammatory response of human osteoblastic cell

425 lines and osteoblast-monocyte interaction upon infection with *Brucella* spp. Infect Immun

426 2009;77:984-95.

427 [9] Giambartolomei GH, Scian R, Acosta-Rodriguez E, Fossati CA, Delpino MV. *Brucella*

428 *abortus*-infected macrophages modulate T lymphocytes to promote osteoclastogenesis via IL-

429 17. Am J Pathol 2012;181:887-96.

430   [10] Scian R, Barrionuevo P, Fossati CA, Giambartolomei GH, Delpino MV. *Brucella*

431   *abortus* invasion of osteoblasts inhibits bone formation. Infect Immun 2012;80:2333-45.

432   [11] Delpino MV, Barrionuevo P, Macedo GC, Oliveira SC, Genaro SD, Scian R, et al.

433   Macrophage-elicited osteoclastogenesis in response to *Brucella abortus* infection requires

434   TLR2/MyD88-dependent TNF-alpha production. J Leukoc Biol 2012;91:285-98.

435   [12] Lanyon LE. Osteocytes, strain detection, bone modeling and remodeling. Calcif Tissue

436   Int 1993;53 Suppl 1:S102-6; discussion S6-7.

437   [13] Pesce Viglietti AI, Arriola Benitez PC, Gentilini MV, Velasquez LN, Fossati CA,

438   Giambartolomei GH, et al. *Brucella abortus* invasion of osteocytes modulates connexin 43

439   and integrin expression and induces osteoclastogenesis via receptor activator of NF-kappaB

440   ligand and tumor necrosis factor alpha secretion. Infect Immun 2016;84:11-20.

441   [14] Gao J, Cheng TS, Qin A, Pavlos NJ, Wang T, Song K, et al. Glucocorticoid impairs cell-

442   cell communication by autophagy-mediated degradation of connexin 43 in osteocytes.

443   Oncotarget 2016;7:26966-78.

444   [15] La Corte R, Trotta F, Adami S. Glucocorticoid receptors and bone. Curr Pharm Des

445   2010;16:3586-92.

446   [16] Weinstein RS, Wan C, Liu Q, Wang Y, Almeida M, O'Brien CA, et al. Endogenous

447   glucocorticoids decrease skeletal angiogenesis, vascularity, hydration, and strength in aged

448   mice. Aging Cell 2010;9:147-61.

449   [17] Clarke BL, Khosla S. Androgens and bone. Steroids 2009;74:296-305.

450   [18] Baulieu EE, Thomas G, Legrain S, Lahlou N, Roger M, Debuire B, et al.

451   Dehydroepiandrosterone (DHEA), DHEA sulfate, and aging: contribution of the DHEAge

452   Study to a sociobiomedical issue. Proc Natl Acad Sci U S A 2000;97:4279-84.

453   [19] Gentilini MV, Velasquez LN, Barrionuevo P, Arriola Benitez PC, Giambartolomei GH,

454   Delpino MV. Adrenal steroids modulate the immune response during *Brucella*

455 *abortus*infection by a mechanism that depends on the regulation of cytokine production.

456 Infect Immun 2015;83:1973-82.

457 [20] Yildiz O, Gokce C, Alp E, Durak AC, Aygen B, Kelestimur F, et al. Investigation of the

458 hypothalamo-pituitary-adrenal axis and changes in the size of adrenal glands in acute

459 brucellosis. Endocr J 2005;52:183-8.

460 [21] Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL. Biochemical and

461 immunological characterization of the secreted forms of human neutrophil gelatinase. J Biol

462 Chem 1985;260:2493-500.

463 [22] Scian R, Barrionuevo P, Giambartolomei GH, De Simone EA, Vanzulli SI, Fossati CA,

464 et al. Potential role of fibroblast-like synoviocytes in joint damage induced by *Brucella*

465 *abortus* infection through production and induction of matrix metalloproteinases. Infect

466 Immun 2011;79:3619-32.

467 [23] Scian R, Barrionuevo P, Giambartolomei GH, Fossati CA, Baldi PC, Delpino MV.

468 Granulocyte-macrophage colony-stimulating factor- and tumor necrosis factor alpha-mediated

469 matrix metalloproteinase production by human osteoblasts and monocytes after infection with

470 *Brucella abortus*. Infect Immun 2011;79:192-202.

471 [24] Ukai T, Yumoto H, Gibson FC, 3rd, Genco CA. Macrophage-elicited osteoclastogenesis

472 in response to bacterial stimulation requires Toll-like receptor 2-dependent tumor necrosis

473 factor-alpha production. Infect Immun 2008;76:812-9.

474 [25] Gentilini MV, Pesce Viglietti AI, Arriola Benitez PC, Iglesias Molli AE, Cerrone GE,

475 Giambartolomei GH, et al. Inhibition of osteoblast function by *Brucella abortus* is reversed

476 by dehydroepiandrosterone and involves ERK1/2 and estrogen receptor. Front Immunol

477 2018;9:88.

478 [26] Verbrugghe E, Boyen F, Van Parys A, Van Deun K, Croubels S, Thompson A, et al.

479 Stress induced *Salmonella Typhimurium* recrudescence in pigs coincides with cortisol induced

480 increased intracellular proliferation in macrophages. Vet Res 2011;42:118.

481 [27] Bongiovanni B, Mata-Espinosa D, D'Attilio L, Leon-Contreras JC, Marquez-Velasco R,

482 Bottasso O, et al. Effect of cortisol and/or DHEA on THP1-derived macrophages infected

483 with *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2015;95:562-9.

484 [28] Hardy R, Cooper MS. Adrenal gland and bone. Arch Biochem Biophys 2010;503:137-

485 45.

486 [29] Bivi N, Nelson MT, Faillace ME, Li J, Miller LM, Plotkin LI. Deletion of Cx43 from

487 osteocytes results in defective bone material properties but does not decrease extrinsic

488 strength in cortical bone. Calcif Tissue Int 2012;91:215-24.

489 [30] Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling

490 mechanisms in health and disease. J Allergy Clin Immunol 2013;132:1033-44.

491 [31] D'Attilio L, Diaz A, Santucci N, Bongiovanni B, Gardenez W, Marchesini M, et al.

492 Levels of inflammatory cytokines, adrenal steroids, and mRNA for GRalpha, GRbeta and

493 11betaHSD1 in TB pleurisy. Tuberculosis (Edinb) 2013

494 93:635-41.

495

496

497

498

499

500

501

502

503   **FIGURE LEGENDS**

504

505   **Figure 1: Effects of cortisol and DHEA on *B. abortus* replication and induction of**

506   **proinflammatory mediators and connexin 43 (Cx43).**Osteocytes were infected at MOI 100

507   and 1,000 in the presence or not of cortisol (1×10^{-6} M), DHEA (1×10^{-8} M), or cortisol plus

508   DHEA (1×10^{-6} M and 1×10^{-8} M, respectively). (A) Intracellular replication was assessed by

509   determining colony forming units (CFUs) after 2, 4, 6, 24, and 48 hof osteocytes infected at

510   MOI 100. ELISA determination of the IL-6 (B) and RANKL (C), TNF-α (D); and MMP-2

511   production by zymography in culture supernatants of 24 h (E). Representative digital images

512   of Cx43 expression revealed by immunofluorescence (F). Quantification of % of Cx43

513   positive cells (G).Data are given as the means ± SEM from at least 3 individual

514   experiments*$P< 0.1$, **$P< 0.01$, and ***$P< 0.001$ cortisol versus untreated. #$P< 0.1$, DHEA-

515   cortisol versus cortisol.

516

517   **Figure 2: Cortisol inhibits osteoclastogenesis induced by supernatants from *B. abortus*-**

518   **infected osteocytes.** Bone marrow-derived monocytes (BMM) cells were either not treated

519   (unstimulated) or stimulated with culture supernatants from osteocytes infected with *B.*

520   *abortus* (*B.a.* infected-Sn MLO-Y4)or with culture supernatants from uninfected osteocytes

521   (Uninfected-Sn MLO-Y4) in the presence or not of cortisol (1×10^{-6} M), DHEA (1×10^{-8} M),

522   or cortisol plus DHEA (1×10^{-6} M and 1×10^{-8} M, respectively) added at 1:2 proportion in

523   conjunction with M-CSF. After 7 days, osteoclastogenesis was determined by the generation

524   of multinucleated TRAP-positive cells. Representative digital images were taken by light

525   microscopy (A), and multinucleated TRAP-positive cells were identified and counted (B).

526   BMM cells were either not treated (unstimulated) or stimulated with culture supernatants

527   from *B. abortus*-infected osteocytes (*B.a.*infected-Sn MLO-Y4) or culture supernatants from

528   unifected osteocytes (Uninfected-Sn MLO-Y4). Culture supernatants were added at 1:2

529   proportion in conjunction or not with cortisol (1×10^{-6} M), DHEA (1×10^{-8} M), or cortisol

530   plus DHEA (1×10^{-6} M and 1×10^{-8} M, respectively) in the presence of M-CSF. RANKL is

531   used as a positive control. After 7 days, osteoclastogenesis was determined by the generation

532   of multinucleated TRAP-positive cells. Representative digital images were taken by light

533   microscopy (C), and multinucleated TRAP-positive cells were identified and counted (D).

534   Data are given as the means ± SEM from at least 3 individual experiments***$P < 0.001$ versus

535   untreated.

536

537   **Figure 3: Cortisol inhibits osteoclast differentiation through OPG increase.** BMM cells

538   were either not treated (unstimulated) or stimulated with culture supernatants from osteocytes

539   infected with *B. abortus* (*B.a.* infected-Sn MLO-Y4) in the presence or not of cortisol (1×10^{-6}

540   ^{6} M), DHEA (1×10^{-8} M), or cortisol plus DHEA (1×10^{-6} M and 1×10^{-8} M, respectively) or

541   with culture supernatants from osteocytes pre-incubated with anti-OPG (a-OPG)-neutralizing

542   antibody or its isotype control. Supernatants were added at 1:2 proportions in conjunction

543   with M-CSF. After 7 days, osteoclastogenesis was determined by the generation of

544   multinucleated TRAP-positive cells (A) and the ability to resorb dentin (B and C). RANKL

545   was used as a positive control. Data are given as the means ± SEM from at least 3 individual

546   experiments *$P < 0.1$, and **$P < 0.01$ versus cortisol.

547

548   **Figure 4: *B. abortus* infection modulates glucocorticoid receptor (GR) and isoenzymes**

549   **11β-hydroxysteroid dehydrogenase (HSD).** GRα, GRβ, 11β-HSD1, and 11β-HSD2

550   expression were determined by RT-qPCR in osteocytes infected by *B. abortus* at

551   multiplicities of infection (MOI) 100 and 1,000 for 24 h. GRα (a), GRβ (B), GRα/β ratio (c),

552   11β-HSD1 (D), 11β-HSD2 (e) and 11β-HSD1/2 ratio (F). N.I.: non-infected. Data are given

553 as the means ± SEM from at least 3 individual experiments *P< 0.1, **P< 0.01, and ***P<

554 0.001 versus untreated.

555

556 **Figure 5: Cortisone mimics the effects of cortisol on osteocytes infected with *B. abortus*.**

557 Osteocytes were infected at MOI 1000 in the presence or not of cortisol (1 x 10^{-6} M) or

558 cortisone (1 x 10^{-6} M) (A) Intracellular replication was assessed by determining colony

559 forming units (CFUs) after 2, 4, 6, 24, and 48 hours. ELISA determination of IL-6 (B),

560 RANKL (C) and TNF-α (D); and MMP-2 production by zymography in culture supernatants

561 of 24 hour infected osteocytes (E). Representative digital images of Cx43 expression revealed

562 by immunofluorescence (F). Data are given as the means SEM of duplicates. Data are given

563 as the means ± SEM from at least 3 individual experiments *P< 0.1, **P< 0.01, and ***P<

564 0.001 versus untreated.

565

566 **Figure 6: Proposed model for the mechanisms involved in the modulation of osteocytes**

567 **by adrenal steroids during *B. abortus* infection.**1. Infection with *B. abortus* induces the

568 secretion of RANKL, TNF-α, IL-6 and MMP-2, inhibits the expression of Cx43, induces

569 osteoclastogenesis and increases GRα/β and 11β-HSD1/2 ratio with the consequent

570 conversion of cortisone in its active form cortisol. 2. When cortisone or cortisol are present,

571 the intracellular CFU is increased respect to untreated cells, the secretion of RANKL, TNF-α,

572 IL-6 and MMP-2 is inhibited. 3. DHEA reverses the increase of CFU counts induced by

573 cortisol. Cortisone and cortisol have no effect on Cx43 expression. 4. Cortisol inhibits

574 osteoclastogenesis in a mechanism that involve OPG. 5. DHEA reverses the effect of *B.*

575 *abortus* infection via the induction of Cx43 expression, inclusive also in the presence of
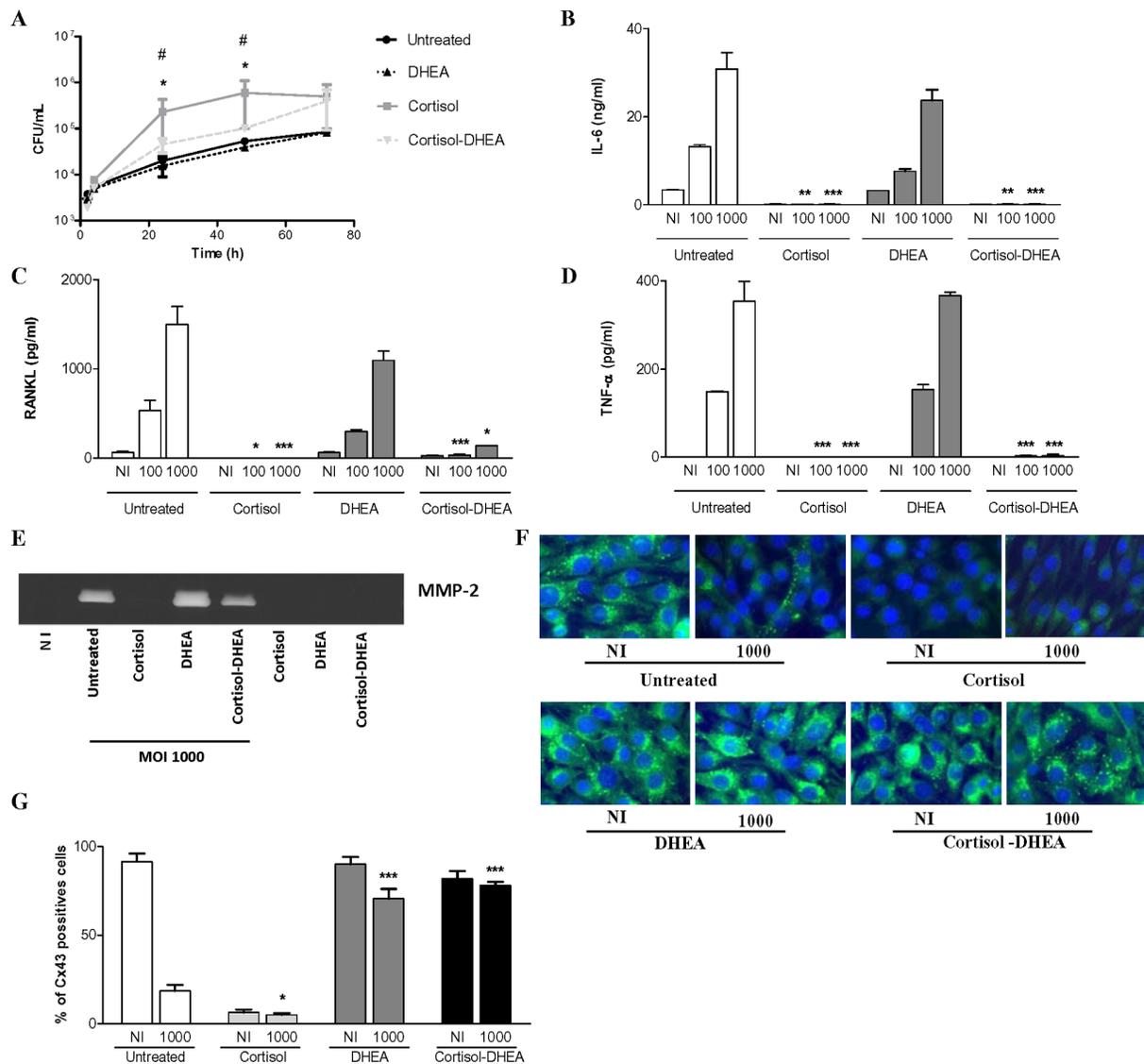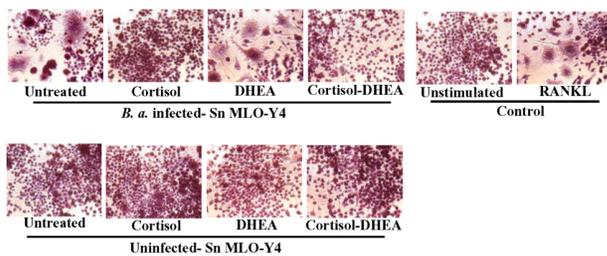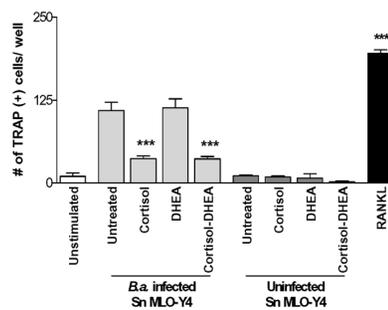
576 cortisol.

577

Figure 1

Supernatants from osteocytes infected with *B. abortus* in the presence or not of adrenal steroids were added to osteoclast precursors.
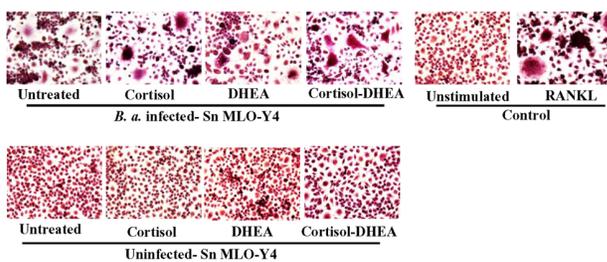
A

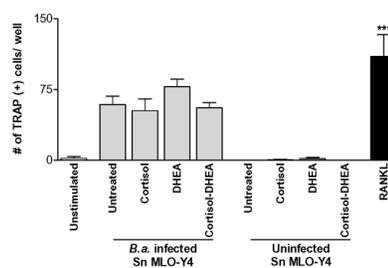
Untreated   Cortisol   DHEA   Cortisol-DHEA   Unstimulated   RANKL
B. a. infected- Sn MLO-Y4   Control

Untreated   Cortisol   DHEA   Cortisol-DHEA
Uninfected- Sn MLO-Y4

B

Supernatants from osteocytes infected with *B. abortus* were added to osteoclast precursors in the presence or not of adrenal steroids exogenously added.

C

Untreated   Cortisol   DHEA   Cortisol-DHEA   Unstimulated   RANKL
B. a. infected- Sn MLO-Y4   Control
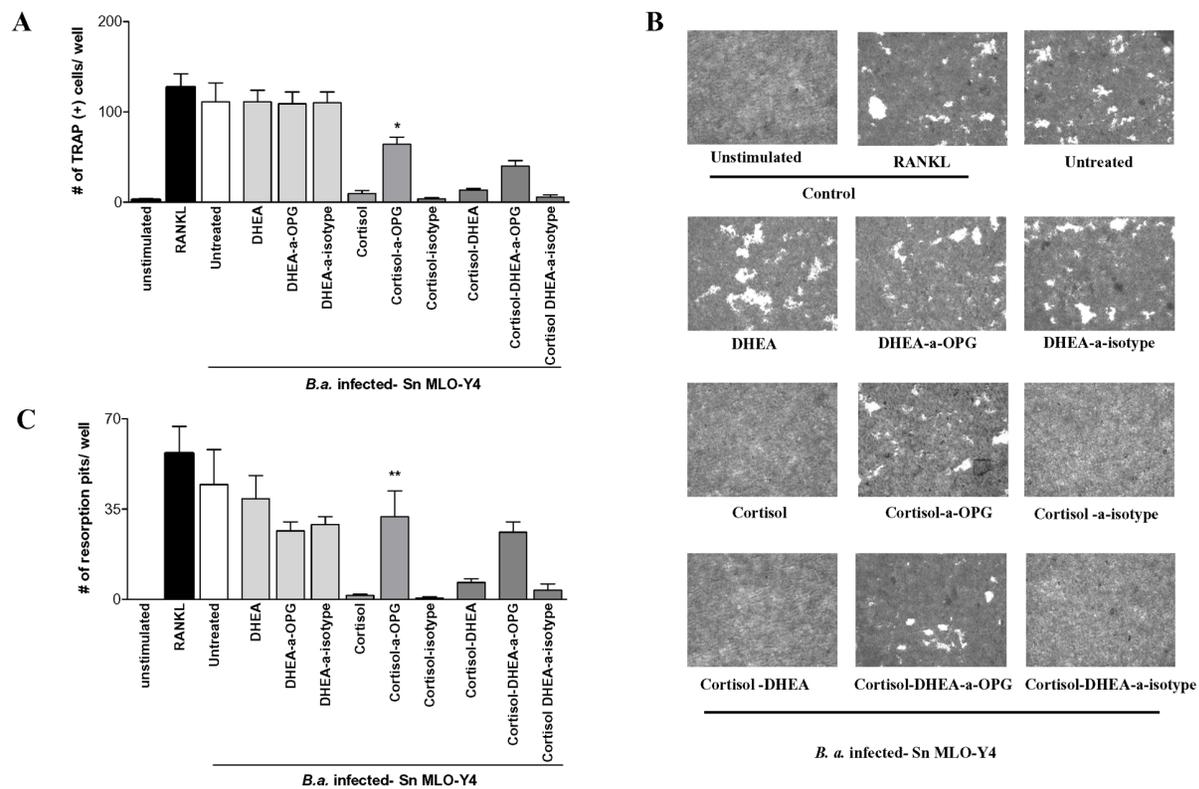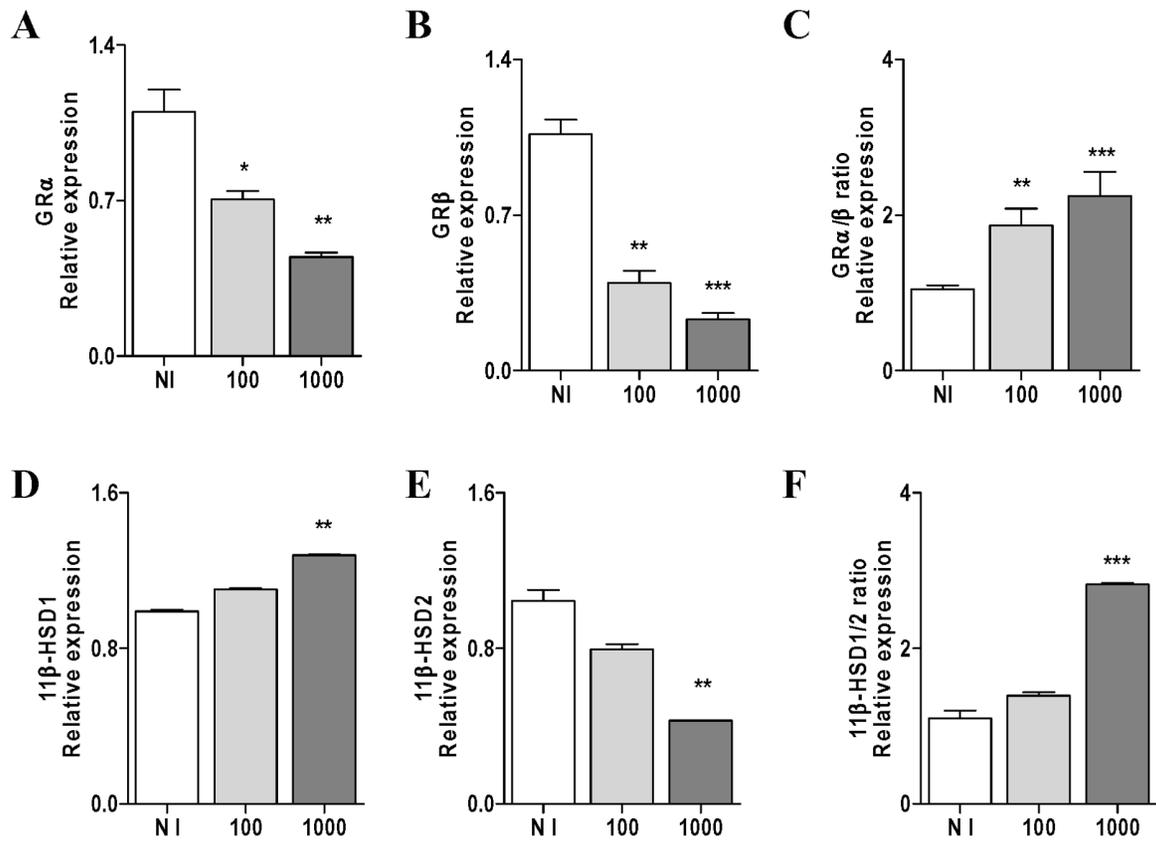
Untreated   Cortisol   DHEA   Cortisol-DHEA
Uninfected- Sn MLO-Y4

D

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6