

1 **Immunomodulatory effects of extract of *Ganoderma lucidum* basidiocarps cultivated
2 on alternative substrate**

3

4 **Running title: Immunopotential of alternatively cultivated *G. lucidum***

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23

24 **Abstract**

25 *Background:* *Ganoderma lucidum* is a medicinal mushroom exhibiting numerous health
26 benefits primarily based on strong immunostimulatory effects. The aim of the study was
27 to investigate if there were differences in effects of extracts of commercially (GC) and
28 alternatively (wheat straw) (GA) cultivated *G. lucidum* basidiocarps on properties of
29 peritoneal macrophages (PM) and monocyte-derived dendritic cells (MoDCs).

30 *Methods:* Differences in immunomodulatory effects of GC/GA extracts were studied.
31 Viability of treated PMs, their adhesive and phagocytic capability and capacity to produce
32 reactive oxygen species (ROS) and NO was tested. Immature MoDCs generated from
33 human monocytes were treated with poly I:C (10.0 µg/ml) and loxoribine (34.0 µg/ml), a
34 selective TLR3 and TLR7 agonists, respectively, and with/without GC/GA extract (100.0
35 µg/ml). The effect of each combination on phenotypic properties, cytokines production by
36 MoDCs, and their proliferation and Th polarizing capacity was studied.

37 *Results:* GA extract stronger stimulated metabolic and phagocytic activity of PMs, their
38 adhesion capability and ability to produce ROS and NO compared to GC. Both tested
39 extracts significantly increased allostimulatory and Th1 polarization capacity of
40 simultaneous TLR3 and TLR7-activated MoDCs, but GA extract was more effective.

41 *Discussion:* The extract of alternatively cultivated *G. lucidum* basidiocarps increased
42 production of ROS and NO by TLR4 stimulated PMs and up-regulated production of
43 certain cytokines as well as allostimulatory and Th1 polarization capacity of MoDCs. GA
44 extract could be a potent immunostimulatory agent for activation of MoDCs with
45 simultaneous engagement of TLRs that seems to be a promising strategy for preparation
46 of DC-based anti-tumor vaccines.

47

48 *Keywords:* Macrophages; Dendritic cells; *Ganoderma lucidum*; Wheat straw
49 Immunomodulators.

50

51 **Introduction**

52

53 *Ganoderma lucidum* (Curtis) P. Karst., known as Reishi, is a popular medicinal
54 mushroom used in traditional medicine for the prevention and treatment of various
55 pathological conditions. Today, there is evidence that bioactive constituents of this
56 species are responsible for numerous health benefits due to strong immunomodulatory,
57 antitumor, antioxidative, anti-inflammatory, antimicrobial and many other activities (Lin
58 et al., 2006a; Yuen et al., 2008; Wasser, 2010; Joseph et al., 2011). Therefore,
59 commercial production of this mushroom is continuously growing and nowadays
60 emphasis is put on creation of system for cheaper, easier, faster and environmental
61 friendly cultivation of biologically more active fruiting bodies. Thus, fruiting bodies with
62 higher immunostimulatory potential could be applied as natural pharmaceutical agents in
63 immunotherapy of primarily patients suffering from various tumor types (Park et al.,
64 2018).

65 The antigen-presenting cells (APCs) are common checkpoint for stimulation of
66 immune system and induction of potent antitumor response in cancer treatment (Martin et
67 al., 2015). They include dendritic cells (DCs), macrophages and B lymphocytes, which
68 participate in capturing, processing and presenting antigens to T lymphocytes (Flaherty,
69 2012). Macrophages and DCs are the powerful phagocytic cells, key players in the innate
70 immune system and link between innate and adaptive immunity, which are derived from
71 peripheral blood monocytes and exist in almost all tissues (Clark et al., 2000; Hirayama et
72 al., 2018). Activated macrophages produce numerous bioactive compounds, including
73 reactive oxygen species (ROS), nitric oxide (NO), an important mediator of innate
74 immune response on various pathological stages, as well as cytokines, primarily
75 interleukins (IL-1, IL-6), tumor necrosis factor α (TNF- α), and interferon- γ (IFN- γ),
76 which are crucial in recruitment and activation of other immune cells and stimulation of
77 adaptive immunity (Hirayama et al., 2018). In the presence of microbes or inflammatory
78 stimuli, DCs undergo a complex process of maturation that includes up-regulation of co-
79 stimulatory molecules, migration to lymph nodes, T lymphocytes priming and cytokine
80 production (Clark et al., 2000). These potent APCs express various pattern recognition

81 receptors (PRRs) and in such a way trigger signaling pathways resulting in their
82 phenotypic changes and functional maturation (Martin et al., 2015). Toll-like receptors
83 (TLRs) present an important group of PRRs on the macrophages and DCs surface and
84 crucial factors for recognition of viruses, bacteria, fungi, and parasites, i.e. they play a key
85 role in innate immunity (Kawai and Akira, 2009). These authors emphasized that ligation
86 of different TLRs by specific TLR agonists presents a powerful tool for induction of DCs
87 maturation. TLR agonists are used as adjuvants or immune modifiers in DC-based trials
88 of tumor immunotherapy (Bhardwaj et al., 2010). However, since single TLR agonist has
89 relatively limited adjuvant effects on DC phenotype and function, current studies are
90 focused on research of synergy between paired TLR agonists (Yheng et al., 2008).

91 Starting from the fact emphasized by Pi and colleagues (Pi et al., 2014) that
92 polysaccharides of *Ganoderma* spp. possess strong immunostimulatory activity based on
93 their recognition as foreign molecules by various PRRs on DCs, and consequently on
94 stimulation of APCs maturation, the aim of this study was defined. In our study we
95 investigated the potential synergism of extracts of commercially (GC) and alternatively
96 (wheat straw) (GA) cultivated *G. lucidum* basidiocarps with different TLRs on different
97 APCs. Namely, we investigated the immunomodulatory effects of GC and GA extracts on
98 functional properties of peritoneal macrophages stimulated by TLR4 and on functional
99 characteristics of human monocyte-derived dendritic cells stimulated by simultaneous
100 engagement of TLR3 and TLR7.

101

102 **Materials and methods**

103 *Organism and growth conditions*

104 The culture of *Ganoderma lucidum* BEOF B 431, isolated from fruiting body collected
105 in Bojčin forest (Belgrade, Serbia), is maintained on Malt agar medium in the culture
106 collection of the Institute of Botany, Faculty of Biology, University of Belgrade. The
107 fruiting bodies were cultivated on alternative substrate consisted of wheat straw under
108 laboratory conditions (Ćilerdžić et al., 2018a). Basidiocarps of a commercial Chinese
109 strain, cultivated on oak sawdust, were purchased at a health food store.

110

111 *Preparation of the basidiocarp extracts*

112 The dried and pulverized commercially and alternatively produced *G. lucidum*
113 basidiocarps (2.0 g) were extracted with 60.0 mL of 96% ethanol by stirring on a
114 magnetic stirrer (150 rpm) for 72 h. The resultant extracts were centrifuged (20 °C, 3000
115 rpm, 10 min) and supernatants were filtered through Whatman No.4 filter paper,
116 concentrated under reduced pressure in a rotary evaporator (Büchi, Rotavapor R-114,
117 Germany) at 40 °C to dryness, and redissolved in 5% dimethyl sulphoxide (DMSO) to an
118 initial concentration of 10.0 mg/mL.

119

120 *Experimental animals*

121 All animal experiments were approved by the Ethics Review Committee for Animal
122 Experimentation of Military Medical Academy and Ministry of Agriculture and
123 Environmental Protection of Republic of Serbia (Veterinary Directorate No. 323-07-
124 7363/2014-05/5). Inbred male Albino Oxford rats (AO; Vivarium for Small Experimental
125 Animals, Military Medical Academy, Belgrade) weighting about 200-220 g were housed
126 in an air-conditioned room at 25 °C on a 12h-light/dark cycle. Animals were provided
127 pelleted food (Veterinary Institute, Subotica) and tap water *ad libitum*. Sacrifice was done
128 with intravenous injection of Ketamin/Xilazyn in a lethal dose. All procedures were done
129 in accordance with the Guide for the care and use of laboratory animals.

130

131 *Peritoneal macrophages isolation and experimental design*

132 The medium used for the cell isolation and incubation was HEPES-buffered Roswell
133 Park Memorial Institute medium (RPMI-1640) supplemented with fetal calf serum (FCS)
134 (Flow, Irvine, Ca, USA), glutamine (ICN Flow, SAD), penicillin, and gentamicin
135 (Galenika a.d.d., Serbia). Peritoneal cells were obtained by sterile lavage with RPMI
136 medium supplemented with 2% FCS and heparin (Galenika a.d.d., Serbia). Enrichment of
137 peritoneal cell exudates with PMs was enabled using density gradient OPTIPREP
138 (Nycomed Pharmas, Norway) with 0.8% NaCl. After centrifugation on gradient,
139 mononuclear cells (highly enriched with PM, >90%) were washed and resuspended in
140 RPMI-1640 supplemented with 10% FCS and cell number was adjusted to 10⁶ cells/mL.

141 Afterward, the cells were seeded in 96-well plate in two ways: *i*) 1×10^5 cells per well for
142 testing the viability and production of phagocytic activity, ROS, NO and cytokine and *ii*)
143 5×10^5 cells per well for assessment of adhesion capacity and.

144 Peritoneal macrophages (PMs) isolated in this way were cultivated under standard
145 conditions (37°C , 5% CO_2) for 24 h, and treated with GC and GA extracts in final
146 concentration of 100.0, 10.0 and 0.1 $\mu\text{g}/\text{ml}$ per well in presence or absence of adequate
147 stimulator (in dependence of evaluated function). Lipopolysaccharide (LPS, Sigma,
148 USA), TLR4 agonist, at final concentration of 100.0 ng/mL per well, was used as a
149 stimulator for assessment of metabolic viability, phagocytic activity, and NO production.
150 Adhesion capacity and ROS production were assessed by phorbol-myristate-acetate
151 (PMA, Sigma, USA) at the final concentration of 250.0 ng/mL per well. Control cells
152 were cultivated under standard conditions, with or without TLR4 agonist and were not
153 treated with GC and GA extracts. All studied functions of PMs were observed after 24 h
154 cultivation *in vitro* and were done in quadruplicate.

155

156 *Cell viability assay*

157 Cell viability was estimated by a quantitative colorimetric assay described for human
158 granulocytes which was based on metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-
159 2,5-diphenyltetrazolium bromide (MTT, Invitrogen) into colored product formazan (Oez
160 et al., 1990a). MTT assay was conducted with 24 h cultivated PMs and MTT which was
161 added in the concentration of 5.0 mg/mL (10.0 μL per well), which were incubated at 37°C
162 in an atmosphere of 5% CO_2 and 95% humidity for 3 h. The absorbance of produced
163 formazan after overnight incubation in the solution composed of sodium dodecyl sulphate
164 (SDS) and HCl (10% SDS with 0.01 N HCl) was measured at dual wavelengths, 570/650
165 nm by an ELISA 96-well plate reader (Behringer, Germany). Cells viability was
166 expressed as absorbance of solubilized formazan at the end of incubation period.

167

168 *Phagocytosis assay*

169 Phagocytic capacity of PMs was determined according to the technique described by
170 Chen and colleagues (Chen et al., 2015). After 24 h cultivation PMs without/with

171 stimulators and GC/GA extract, the supernatants were collected and 50 μ l /well of neutral
172 red (1:300) was added and incubated for 4 h. After incubation, supernatants were
173 discarded, cells were washed with phosphate-buffered saline (PBS) three times and lysed
174 by adding 100.0 μ L/well of cell lysing solution (ethanol and 1% acetic acid at the ratio of
175 1:1), and absorbance of the solution was measured at 540/650 nm using Microplate
176 Reader (Behringer, Germany).

177

178 *Adhesion capacity assay*

179 Adhesion capacity of PMs was assessed by a method of Oez and colleagues (Oez et
180 al., 1990b) based on the cell ability to adhere to plastic matrix. After the 24 h cultivation
181 without/with stimulators and GC/GA extract, supernatants were removed and cells were
182 washed three times with warm PBS in order to remove non-adhered cells. Then in the
183 each well added methanol (100.0 μ L/well) and it was incubated for 7 minutes. Attached
184 cells were dyed with 0.1% solution of methyl blue (100.0 μ L/well) for 15 minutes and
185 washed three times with tap water. Plates were left to dry on air over night and color was
186 dissolved by adding 0.1 N HCl (200.0 μ L/well). Absorbance of the solution in the each
187 well was measured at 650/570 nm using Microplate Reader (Behringer, Germany).

188 *NBT reduction assay*

189 NBT assay was used to evaluate generation of superoxide anion (O_2^-) produced by
190 PMs (Pick et al., 1981). Briefly, nitroblue tetrazolium (NBT, Invitrogen), in final
191 concentration of 0.5 mg/mL per well, was added to PMs suspension after 24 h treatment
192 of PMs without/with stimulators and GC/GA extracts and the mixture was incubated at
193 37 °C in an atmosphere of 5% CO₂ and 95% humidity for one hour. Formed diformazan
194 crystals were dissolved by adding SDS-HCl mixture (100.0 μ L/well) and optical density
195 was measured at 570/650 nm by Microplate reader (Behringer, Germany).

196

197 *Determination of NO production*

198 Production of NO was quantified by the accumulation of nitrite as a stable end-product
199 and determined by a Greiss reaction assay (Green et al., 1982). Equal volumes of the
200 supernatants and Griess reagent [0.35% 4-aminophenyl sulfone (Sigma-Aldrich,

201 Germany), 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 1M HCl (POCh,
202 Poland)] were incubated at room temperature (22 ± 2 °C) for 10 minutes. Optical density
203 of solution was measured at 540/650 nm using Microplate Reader (Behringer, Germany).
204 The nitrite concentration (μM) was calculated from the prepared standard curve for the
205 known NaNO₂ concentrations.

206

207 *Preparation and treatment of human monocyte-derived dendritic cells*

208 Immature monocyte-derived dendritic cells (MoDCs) were generated from adherent
209 fraction of human peripheral blood mononuclear cells (PBMCs). Namely, PBMCs from
210 buffy coats of healthy volunteers (upon written an informed consent) were isolated by
211 density centrifugation in Lymphoprep (Nycomed, Oslo, Norway), re-suspended in 5.0 mL
212 of 10% FCS with 2-Mercaptoethanol (2-ME) in RPMI medium and allowed to adhere to
213 plastic flasks. After incubation at 37 °C for 90 minutes, non-adhered cells were removed
214 and adhered cells were cultured in 5.0 mL of RPMI medium containing granulocyte-
215 macrophage colony-stimulating factor (GM-CSF; 100.0 ng/mL) and IL-4 (20.0 ng/mL).
216 On day three, a half of medium volume was removed and replaced with the same volume
217 of fresh medium containing GM-CSF and IL-4 and it was incubated for next two days. At
218 the end of incubation period (on day 5), MoDCs (5×10^5 cells/well) were moved in 24-
219 well plate, in RPMI medium containing GM-CSF/IL4. After sixt days, immature MoDCs
220 were replated (5×10^5 cells/ml) in medium with different combination of TLR3 agonist
221 (Poly (I:C), Sigma-Aldrich, Germany, 10.0 μg/mL) and TLR7 agonist (Loxoribine
222 Sigma-Aldrich, Germany, 34.0 μg/mL) with GC and GA extracts (100.0 μg/mL), and
223 incubated for 24 h. Afterwards, cell-free supernatants were collected for cytokine
224 analysis, while cells were detached and their phenotype was observed. Cell-free
225 supernatants were collected and stored at – 20 °C for the subsequent determination of
226 cytokine levels. The cells were used for further studies.

227

228 *Flow cytometry analysis of MoDCs for immunophenotyping*

229 Control and treated MoDCs (2×10^5 cells/tube) were washed in PBS supplemented
230 with 2% FCS and 0.1% NaN₃, and incubated at 4 °C for 45 min with one of the following

231 monoclonal antibodies (mAbs): HLA-DR coupled with phycoerythrin (PE), CD83
232 conjugated with fluorescein isothiocyanate (FITC), CD86-PE, CD40-FITC, CD54-PE
233 (Serotec, Oxford, UK), and CCR7-FITC (R&D Systems, Minneapolis, MN, USA). The
234 control MoDCs incubated with irrelevant mouse Mab reactive with rat antigens were used
235 as the irrelevant control. The cells were incubated at 4 °C for 45 min, washed and fixed
236 with 1% paraformaldehyde. For cell fluorescence analysis, at least 5×10^3 cells per sample
237 were analyzed using EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany). Cell-
238 surface expression on MoDCs was determined by means of a forward versus side scatter
239 gate.

240

241 *Mixed leukocyte reaction with allogeneic CD4⁺ T lymphocytes*

242 Conventional CD4⁺ T lymphocytes were isolated from PBMCs using negative
243 immunomagnetic sorting with CD4⁺ T lymphocytes isolation MACS kit (Mytenyi
244 Biotec, Germany). According to flow cytometry analysis, purity of isolated CD4⁺ T
245 lymphocytes was higher than 95%. Purified allogenic CD4⁺ T lymphocytes (10^5
246 cells/well) were placed in 96-well plates and cultivated with MoDCs pre-treated with
247 TLR agonists and *G. lucidum* extracts in RPMI medium with 10% FCS. Cell proliferation
248 was detected after five days of cultivation. Cells were pulsed with [³H] thymidine (1.0
249 μ Ci/well; Amersham, UK) for the last 18 h of cultivation, then harvested onto glass fiber
250 filters, and [³H] thymidine incorporation into DNA was measured by β -scintillation
251 counting (LKB-1219; Rackbeta, Finland). Results were expressed as counts per minute
252 (c.p.m.).

253

254 *Evaluation of cytokine production*

255 The levels of TNF- α , IL-12, IL-6, and IL-10 were measured in the cell-free
256 supernatants of control and treated MoDCs cultures (4×10^5 cells/mL) by ELISA assays
257 (R&D Systems, Minneapolis, USA). The levels of cytokines produced by CD4⁺ T
258 lymphocytes in co-cultures with MoDCs were evaluated in the cell-free supernatants of
259 co-cultures. Briefly, purified allogenic CD4⁺ T cells (1×10^5 cells/well) were cultivated
260 with MoDCs (1×10^4 cells/well) pre-treated with TLR agonists and *G. lucidum* extracts in

261 RPMI medium with 10% FCS in 96-well plates. Phorbol-myristate-acetate (20.0 ng/mL)
262 and ionomycin (500.0 ng/mL) (Merck, Austria) were added to the wells after five days.
263 Cells were incubated for an additional 8 h and then harvested, and cell-free supernatants
264 were collected after centrifugation and stored at -20 °C for the subsequent determination
265 of the studied cytokine levels.

266

267 *Statistical analysis*

268 All measurements were done in quadruplicates in double experiments and obtained
269 results were reported as mean \pm standard error. Data were analyzed for significant
270 differences using Student's paired *t*-test. A *p* value less than 0.05 was considered to be
271 statistically significant.

272

273 **Results**

274

275 *Effect of Ganoderma lucidum extracts on metabolic activity/viability of PMs*

276 The extracts of commercially and alternatively cultivated *G. lucidum* basidiocarps (GC
277 and GA, respectively) have no effect on metabolic activity/viability of non-stimulated
278 PMs, except GC extract in concentration of 100.0 μ g/mL which affected inhibitory (Fig.
279 1A). However, the all tested extracts (except GC in a concentration of 100.0 μ g/mL)
280 showed stimulatory effect on metabolic activity/viability of LPS-stimulated PMs.
281 Furthermore, in comparison with GC, GA has the stronger effect in concentration 10
282 μ g/ml and 100 μ g/ml.

283

284 *Effect of Ganoderma lucidum extracts on adhesive capability and phagocytic activity of
285 PMs*

286 Effect of *G. lucidum* extracts cultivated on different substrates (commercially and
287 alternatively) on adhesion capacity of non-stimulated and PMA-stimulated PMs are
288 shown on Fig. 2. Treatment of non-stimulated PMs with GC extract did not modulate
289 their adhesive capability, while treatment with GA extract, in all tested concentrations,
290 enhanced their adhesive function compared to ability of non-treated and GC treated PMs

291 (Fig. 2A). Comparing with a control, GC extract had no effect on adhesive capacity of
292 PMA-stimulated PMs, while GA extract (in all tested concentrations) enhanced this
293 ability (Fig. 2B).

294 GC and GA extracts showed no significant effect on phagocytic function of non-
295 stimulated PMs (data not shown). However, in the case of LPS-stimulated PMs, GA
296 extract (in all tested concentrations) statistically considerable up-regulated this activity in
297 comparison with non-treated PMs and PMs treated with GC extract (Fig. 3).

298

299 *Effect of Ganoderma lucidum extracts on ROS and NO production by PMs*

300 The treatment of non-stimulated PMs with GC and GA extracts no affected their
301 potential of ROS production (data not shown). Comparing with adequate control cells,
302 GC extract had no effect on ROS production by LPS-stimulated PMs, contrary to GA
303 extract which statistically significant increased this ability (Fig. 4).

304 Treatment of non-stimulated and LPS-stimulated PMs with GC and GA extracts, in all
305 tested concentrations (except treatment of non-stimulated cells with 100.0 µg/mL of GC
306 extract) caused increase of NO production. Furthermore, GA extract was statistically
307 stronger inducer of NO production by tested PMs than GC extract (Fig. 5).

308

309 *Effect of Ganoderma lucidum extracts on phenotype and cytokine production by MoDCs*

310 Phenotype analysis showed that GC and GA extracts did not affect MoDCs phenotype
311 measured by percent of HLA-DR, CD83, CD86, CD40, and CCR7 positive cells (data not
312 shown). Simultaneous treatment of MoDCs with treatment with Poly (I:C), Loxoribine
313 and GC or GA extracts up-regulated mean of fluorescence (MnI) of CD83 and HLA-DR.
314 Additionally, thus simultaneous treatment of TLR 3 and TLR 7-stimulated MoDCs with
315 GA extract also affected the expression of co-stimulatory molecules, CD86 and CD40, as
316 well as chemokine receptor, CCR7 (Fig. 6A).

317 The additions of GC extract stimulated production of IL-6 and TNF-α, had no effect
318 on production of IL-12, and inhibited the production of IL-10 by TLR3 and 7-stimulated
319 MoDCs. On the other hand, GA extract inhibited the production of IL-6 and IL-10 and

320 had no effect on TNF- α and IL-12 production by studied MoDCs compared to MoDC
321 treated only with TLR3 and TLR7 antagonist (Fig. 6B).

322

323 *Effect of Ganoderma lucidum extracts on alostimulatory and Th cells polarization*
324 *capacity of MoDCs*

325 Ability of TLR3 and TLR7-stimulated MoDCs to induce the proliferation of allogenic
326 CD4 $^{+}$ T lymphocytes was not changed by addition of GC extract, while GA extract
327 significantly enhanced this ability (Fig. 7A). MoDCs treated with TLR3 and TLR7
328 agonists and with GC extract down-regulated production of IFN- γ and had no effect on
329 IL-17 production by allogenic CD4 $^{+}$ T lymphocytes compared to effect of MoDCs treated
330 only with TLR3 and TLR7 agonists. On the other side, GA extract in combination with
331 TLR3 and TLR7 changed the properties of MoDCs and thus stimulated MoDCs up-
332 regulated IFN- γ synthesis and had no effect on IL-17 generation by allogenic CD4 $^{+}$ T
333 lymphocytes in comparison with TLR3 and TLR7-stimulated MoDCs (Fig. 7B).
334 Additionally, GA extract in combination with TLR3 and TLR7 up-regulated IL-17
335 synthesis by allogenic CD4 $^{+}$ T lymphocytes in comparison with GC, TLR3 and TLR7-
336 stimulated MoDCs (Fig. 7B).

337

338 **Discussion**

339 Cooperation of different PRR signals in APC during the induction of immune
340 responses is an emerging field in innate immune research. Activation of two or more
341 TLRs, or other PRRs at the same time, which mimic the actual situation during host cell–
342 microbe interaction, may lead to synergistic, antagonistic, or additive effects (Mäkelä et
343 al., 2009). On the other side, *Ganoderma* spp. extracts and metabolites (derived from
344 wild and/or traditionally cultivated basidiocarps) express strong immunomodulatory
345 characteristics and present an effective modifiers of different biological processes (Lin et
346 al., 2006b; Boh, 2013; Shi et al., 2013; Liu et al., 2016a,b). According to available
347 literature, there is no knowledge about the immunomodulatory potential of crude extracts
348 of *G. lucidum* basidiocarps cultivated on any alternative and environmentally friendly
349 substrate. This is the first report on it and our findings showed that crude extract of *G.*

350 *lucidum* basidiocarps cultivated on nutritionally poor wheat straw contained molecules
351 with higher immunomodulatory potentials that in cooperation with agonists of different
352 TLRs induced significantly better activation of APCs compared to effect of TLRs alone.
353 Thus, the GA extract expresses better immunostimulatory potential in cooperation with
354 TLR4 agonist in up-regulation of PMs functional characteristics compared to GC extract
355 as well as induction of better allostimulatory and Th1 polarization capacity of MoDCs
356 treated in cooperation with TLR3 and TLR7 antagonists. Therefore, extract of
357 alternatively obtained basidiocarps could be an effective additional agent during
358 simultaneous engagements of different TLRs on APCs for *in vitro* preparation of APCs as
359 tool for anti-tumor therapy.

360 Today, it is well known that fungi possess potential for production of high diversity
361 low molecular weight products (secondary metabolites) with various biological activities
362 that are mainly important for their interactions with other organisms (Brakhage and
363 Schroeckh, 2011). These secondary metabolites are present in a small amount and their
364 composition/combination depends on the type of substrate on which they are cultivated. It
365 may be a reason for different immunomodulatory effects of GC and GA extracts noted in
366 this study. The literature data and data of this study indicate that the study of the *in vitro*
367 cultivation conditions that could possibly optimize their production seems of major
368 importance. Another very important fact in recent modern period is the need to find a
369 cost-effective and environmental friendly alternative substrate for *G. lucidum* cultivation
370 which classically was grown on sawdust of sheesham, mango, and poplar.

371 The genus Ganoderma (especially *G. lucidum*), has been used since ancient times in
372 Eastern traditional medicine. In recent years, the precise effect and their mechanisms
373 were investigated and *G. lucidum* in modern days is used in the treatment and prevention
374 of various pathological conditions. The numerous studies have already demonstrated that
375 various *Ganoderma* spp. extracts and metabolites possess strong immunostimulatory
376 activity and present effective modifiers of some biological response (Lin et al., 2006b;
377 Boh, 2013; Shi et al., 2013; Liu et al., 2016a,b). Also, Pi et al. (2014) and Lin et al.
378 (2006b) noted remarkable activation of Th1 and Th2 cells and certain cytokines synthesis
379 in mice treated with *G. tsugae* hot water extract and *G. formosanum* polysaccharide

380 fraction. Similarly, *G. lucidum* polysaccharide, besides effective stimulation of Th1/Th2
381 immune response, caused stronger proliferation of murine macrophages and significantly
382 higher synthesis of NO as well as IFN- γ , TNF- α , IL-4 and IL-6 in comparison with the
383 control group (Shi et al., 2013; Liu et al., 2016a,b).

384 Regarding to the crucial role of macrophages during establishing and maintaining
385 homeostasis and defending against pathogens and transformed cells, these cells are
386 involved in pathogenesis in many diseases (Jung et al., 2015; Ampem et al., 2016; Da
387 Silva and Barton, 2016). In response to differences in soluble characteristics of
388 microenvironment and different signals from microorganism-associated molecular
389 patterns, macrophages can polarize into pro-inflammatory, M1, or anti-inflammatory, M2,
390 phenotype (Lawrence and Natoli, 2011; Murray and Wynn, 2011). *In vitro* cultivation of
391 PMs from mice/rats represents an exceptionally powerful technique to investigate
392 macrophage functions in response to different stimuli, resembling as much as possible the
393 conditions observed in various pathophysiological conditions or as potential therapeutic
394 agents. Thus, glucan isolated from *G. lucidum* spores has also stimulated cytokine
395 production by PMs in culture (Guo et al., 2009). The considerable immunostimulatory
396 effect was also exhibited by *G. atrum* polysaccharide as well as its acetylated form, which
397 caused increased viability of T lymphocytes and level of IL-2 and TNF- α in serum of
398 immunosuppressive mice treated with cyclophosphamide overdoses, by regulation of
399 ROS production and NF- κ B activity (Chen et al., 2014; Yu et al., 2014; Li et al., 2017). In
400 this study, the immunomodulatory potential of GC and GA extracts was evaluated on
401 PMs with or without TLR4 cooperation (LPS). Macrophages stimulated with LPS are
402 termed as classical activation macrophages (M1 macrophages) and they are involved in
403 the inflammation, pathogen clearance, and anti-tumor immunity (Shapouri-Moghaddam
404 et al., 2018; Atri et al., 2018). Results of this study show that GC extract with LPS as
405 TLR4 agonist expressed potential for stimulation of metabolic activity/viability of PMs
406 and stimulation of NO production by PMs, while do not express potential for modulation
407 of adhesive and phagocytic potential and ROS production by PMs. On the other hand, GA
408 extract in combination with TLR4 signals induced by LPS induce intensive stimulation of
409 all investigated functions of PMs including metabolic activity, phagocytic capacity, and

410 production of ROS and NO compared to effect of GC. Additionally, GA increased
411 adhesive capacity of PMA-stimulated PMs. These results may indicate that GA extract
412 with TLR4 agonist induce stronger signaling that is responsible for stimulation of very
413 significant characteristics of M1 macrophages such as their metabolic activity, phagocytic
414 activity, ROS and NO production compared to TLR4 signaling alone. Also, adhesive
415 capacity of PMA-stimulated PMs was additionally increased by GA extracts. A few
416 studies demonstrated mechanism of mushroom polysaccharides action on cytokine
417 production. Namely, Kim et al. (2012) and Pi et al. (2014) showed that augmentation of
418 TNF- α synthesis by macrophages was based on the polysaccharide binding for TLR4
419 sited on macrophage membrane and its recognition as pathogen-associated molecular
420 patterns. However, detailed analyses demonstrated that induction of mRNA expression in
421 Sarcoma 180-bearing mice is the main mechanism of the polysaccharide action (Huang et
422 al., 2016). Since these studies demonstrated that intracellularly generated ROS (as a
423 response to pathological stimuli) affect NF- κ B activation and in a such way cytokine
424 production by macrophages, it can be concluded that ethanol extract of alternatively
425 cultivated *G. lucidum* basidiocarps which significantly increased ROS production in PMs,
426 could be a potent immunostimulatory and anti-tumor agent.

427 Induction of effective adaptive immune responses depends on signals from innate
428 immunity especially from level of maturation of DCs and their characteristics (Abbas et
429 al., 2017). In our study, the combination of poly I:C, loxoribine and GC/GA extract
430 induced phenotypic maturation of MoDCs as determined by up regulating the surface
431 molecules, including HLA-DR and CD83. CD83 acts as a key DC maturation marker
432 (Prechtel et al., 2007). However, combination of poly I:C, loxoribine and GA extract
433 induced up regulation of CD86, CD40 and CCR7. CD86 is a main co-stimulatory ligand
434 for T cells, providing the second signal for proliferation and clone expansion of antigen-
435 specific T cells (Jeannin et al., 2000). CD40 is also an indicator of activation state of
436 MoDCs whose up-regulation acts in favor of enhanced T cell activation (Sheikh and
437 Jones, 2008). Interaction of this molecule with its ligand (CD40L), expressed by activated
438 T cells is important for up-regulation of co-stimulatory molecules on DCs and enhanced
439 capacity of DCs to trigger proliferative responses and for regulation of DC functions

440 (Cella et al., 1999). It is in line with our results which showed significantly higher
441 proliferation of CD4 T lymphocytes co-cultivated with MoDC treated with combination
442 of poly I:C, loxoribine and GA extract. It is important to mention that solely maturation
443 of DCs, expressing high levels of co-stimulatory and maturation markers, is not sufficient
444 for an adequate immune response. Namely, various cytokine production and subsequent
445 CD4⁺ T lymphocytes polarization by DCs is of great significance in the induction of
446 proper immune response as well (Zobylawski et al., 2007). Poly I:C is known as a potent
447 stimulator of bioactive IL-12 production and subsequent activator of the Th1 immune
448 response (Rouas et al., 2004). Our findings confirm these published results because Poly
449 I:C in combination with loxoribine and GC/GA extract induces the up-regulation of IL-12
450 level. Important finding of this study was the intensive promotion of Th1 and slightly
451 promotion of Th17 polarizing capability of MoDCs by simultaneous engagement of poly
452 I:C, loxoribine and GA compared to capability of MoDCs by simultaneous engagement
453 of poly I:C, loxoribine and GC. Regarding IL-10 production, obtained results are also
454 interesting. Namely, IL-10 is an immunoregulatory cytokine, responsible for tolerogenic
455 properties of DCs (Smits et al., 2005), which participates in balancing of immune
456 response (Saraiva et al., 2009). Production of IL-10 could be relevant as a down-regulator
457 of an extensive production of immunostimulatory cytokines, knowing that the balance
458 between stimulatory and inhibitory cytokines is important for critical point during
459 immune response. The decreased level of IL-10, in co-culture of CD4⁺ T lymphocytes
460 and MoDCs treated simultaneous with poly I:C and loxoribine and GC/GA, could be
461 explained by mutual antagonistic effects of Th1 cells on Th2 cells and Tregs as showed
462 by Glimcher and Murphy (2000) [49]. Therefore, down-regulation of IL-10 production
463 may serve as an additional mechanism for promotion of the Th1 immune response.

464 Bearing in mind the significance of anti-tumor vaccines, this study performed to find
465 optimal protocol for development of DCs able to induce adequate immune response. The
466 results that we obtained in this study suggest that simultaneous TLR3 and TLR7 signaling
467 with signaling induced by components of crude extract of *G. lucidum* cultivated on wheat
468 straw may provide a previously un-described approach for DC-based vaccine
469 development by using synergistic TLR ligands and *G. lucidum* extract. Overall, these

470 results point to that natural immunomodulators possible mechanism for enhancement of
471 effects of known TLR agonists.

472

473 **Conclusion**

474 The importance of the presented results can be realized by taking into account several
475 facts: (i) immunomodulatory activity is known only for wild and traditionally cultivated
476 *G. lucidum* basidiocarps; (ii) traditional cultivation of *G. lucidum* basidiocarps on various
477 hardwood sawdusts is not ecologically and economically friendly; (iii) substrate
478 composition and cultivation conditions significantly affected type and content of
479 bioactive metabolites and their activities [50]; (iv) wheat straw induced a synthesis of
480 numerous bioactive molecules, primarily triterpenoids (Ćilardžić et al., 2018b), which
481 could be considered as the one of strong modulators of APCs activity; (v) there is
482 increasing need for natural immunomodulators, without any side effect; (vi) modern
483 vaccines based on highly purified antigen induce insufficient immune protection and a
484 need for natural vaccine adjuvants is continuously growing.

485

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490

491 **Disclosure of interest**

492 The authors declare that there is no conflict of interest.

493

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639

640 **Legends of Figures**

641 **Fig. 1.** Effect of *Ganoderma lucidum* extracts on metabolic activity/viability of PMs

642

643 **Fig. 2.** Effect of *Ganoderma lucidum* extracts on adhesive capability of PMs

644

645 **Fig. 3.** Effect of *Ganoderma lucidum* extracts on phagocytic activity of LPS-stimulated
646 PMs

647

648 **Fig. 4.** Effect of *Ganoderma lucidum* extracts on ROS production by LPS-stimulated
649 PMs

650

651 **Fig. 5.** Effect of *Ganoderma lucidum* extracts on NO production by PMs

652

653 **Fig. 6.** Effect of *Ganoderma lucidum* extracts on phenotype (**A**) and cytokine production
654 (**B**) by MoDCs

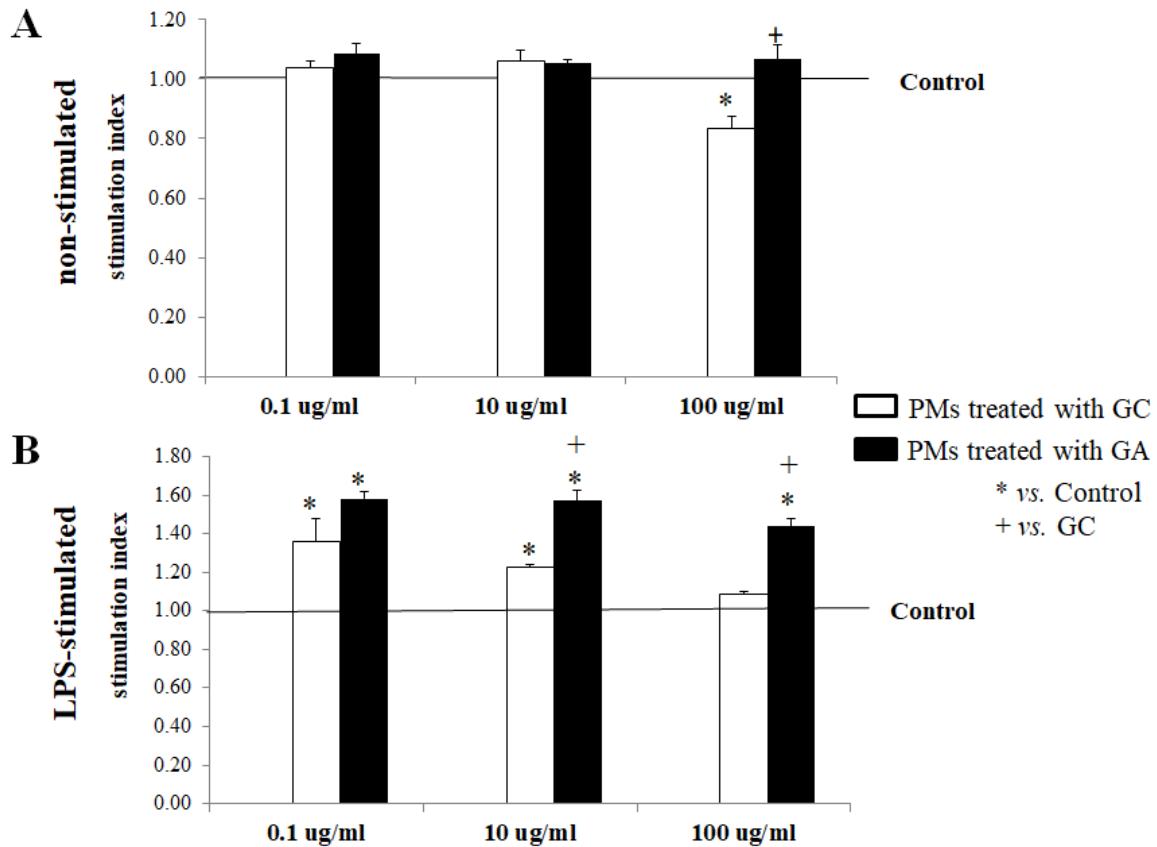
655 *Phenotypic characteristics of MoDC. MoDC were obtained by cultivation of human*
656 *monocytes for 6 days with GM-CSF (100.0 ng/mL) and IL-4 (20.0 ng/mL) and then*
657 *stimulated with Poly (I:C) (10.0 µg/mL), Loxoribine (34.0 µg/mL) and GC/GA (100.0*
658 *µg/mL). Non-adherent cells were collected and stained for key DC markers using MAb*
659 *(anti-HLA-DR – PE, CD86 – PE, CD83 – FITC, CD40 – FITC, CD54 – PE and CCR7 –*
660 *FITC) and analyzed by flow cytometry. Results are presented as over-laid histograms*
661 *within the gated population of one experiment. These data are representative of three*
662 *independent experiments. Red and Blue line for MoDC TLR3/7 + GA, Full gray*
663 *histogram for MoDC TLR3/7.*

664

665 **Fig. 7.** Effect of *Ganoderma lucidum* extracts on alostimulatory (**A**) and Th polarization
666 capacity (**B**) of MoDCs

667 *These data are representative of three independent experiments.*

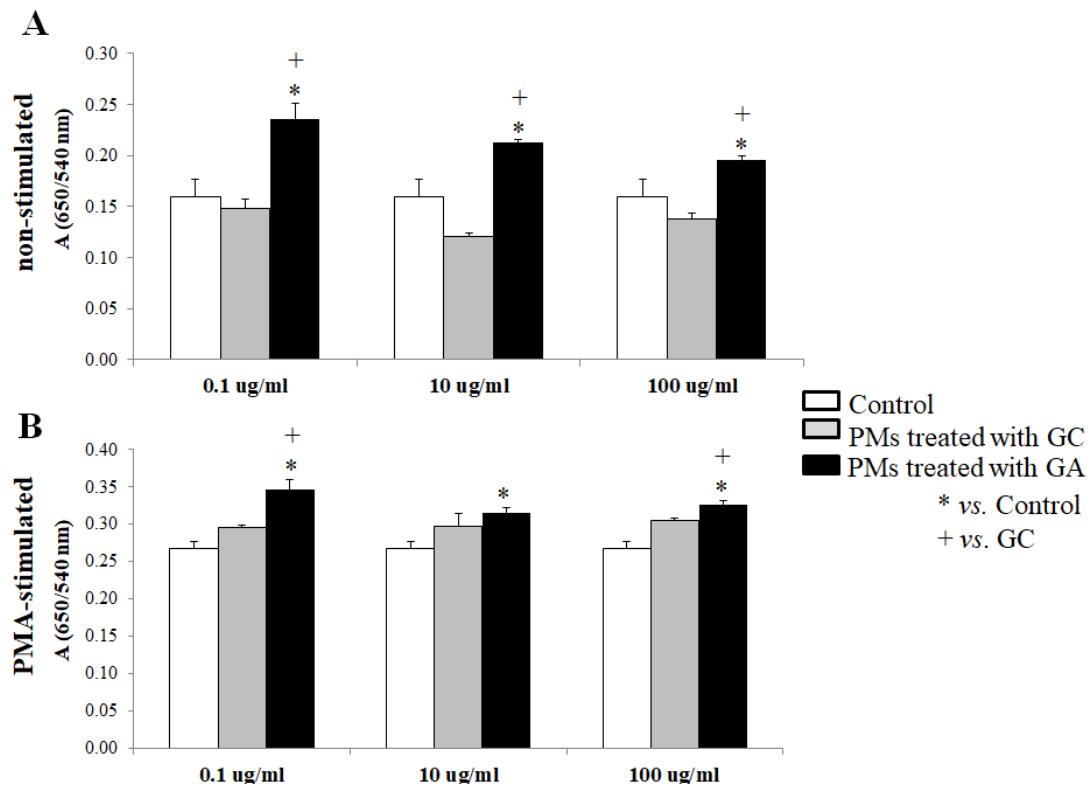
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669

670 Fig.1

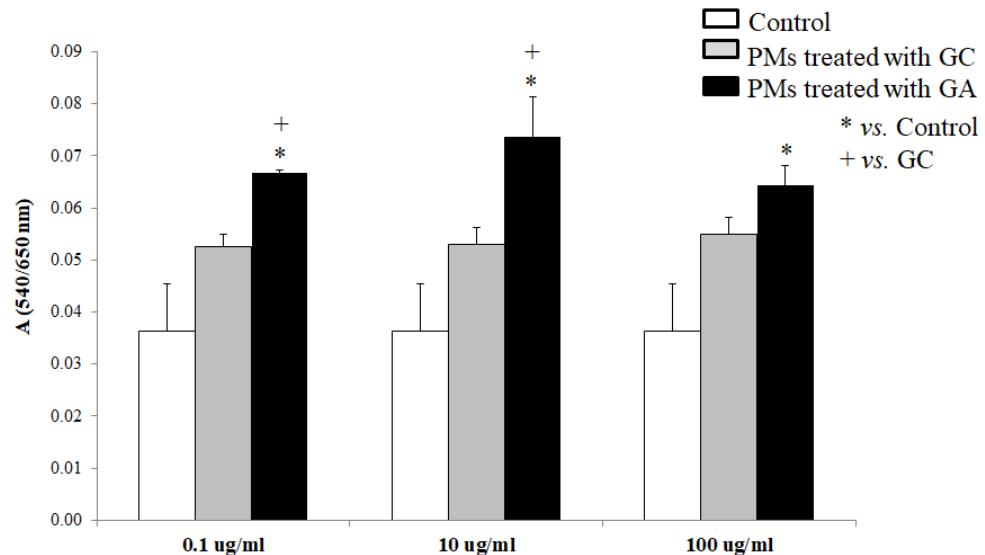
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673 Fig 2.

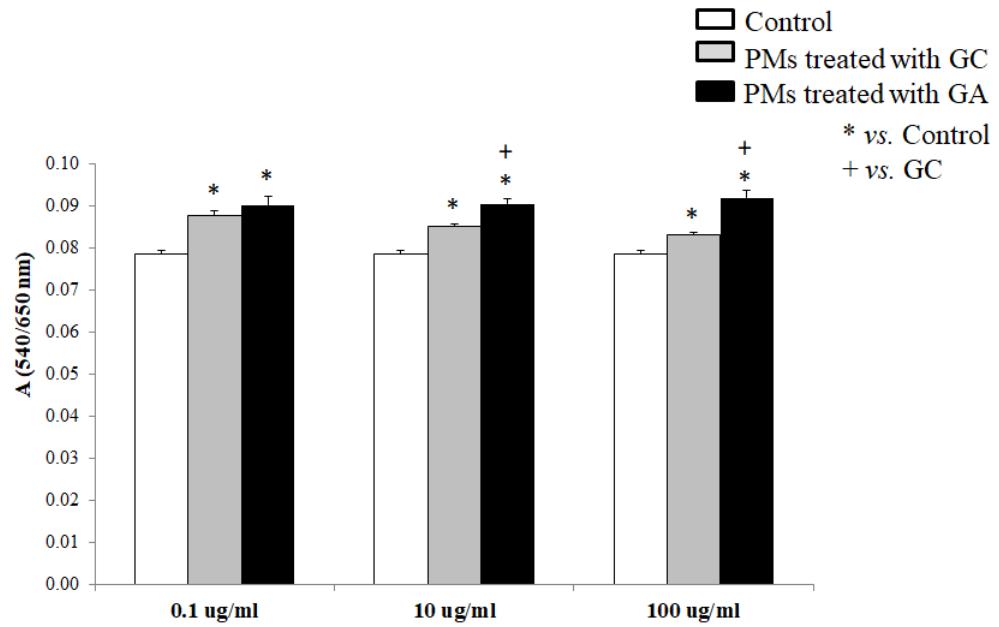
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676 Fig 3.

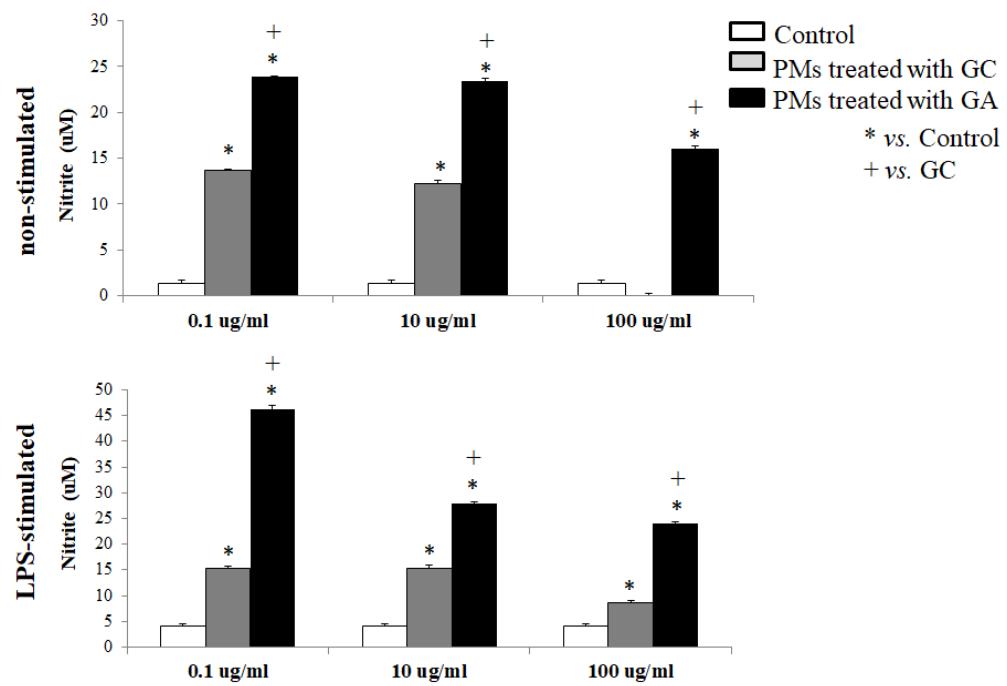
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679 Fig 4.

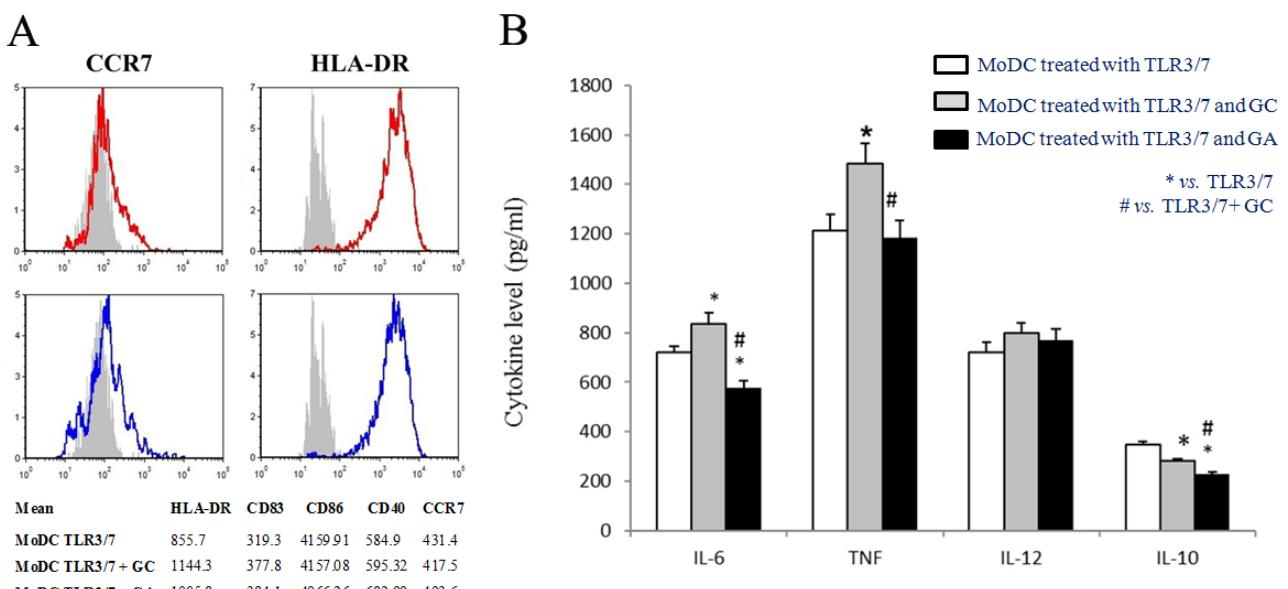
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682 Fig 5.

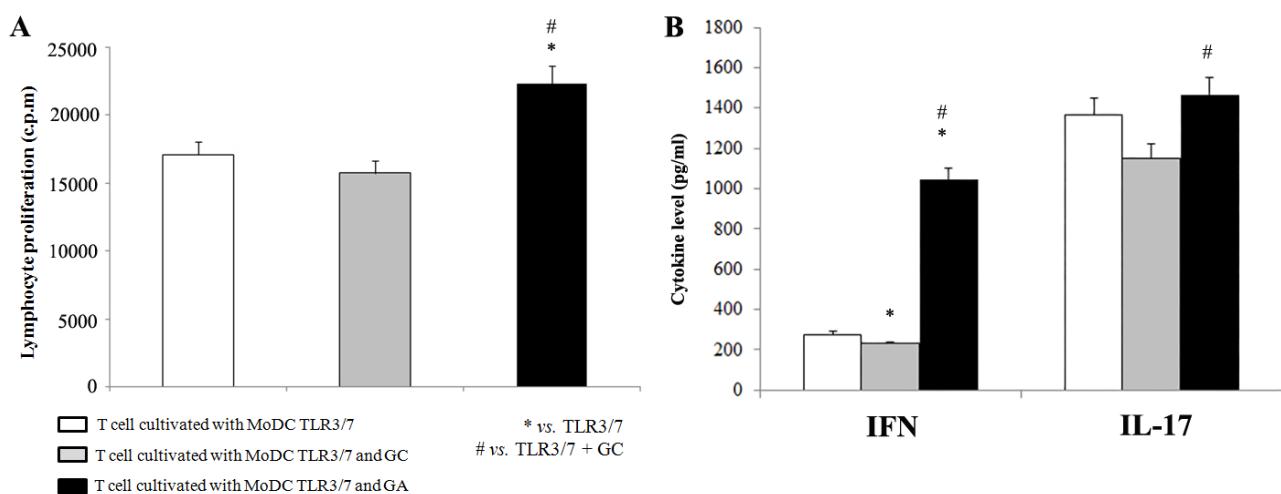
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685 Fig 6.

686



687

688 Fig 7.