1 **Original paper**

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3 **Vitamin D–dependent Cathelicidin Inhibits *Mycobacterium marinum* Infection in**

4 **Human Monocytic Cells**

5

6 **Running title**: Vitamin D–dependent CAMP inhibits *M. marinum* infection

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17 **Abstract**

18 **BACKGROUND**: 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) up-regulates the
19 production of human cathelicidin antimicrobial peptide (CAMP) from
20 monocytes/macrophages infected with *Mycobacterium tuberculosis* (*M. tbc*). CAMP
21 facilitates the co-localization of autophagolysosomes with *M. tbc*, promoting the
22 antimicrobial activity of monocytes. *Mycobacterium marinum* (*M. marinum*) is an
23 acid-fast bacillus that causes less severe granulomatous skin lesions compared with *M.*
24 *tbc*.

25 **OBJECTIVE**: We investigated whether autophagic antimicrobial activity is promoted
26 by 1,25(OH)2D3 or C-terminal of cathelicidin LL-37 in human monocytes upon
27 infection with *M. marinum*.

28 **METHODS**: Human monocytes (THP-1) were infected with *M. marinum*. Effects of
29 simultaneous treatments of 1,25(OH)2D3, exogenous LL-37 peptide,
30 autophagolysosome inhibitors, 3-methyladenine or chloroquine, were examined.

31 **RESULTS**: CAMP was strongly induced by adding 1,25(OH)2D3 to the culture of
32 THP-1 cells. In the absence of 1,25(OH)2D3 *M. marinum* infection alone did not induce
33 CAMP, however, simultaneous addition of 1,25(OH)2D3 to *M. marinum* infection
34 accelerated CAMP production more than 1,25(OH)2D3 alone. Proliferation of *M.*

35 *marinum* was markedly decreased in the presence of 1,25(OH)2D3 or exogenous LL-37

36 in THP-1 cells. Co-localization of CAMP with autophagolysosome was evident in

37 1,25(OH)2D3 and LL-37 treated THP-1cells after *M. marinum* infection.

38 Autophagolysosome inhibitors abrogated the antimicrobial effects of 1,25(OH)2D3 and

39 exogenous LL-37 against *M. marinum* infection in THP-1 cells.

40 **CONCLUSIONS**: Human monocytic cells, whose CAMP production is up-regulated

41 by 1,25(OH)2D3-vitamin D receptor pathway, accelerate antimicrobial function of

42 autophagolysosome in *M. marinum* infection.

43

44 Abbreviations: CAMP, cathelicidin antimicrobial peptide; VDR, vitamin D receptor;

45 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; *ATG*, autophagy-related genes: CQ,

46 chloroquine; 3-MA;3-methyladenine

47

48 **Keywords:** cathelicidin antimicrobial peptide; vitamin D3; autophagy; *Mycobacterium*

49 *marinum*

50 **INTRODUCTION**

51 The human cathelicidin antimicrobial peptide (CAMP) has been shown to
52 exhibit broad-spectrum antimicrobial activity against a range of Gram-positive and
53 Gram-negative bacterial species. CAMP gene is a direct target of the vitamin D receptor
54 (VDR) and is strongly up-regulated in various cells such as neutrophils, macrophages
55 and epithelial cells by 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3), an active form of
56 vitamin D[1,2]. CAMP becomes its mature extracellular form of 37 amino acid peptide,
57 LL-37, after truncation by protease, and executes its antimicrobial activity directly
58 against invading pathogens.

59 It has been shown that CAMP plays intracellular roles in addition to direct
60 antimicrobial activity against pathogens. Jo et al. have recently shown that
61 1,25(OH)2D3 can up-regulate the production of CAMP from monocytes/macrophages
62 infected with *Mycobacterium tuberculosis (M. tbc)*, and CAMP induces autophagy and
63 facilitates the co-localization of autolysosomes with *M. tbc* to promote the antimicrobial
64 activity of monocytes[3]. Autophagy is a generic term for all pathways by which
65 cytoplasmic materials are delivered to the lysosome in animal cells or the vacuole in
66 plant and yeast cells[4,5]. Autophagy occurs when an autophagosome (a double-membrane
67 vacuole) containing cytoplasmic material, fuses with a lysosome to deliver sequestered

material for lysosomal degradation[6], and multiple autophagy-related genes (*ATG*) proteins govern autophagosome formation[7]. Growing evidence suggests that autophagy contributes to the intracellular killing of *M. tbc* by facilitating phagolysosome fusion and thereby providing a mechanism to counteract the ability of *M. tbc* to evade the host response[8-10].

Mycobacterium marinum (*M. marinum*) is a nontuberculous photochromogenic mycobacterium species belonging to group I of Runyon's classification[11], and causes granulomatous skin lesions. The cutaneous infection caused by *M. marinum*, which mostly affects those who own aquariums or are in contact with fish, is the most common. The lesion, usually at the hand or forearm, is initially nodular but may subsequently ulcerate, whereas the sporotrichoid form is characterized by small nodules along lymphatic ducts[12]. *M. marinum* has been increasingly studied as a model of *M. tbc* due to its relative safety and its shared mechanisms of pathogenesis[13-15]. However it still remains to be determined whether 1,25(OH)2D3 and/or LL-37 can augment antimicrobial activity against *M. marinum* infection like against *M. tbc* infection as recently shown. Here we show that antimicrobial activity is promoted by autophagy via 1,25(OH)2D3 or LL-37 in human monocytes infected with *M. marinum*.

85 **Materials and Methods**

86 **Cells and Reagents**

87 THP-1 cells and U937 cells were purchased (American Type Culture Collection) and
88 maintained in RPMI 1640 (Wako, Osaka, Japan) with 10% FBS (GIBCO, Carlsbad, CA,
89 USA). Autophagy-related gene 5 deficient (*ATG5*$^{-/-}$) MEF cells were obtained from
90 Riken BioResource Center, Cell Bank (Ibaraki, Japan) and maintained in DMEM with
91 10% FBS[16]. 1,25(OH)2D3 (BIOMOL International) was added to the culture at 10 nM
92 and synthetic LL-37 peptide (Innovagen, Lund, Sweden) was added at 10 μg/ml.
93 Autophagy antagonists 3-methyladenine (3-MA) and chloroquine (CQ) (Sigma-Aldrich,
94 St Louis, MO, USA) were added to the culture at 1 μg/ml and 5 μM, respectively.
95

96 **Pathogens and Colony-Forming Unit (CFU) Assay**

97 *M. marinum* strain NJB0419 was obtained from Japan Anti-Tuberculosis Association
98 (Tokyo, Japan) and cells were infected with *M. marinum* at multiplicity of infection
99 (MOI) of 1–10. The efficiency of infection was determined by Ziehl-Neelsen staining.
100 Infected cells were treated with 1,25(OH)2D3 or LL37 in triplicate wells, and then
101 collected between 1–3 days. Cells were centrifuged at 2000 rpm for 5 minutes to form a
102 pellet and then the supernatant was aspirated. Intracellular *M. marinum* were obtained

103 by lysing the cells with 0.5% Triton-X100 in PBS. *M. marinum* isolates were 10–fold
104 serially diluted and plated on Middlebrook 7H10 (BD Bioscience, San Jose, CA, USA)
105 agar plates supplemented with 10% OADC Enrichment, then incubated at 30°C for 10
106 days. Colonies formed were counted as CFU for quantification of the mycobacteria. *M.*
107 *marinum* optical density (OD_{570}) is measured by Model 680 Microplate Reader
108 (BIO-RAD, CA, USA)
109

110 **Isolation of total RNA and quantitative RT-PCR**

111 Total RNA was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA, USA).
112 First-strand cDNA was synthesized from 1 μg of the total RNA using a SuperScript™
113 III First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR
114 (qRT-PCR) was performed using SYBR Premix DimerEraser™ (TaKaRa, Siga, Japan)
115 in a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The
116 gene-specific primer sequences are shown in Table 1. β-actin was amplified as an
117 internal control each time qRT-PCR was performed, and the ΔΔCt method was
118 employed to quantify the relative amounts of transcripts. The Student's *t*-test was used
119 for statistical analyses. A p value < 0.05 was considered statistically significant.
120

121 **Enzyme-linked immunosorbent assay (ELISA)**

122 Interleukin (IL)-12/23 p40, interferon (IFN)-β or tumor necrosis factor (TNF)-α in the
123 supernatants of treated cells was measured using commercially prepared ELISA plates
124 according to the manufacturer's suggestion (IL-12/23 p40 and TNF-α, R&D Systems,
125 Minneapolis, MN, USA; IFN-β, Peprotech, Rocky Hill, NJ, USA).

126

127 **Western blot**

128 2×10^5 cells were lysed and separated on 15% SDS-PAGE gels and transferred onto a
129 polyvinylidene difluoride membrane as described previously[17]. Membranes were
130 blocked in PBS containing 5% skim milk at room temperature for 1 h. The specific
131 proteins were determined by incubation with specific antibodies against human β-actin
132 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VDR or LL-37 (1:200,
133 Santa Cruz Biotechnology) at 25°C for 1h. After washing, the membrane was incubated
134 in a 1:3,000 dilution of a secondary antibody (sheep anti-mouse IgG-HRP conjugate;
135 GE Healthcare, Little Chalfont, UK) at room temperature in the washing buffer (PBS
136 containing 0.5% Tween 20) for 30min. The protein bands were visualized using ECL
137 Western Blotting Detection Reagents (GE Healthcare). Densitometry LAS-3000
138 software (FUJIFILM, Tokyo, Japan) was used to quantify the LL-37 protein levels, and

139 the levels were normalized to β-actin.

140

141 **Immunofluorescent staining**

142 Cells were seeded onto glass cover slips in 24-well plates. Cells were fixed in 3.7%

143 paraformaldehyde, incubated in 50 mM glycine for 5 minutes, permeabilized and

144 blocked with 1.5% BSA for 30 minutes. Immunostaining was performed using

145 polyclonal anti-LL-37 antibodies (1:1000, Innovagen) or monoclonal anti-LC3 (1:200,

146 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and LAMP-1 antibodies (1:200,

147 Santa Cruz Biotechnology). Secondary Alexa Fluor 488 and 546-conjugated antibodies

148 were obtained from Invitrogen and used by 1:2000. Cells were washed with PBS, and

149 cover slips were mounted using ProLong® Gold antifade Reagent with DAPI

150 (Invitrogen). Fluorescence images were acquired by BIOREVO BZ-9000 (Keyence,

151 Japan). Presented are representative results observed in the majority of cells from

152 several repeats.

153

154 **Lentiviral shRNA transduction to THP-1 cells**

155 VDR and control shRNA Lentiviral Particles were purchased from Santa Cruz

156 Biotechnology. RPMI 1640 containing virus (MOI:1) and polybrene (8 µg/ml) was

157 added to THP-1 cells, and seeded onto 24-well plates. The plate was centrifuged at 2000

158 rpm for 90 minutes, then RPMI 1640 was added and the cells incubated at 37°C

159 overnight. The next day, cells were washed and infected with virus using the same

160 protocol, and incubated for 24 hours. Then, medium was changed and the cells were

161 incubated for 72 hours. Single colony isolation was performed by growth in 50%

162 methylcellulose mixed-RPMI 1640 containing puromycin (4 μg/ml) for 4-5 weeks. The

163 protocol for transduction into THP-1 cells was a gift from the Department of Virology,

164 Kyushu University School of Medicine, Fukuoka, Japan.

165 **Results**

166 **LL-37 does not directly inhibit the growth of *M. marinum in extracellular culture.***

167 First, we tested the direct antimicrobial activity of the externally added LL-37

168 (10μg/mL) in *M. marinum in vitro axenic* culture in the absence of THP-1 cells. OD_{570},

169 which reflects the concentration of *M. marinum* did not show any difference between

170 cultures in the presence or absence of LL-37 and in fact there was no difference in CFU

171 at 72 hours, indicating that LL-37 does not directly inhibit the growth of *M. marinum* in

172 culture medium(Fig. 1A, 1B).

173

174 ***M. marinum* growth in THP-1 cells were suppressed by treatment with**

175 **1,25(OH)2D3 or LL-37**

176 Next, we investigated whether 1,25(OH)2D3 or extracellular LL-37 could suppress *M.*

177 *marinum* growth in THP-1 cells. THP-1 cells were infected with *M. marinum* at MOI of

178 1 in the presence or absence of 1,25(OH)2D3 (10 nM) or LL-37 (10 μg/ml) and

179 incubated for 24 to 72 hours (Fig. 2A, 2B). Intracellular *M. marinum* growth occurred

180 vigorously in THP-1 cells in the absence of 1,25(OH)2D3 or LL-37. Addition of

181 1,25(OH)2D3 or LL-37 significantly suppressed *M. marinum* growth as confirmed by

182 CFU (Fig. 2A). Similar results were obtained using other human monocyte cell line,

183 U937 cells (data not shown). Morphological analysis (Ziehl-Neelsen staining) revealed
184 that numerous live bacilli in the cytoplasm in THP-1 cells at 24hours after infection
185 with *M. marinum*, while simultaneous treatment of 1,25(OH)2D3 or LL-37 caused
186 marked degradation and decrement of bacilli inside the cells (Fig. 2B).
187

188 **Intracellular CAMP is increased in 1,25(OH)2D3–treated THP-1 cells**

189 We further investigated whether 1,25(OH)2D3 treatment could induce cathelicidin in
190 THP-1 cells. Analyses by qRT-PCR showed that CAMP mRNA was strongly up
191 regulated in uninfected THP-1 cells, and further up regulated in *M. marinum*-infected
192 cells following the addition of 1,25(OH)2D3 (10 nM) (Fig. 3A). In contrast, CAMP was
193 not induced by *M. marinum* infection alone or *M. marinum* supplemented with
194 exogenous LL-37. The presence of LL-37 was detected in the culture supernatant of the
195 1,25(OH)2D3–treated control culture, and was further increased when infected with *M.*
196 *marinum* (Fig. 3B). Similar results were observed at 72 hours after infection (data not
197 shown). IFN-β, TNF-α and IL-12p40 mRNA expressions were strongly induced by *M.*
198 *marinum* infection, and suppressed by simultaneous treatment with 1,25(OH)2D3 or
199 exogenous LL-37 (Fig. 3A). Concentrations of IFN-β, TNF-α, IL-12p40 in the
200 supernatant measured by ELISA showed comparable changes consistent with mRNA

201 data (Fig. 3B). These results suggest an anti-inflammatory function of cathelicidin
202 LL-37 peptide.
203
204 **Autophagy is induced in 1,25(OH)2D3 and LL-37–treated THP-1 cells 24 hours**
205 **after *M. marinum* infection**
206 Intracellular CAMP (green) levels increased in 1,25(OH)2D3–treated THP-1 cells, and
207 partly co-localized with LC3 protein (red) located on autophagosome membranes (Fig.
208 4A). LC3 (green) and LAMP1 (red), the main glycoprotein in lysosomal membranes,
209 were co-localized in 1,25(OH)2D3 or LL-37–treated THP-1 cells after *M. marinum*
210 infection (Fig. 4B). These results suggest that autophagolysosome is induced in THP-1
211 cells by simultaneous treatment with 1,25(OH)2D3 or LL-37 upon infection with *M.*
212 *marinum*.
213
214 **Blocking VDR by shRNA suppresses intracellular killing of *M. marinum* but**
215 **rescued by addition of external LL-37**
216 VDR–specific shRNA was designed to interfere with the transcription of VDR. Fig. 5A
217 showed a marked reduction of CAMP (18kDa) levels in VDR–shRNA treated cells in
218 the presence of 10 nM 1,25(OH)2D3 compared to control–shRNA treated cells. A

219 truncated form of CAMP (arrow head, 12kDa) was abundant in control cells infected

220 with *M. marinum*, but not in uninfected controls treated with 1,25(OH)2D3.

221 VDR-knock down cells showed increased CFU of *M. marinum* (Fig. 5B) in the presence

222 of 10nM 1,25(OH)2D3, suggesting that 1,25(OH)2D3 signaling through VDR is

223 required for the intracellular killing of *M. marinum*. We further investigated whether

224 reduced antimicrobial activity against *M. marinum* in shVDR-THP-1 cells can be

225 rescued by adding external LL-37. As expected, addition of LL-37 in shVDR-THP-1

226 cells recovered the killing activity against *M. marinum* (Fig. 5B).

227

228 **Autophagy antagonists or *ATG5* deficiency could not suppress the growth of *M.***

229 ***marinum*, and addition of external LL-37 did not recover the bactericidal activity**

230 Next, we tested whether blocking autophagy using autophagy antagonists resulted in the

231 suppression of intracellular killing of *M. marinum*. Growth of *M. marinum* in THP-1

232 cells was facilitated by two different autophagy antagonists, 3-MA or CQ (Fig. 6A).

233 And this loss of activity cannot be rescued by addition of external LL-37, suggesting

234 that autophagy is required for LL-37 to work. We also assessed the protective ability of

235 autophagy against *M. marinum* infection using cells from *ATG5*–deficient mice. Since

236 the *ATG5* deficiency in mice is lethal, *ATG5*$^{-/-}$ mouse embryonic fibroblasts (MEFs)

237 were used. In *ATG5*$^{-/-}$ MEF, increased *M. marinum* growth was observed compared to

238 wild type MEF (Fig. 6B).

239

240 **DISCUSSION**

241 In this study we demonstrated that 1,25(OH)2D3 treatment reduced *M.*
242 *marinum* survival through elevated intracellular and extracellular levels of CAMP
243 protein production in human monocytic cell lineTHP-1. Strikingly CAMP induction by
244 1,25(OH)2D3 was almost completely shut down by shRNA VDR knocked down in
245 human monocytic cells line THP-1 and antimicrobial activity of 1,25(OH)2D3 was
246 diminished by shRNA VDR knocked down in THP-1, while antimicrobial activity
247 induced by exogenous LL-37 remains intact with the same shRNA VDR knocked down
248 THP-1 cells. Furthermore addition of LL-37 to shRNA VDR knocked down THP-1cells
249 recovered antimicrobial activity against *M. marinum* infection. These results strongly
250 suggest that antimicrobial activity of 1,25(OH)2D3 against *M. marinum* is mediated by
251 induction of endogenous CAMP, which confirms the previous reports of studies using
252 *M. tbc*[3].

253 In this study we also explored the possible mechanism of antimicrobial activity
254 of CAMP in human monocytic cells against *M. marinum* infection. First we showed that
255 CAMP induced by 1,25(OH)2D3 co-localizes with autophagolysomes (LL-37/LC3,
256 LC3/LAMP1) in THP-1 cells after infection with *M. marinum*. In line with this it is of
257 note to mention the appearance of truncated form of CAMP (12KDa) in 1,25(OH)2D3

258 treated THP-1 cells after *M. marinum* infection (Fig.5A), which is consistent with the

259 findings of CAMP localization in autophagolysosome compartments. Furthermore we

260 found that 3-MA (autophagy inhibitor) and chloroquine (lysosomotropic agent that

261 prevents endosomal acidification and thus inhibits the formation of autophagolysosome)

262 prevent antimicrobial activity induced by CAMP against *M. marinum* infection in

263 THP-1 cells. In fact autophagy deficient cells (*ATG5*-/- MEF) failed to show the

264 antimicrobial activity induced by CAMP (endogenous CAMP induced by 1,25(OH)2D3

265 and exogenously LL-37) against *M. marinum* infection in THP-1 cells. These results

266 clearly indicate that both endogenous CAMP induced by 1,25(OH)2D3 and

267 exogenously added LL-37 exert antimicrobial activity against *M. marinum* by induction

268 of autophagolysosome.

269 Another finding of particular interest in this study is effect of 1,25(OH)2D3

270 treatment or exogenous LL-37 on inflammatory cytokine production in THP-1 cells

271 after *M. marinum* infection. As shown in Fig.3 we found that treatment with

272 1,25(OH)2D3 or LL-37 suppress IFNβ, TNFα and IL-12p40, suggesting the

273 anti-inflammatory role of VitD-CAMP in *M. marinum* infection. The intracellular roles

274 of CAMP have been sought in many aspects, and previous studies using various cell

275 types, including macrophages, dendritic cells[18-20], fibroblasts[21,22], keratinocytes[23],

276 pancreatic islet cells[24], and kidney cells[25] indicated that vitamin D can inhibit NF-κB
277 signaling, suggesting an immune regulatory function of cathelicidin. Several
278 mechanisms have been proposed, including vitamin D–induced increased levels of
279 IκBα[22-24], which interferes with the binding of NF-κB subunits to promoter regulatory
280 areas[20,21,25]. In addition to this, it has been shown that 1,25(OH)2D3 can reduce the
281 transcription and secretion of protective IFN-γ, IL-12p40 and TNF-α in *M. tbc* infected
282 peripheral blood mononuclear cells (PBMCs) and macrophages by regulation of RelB[18].
283 LL-37 suppresses the LPS-induced TNF-α response in PBMCs via NF-κB down
284 regulation and RelB involvement[19,20]. Activation of autophagy by 1,25(OH)2D3 or
285 LL-37 may also limit proinflammatory cytokine production by targeting inflammasome
286 or inflammasome-independent modulation of cytokine response[26-28]. Exogenous LL-37
287 suppressed the LPS-induced TNF-α and chemokine MCP-1 responses in peripheral
288 blood mononuclear cells (PBMCs)[19,20,29]. LL-37 also functions as an immune regulator
289 to control inappropriate immune responses to pathogen infection such as septic shock.
290 Furthermore, LL-37 inhibits the binding of LPS and lipopolysaccharide-binding protein
291 (LBP) thereby suppressing the production of proinflammatory cytokines such as
292 TNF-α[24,25]. In line with these studies, our current results of CAMP in *M. marinum*
293 infected THP-1 cells also suggest that cathelicidin may have unique suppressive role on

294 innate immune mediator such as IFNα, TNFα and IL-12, although further studies will

295 be needed to explore the precise mechanism of this anti-inflammatory role of CAMP in

296 human monocytic cells.

297 In summary we showed that both intracellular/extracellular CAMP/LL-37

298 suppress *M. marinum* infection via autophagolysosome in human monocytes and

299 1,25(OH)2D3 plays an important role in *M. marinum* infection. 1,25(OH)2D3

300 insufficiency is known to cause infection and relapse of tuberculosis[30]. Current study

301 demonstrates that 1,25(OH)2D3 has an important protective role against the infection

302 with another mycobacterium species, *M. marinum,* in addition to *M. tbc*. Analogues of

303 1,25(OH)2D3, such as calcipotriol, is used for the treatment of dermatologic diseases

304 such as psoriasis. Also, ultraviolet-light (UV) therapy is a common practice for various

305 dermatologic diseases. Our results might suggest that topical 1,25(OH)2D3 analogues

306 as well as UV therapy could be useful supplemental therapies for superficial infection of

307 *M. marinum* in combination with regular antibiotic therapy. Considering that activated

308 Vit D3 induced CAMP increases antimicrobial activity via autophagolysosome, while

309 down regulating pro-inflammatory cytokine production, topical therapy with active Vit

310 D3 may benefit to the prevention of immunohistopathological tissue damages.

311 **Figure legends**

312 **Figure 1. LL-37 did not directly kill the cultured *M. marinum.* A.** Growth curves of

313 *M. marinum* in 7H9 broth medium containing LL-37 (10⌈g/mL), or DMSO (0.1%).

314 OD_{570} was measured at time indicated. Data points represent the mean of five separate

315 experiments. **B.** Comparison of CFU at 72 hours after addition of LL-37. *M. marinum*

316 growth in 7H9 broth was not suppressed by LL-37 directly treatment (n=3).

317

318 **Figure 2. *M. marinum* growth in THP-1 was suppressed by treatment with**

319 **1,25(OH)2D3 or exogenous LL-37. A.** Comparison of CFU 72 hours after infection of

320 *M. marinum* (MOI: 1) in THP-1 cells. *M. marinum* growth in THP-1 was suppressed by

321 1,25(OH)2D3 or LL-37 treatment (n=3, *, **p<0.01). **B.** THP-1 cells did not suppress

322 intracellular *M. marinum* growth and live bacilli were observed in the cytoplasm. *M.*

323 *marinum* bodies (seen in control cells in the leftmost panel) were degraded in the cells

324 cultured with 1,25(OH)2D3 or LL-37 (Ziehl-Neelsen staining).

325

326 **Figure 3. Intracellular CAMP is increased in 1,25(OH)2D3–treated THP-1 cells 24**

327 **hours after *M. marinum* infection. A.** mRNA of cathelicidin is strongly up regulated

328 by 1,25(OH)2D3 in uninfected THP-1 cells. *M. marinum* infection (MOI: 1) in the

329 presence of 1,25(OH)2D3 further promoted the induction of cathelicidin. IFN-β, TNF-α,

330 and IL-12p40 induced by *M. marinum* infection was suppressed by the addition of

331 1,25(OH)2D3, or LL-37. **B.** Proteins in culture supernatants were measured by western

332 blotting or ELISA. CAMP expression was measured by western blotting and *M.*

333 *marinum* infected THP-1 cells secreted higher levels of LL-37 in culture supernatant

334 compared to controls. Concentrations of IFN-β, TNF-α, IL-12p40 in the supernatant

335 wee measured by ELISA and showed proportional changes consistent with the mRNA

336 data.

337

338 **Figure 4. Autophagy is induced in 1,25(OH)2D3 and LL-37–treated THP-1 cells 24**

339 **hours after *M. marinum* infection. A.** Immunofluorescence staining 24 hours after *M.*

340 *marinum* infection. Intracellular CAMP (green) was increased in 1,25(OH)2D3–treated

341 THP-1 cells, and partly co-localized with LC3 (red), a protein localized on the

342 autophagosome membrane. **B.** Immunofluorescence staining 24 hours after *M. marinum*

343 infection. LC3 (green) and LAMP1 (red), a main glycoprotein in the lysosomal

344 membrane, were co-localized in 1,25(OH)2D3 or LL-37–treated THP-1 cells after *M.*

345 *marinum* infection.

346

347 **Figure 5. Blocking VDR by shRNA suppressed intracellular killing of *M. marinum*.**

348 **A.** Western blotting of THP-1 cell lysates treated with VDR–shRNA showed marked

349 reduction of CAMP in cells in the presence of 10 nM 1,25(OH)2D3 compared to

350 control–shRNA treated cells. In control cells infected with *M. marinum* (MOI: 1), a

351 truncated form of CAMP (arrow head) was abundant compared with

352 1,25(OH)2D3–treated uninfected controls. **B.** Cells treated with VDR–shRNA showed

353 increased CFU upon infection with *M. marinum* (MOI: 10) in the presence of 10nM

354 1,25(OH)2D3 but rescued by external LL37 (n=3, *,** p<0.05).

355

356 **Figure 6. Autophagy antagonists or *ATG5* deficiency suppressed intracellular**

357 **killing of *M. marinum*. A.** Comparison of CFU 72 hours after infection of *M. marinum*

358 (MOI: 10) in THP-1 cells. *M. marinum* growth in THP-1 was significantly promoted by

359 3-MA or CQ and cannot be rescued by addition of external LL-37. (n=3, *, **p<0.01).

360 **B.** Comparison of CFU 72 hours after infection of *M. marinum* (MOI: 10) in mouse

361 embryonic fibroblast (MEF) cells. *M. marinum* growth in *ATG5*$^{-/-}$ MEFs was

362 significantly increased compared to controls (n=3, *p<0.05).

363

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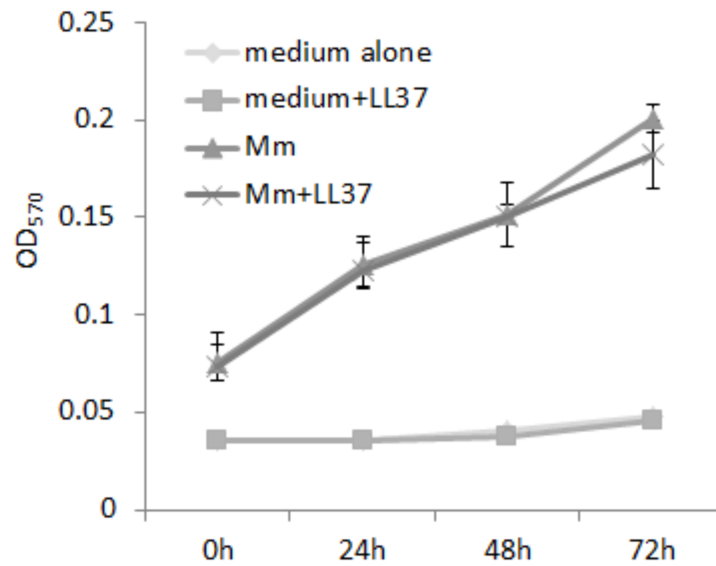
Figure 1.



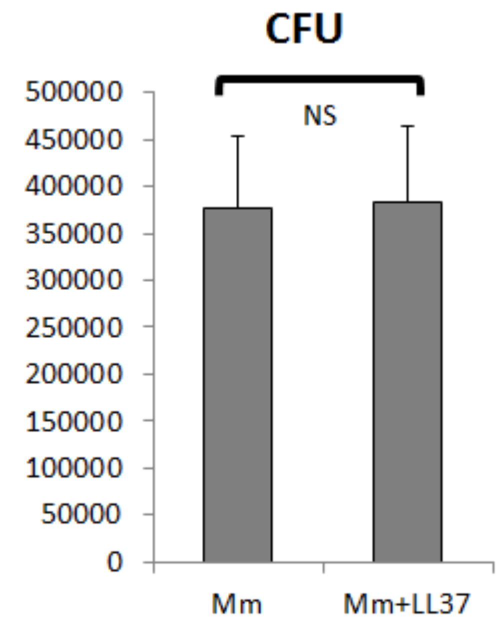


Figure 2.

A)

M. marinum

1200000
1000000
800000
600000
400000
200000
0

CFU/well

*
**

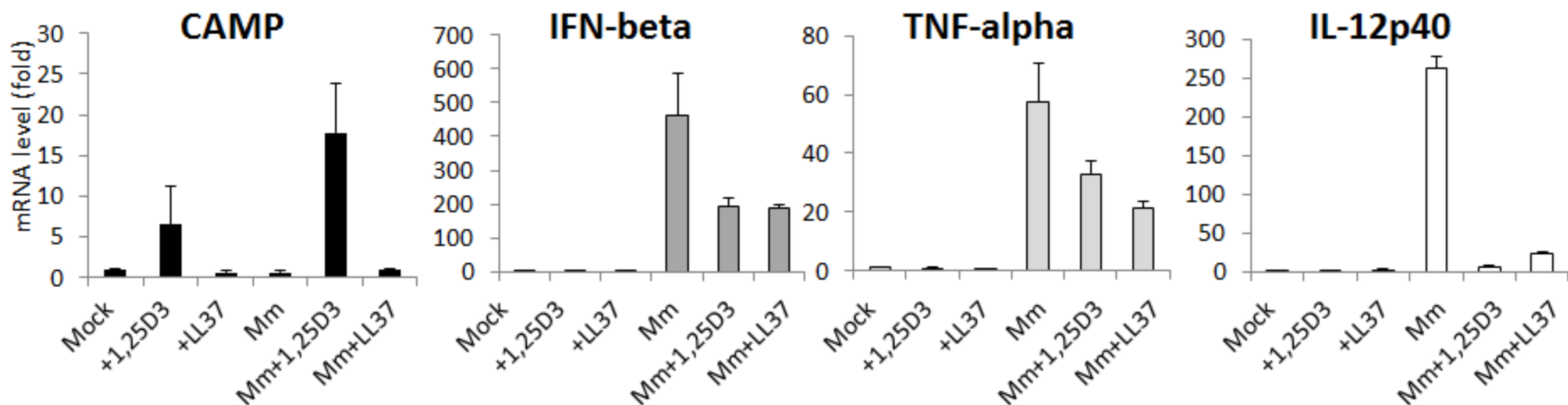
Mm
Mm+1,25D3
Mm+LL37

B)

Mm
Mm+1,25D3
Mm+LL37

Figure 3.

A)



B)

LL37

IFN-beta

TNF-alpha

IL-12p40

A.U.

300000 250000 200000 150000 100000 50000

pg/mL

250 200 150 100 50 0

80 70 60 50 40 30 20 10 0

250 200 150 100 50 0

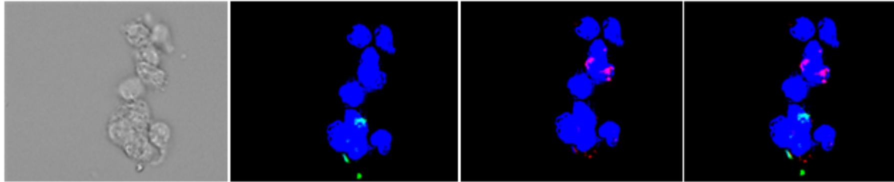
Mock +1,25D3 Mm Mm+1,25D3

Mock +1,25D3 +LL37 Mm Mm+1,25D3 Mm+LL37

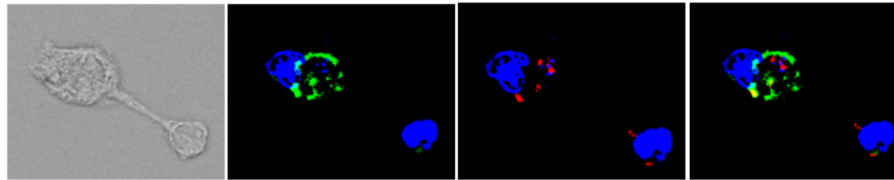
Figure 4.

A)

Mm



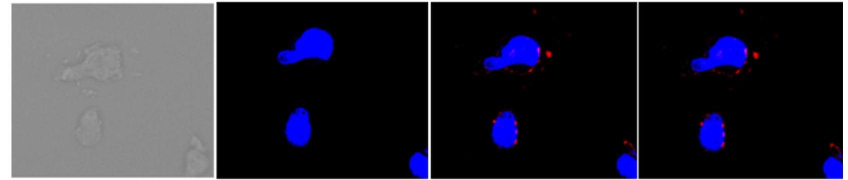
Mm+1,25D3



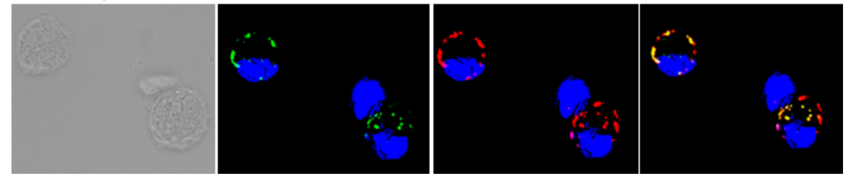
LL37 LC3 Merge

B)

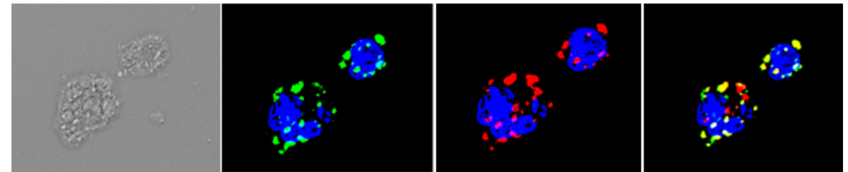
Mm



Mm+1,25D3

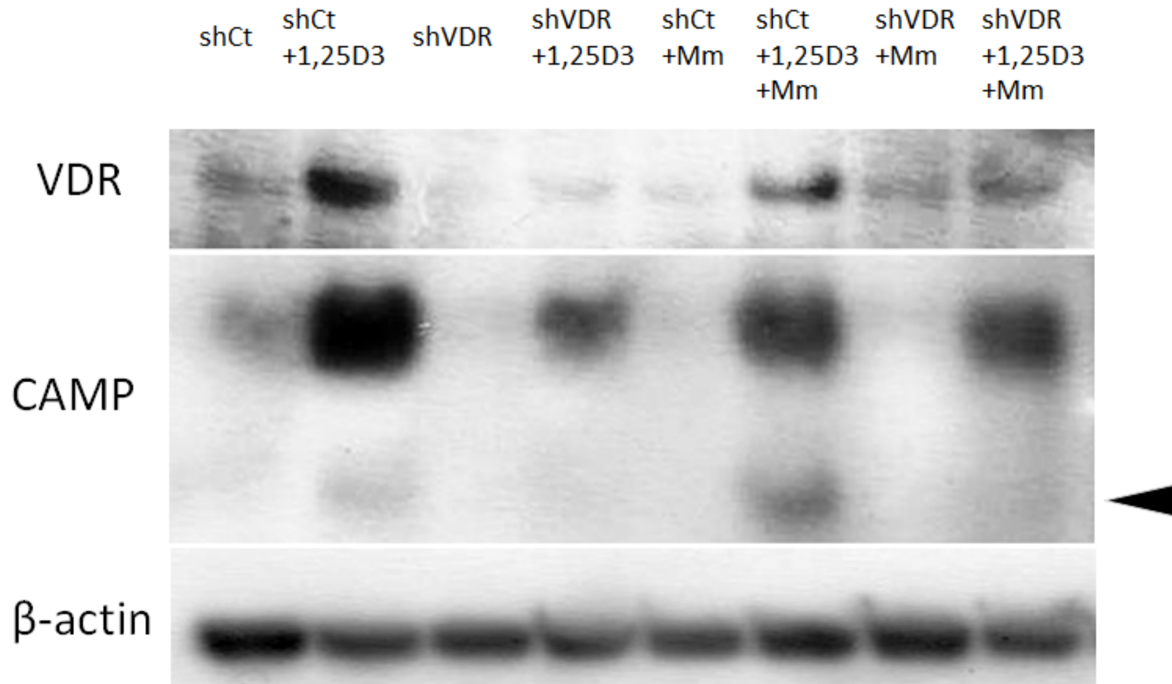


Mm+LL37



LC3 LAMP1 Merge

Figure 5.



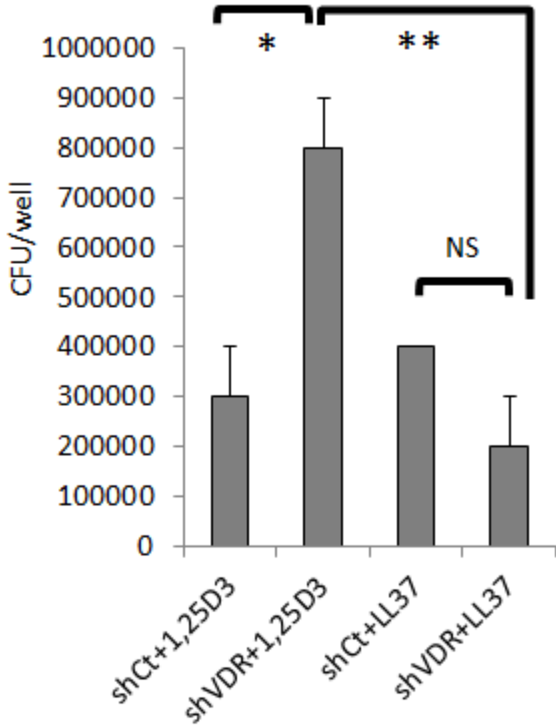


Figure 6.