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Therapeutic efficacy of the live-attenuated *M. tuberculosis* vaccine MTBVAC in a preclinical model of bladder cancer

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Running title: MTBVAC vaccine as bladder cancer immunotherapy

Keywords: MTBVAC, BCG, bladder cancer, immunotherapy, live tuberculosis vaccines

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29 BACKGROUND

30 Live attenuated BCG is a first-line treatment for non-muscle invasive
31 bladder cancer. Nevertheless, BCG treatment fails in a significant percentage of
32 patients. Additionally, periodic shortages of BCG supply represent a serious
33 problem that obligates the use of less efficient alternative chemotherapeutic
34 treatments.

35 TRANSLATIONAL SIGNIFICANCE

36 Our data demonstrate an antitumor effect of the novel live attenuated *M.*
37 *tuberculosis* vaccine MTBVAC in an orthotopic murine model of bladder cancer.
38 Notably, MTBVAC is currently under clinical evaluation as tuberculosis vaccine.
39 Our results suggest that MTBVAC could be a promising candidate and support its
40 further exploration as a novel bladder cancer immunotherapy.

41

42 ABSTRACT

43 Intravesical instillation of Bacillus Calmette–Guérin (BCG) has been a first-
44 line therapy for non-muscle invasive bladder cancer for the last four decades.
45 However, this treatment causes serious adverse events in a significant number of
46 patients and a substantial percentage of recurrence episodes. MTBVAC is a live
47 attenuated vaccine derived from a *Mycobacterium tuberculosis* clinical isolate and
48 is currently under evaluation in clinical trials to replace BCG as a tuberculosis
49 vaccine. Here, we describe for the first time the potential of MTBVAC as a bladder
50 cancer therapy *in vitro* and *in vivo* in a preclinical model. MTBVAC colonized
51 human bladder tumor cells to a much greater extent than BCG via a mechanism
52 mediated by macropinocytosis and induced cell growth inhibition following
53 internalization. *In vivo* testing in an orthotopic murine model of bladder cancer
54 demonstrated a higher antitumor effect of MTBVAC in experimental conditions in
55 which BCG did not work. Our data encourage further studies to support the

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56 possible application of MTBVAC as a new immunotherapeutic agent for bladder
57 cancer.

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59 INTRODUCTION

60 Bladder carcinoma (BC) is one of the most frequently occurring types of
61 cancer worldwide, especially in developed countries (1). Established more than
62 four decades ago (2), intravesical instillation of the tuberculosis vaccine
63 *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) following tumor resection is
64 still a first-line therapy for high-risk non-muscle-invasive bladder cancer to
65 prevent tumor progression and recurrence (2). The current generally accepted
66 regimen consists of an induction phase of six weekly instillations of BCG, followed
67 by a maintenance regimen of three weekly instillations every three and six months
68 over three years (3). Recurrence-free survival at five years with this regimen
69 ranges from 60% to approximately 90% depending on the study (4, 5). In the case
70 of patients who received only BCG at the induction phase, this percentage drops to
71 40% (3). BCG is considered a therapy that is well tolerated by many patients.
72 However, there are still a significant number of people who must interrupt the
73 treatment due to severe adverse events (6).

74 The mechanism of action of BCG against BC has not been completely
75 elucidated. The possible involvement of BC cells includes fibronectin-mediated
76 attachment and internalization of BCG (7), secretion of cytokines and chemokines
77 by tumor cells, and presentation of BCG and/or cancer cell antigens to the immune
78 system (8). There is broad consensus that a competent immune system is required
79 to obtain an effective response, and many cell types from both the innate and
80 adaptive systems, including CD4+ and CD8+ T cell lymphocytes as well as natural
81 killer cells, granulocytes, macrophages, and dendritic cells, have been implicated in
82 this process (8).

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83 MTBVAC is a live vaccine based on rational attenuation of a clinical isolate
84 of *Mycobacterium tuberculosis* that conserves the whole gene repertoire absent in
85 BCG (derived from *M. bovis* originally isolated from cattle) (9). MTBVAC
86 attenuation is conferred by two independent unmarked deletions in the *phoP* and
87 *fadD26* virulence genes. PhoP is a transcription factor that controls approximately
88 2% of the coding capacity of the *M. tuberculosis* genome and is mainly involved in
89 virulence. Deletion of *fadD26* leads to complete abolishment of
90 phtioceroldimycocerosates (PDIM) synthesis, known to be essential for virulence
91 (10). Intradermal MTBVAC administration has shown an excellent safety profile in
92 healthy human adults (11), and currently, it is being evaluated in newborns
93 (clinical trial identifier: NCT02729571).

94 MTBVAC has shown better immunogenic properties compared to BCG in
95 different preclinical models (10). As a result, we hypothesize that this live vaccine
96 could be a good approach to treat BC. In the present work, we studied the ability of
97 MTBVAC to infect human bladder cancer cells and its cytotoxic effect *in vitro*
98 compared with BCG. In addition, we evaluated the efficacy of MTBVAC *in vivo* in an
99 orthotopic mouse model of BC. To our knowledge, this study is the first in which a
100 live attenuated *M. tuberculosis* strain has been evaluated as a BC treatment.

101 METHODS

102 *Bacteria*

103 BCG stock used in the present study was prepared from a commercial vial of
104 BCG-MEDAC, which is one of the BCG formulations licensed for bladder cancer
105 treatment. As indicated by the manufacturer, BCG-MEDAC was obtained from a
106 seed of BCG Pasteur (strain 1173P2, Institut Pasteur Paris, France). MTBVAC
107 vaccine was generated in our laboratory by deletion of the virulence factors *phoP*
108 and *fadD26* in the clinical isolate *M. tuberculosis* Mt103 (9). Both vaccine strains
109 were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with ADC
110 10% (Difco) and 0.05% (v/v) Tween-80 (Sigma) or on solid Middlebrook 7H10
111 (Difco) supplemented with ADC 10%. For *in vitro* experiments, BCG and MTBVAC
112 were transformed by electroporation with an integrative pMV361 plasmid
113 encoding green fluorescent protein (GFP) (a kind gift from Christophe Guilhot,
114 Toulouse, France).

115 *Cells and infections*

116 Human J82 and T24 cells (12), murine MB49-luc cells (13) and murine MH-
117 S cells (HPA Culture Collections, Cat number 95090612) were used in the present
118 study. All the experiments were performed with cells thawed from the original
119 frozen stocks prepared following cell lines acquisition. Cells were cultured at 37°C
120 and 5% CO_2 in DMEM supplemented with 10% inactivated foetal bovine serum
121 (Biological Industries), 2 mM glutamine (Biological Industries) and antibiotics
122 (penicillin/streptomycin/ciprofloxacin) (Sigma). All the experiments were done
123 using cells with less than five passages from its thawing. Cells were routinely
124 cultured in the presence of ciprofloxacin (10 μg/ml) to prevent mycoplasma

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125 contamination. Mycoplasma absence was confirmed after finalizing the
126 experiments using the MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

127 For *in vitro* experiments, cells were seeded in complete medium without
128 antibiotics the day prior to infection. 10^4 cells were seeded in 96-well plates and
129 allowed to attach to plastic overnight. Log-phase bacterial cultures were
130 centrifuged at 100 g to remove clumps, and bacterial density in the supernatant
131 was determined by optical density. Then, bacterial suspensions for the indicated
132 MOIs were prepared in complete medium (without antibiotics) and added to cell
133 cultures during the indicated times. The inhibitors Wortmannin (Millipore), IPA-3
134 (Millipore), EIPA (Sigma) and Nystatin (Sigma) were added to the cultures at the
135 indicated concentrations one hour before the bacterial suspensions.

136 To monitor infected cells by flow cytometry, infections were performed
137 with GFP-expressing BCG and MTBVAC. At the indicated time points, cells were
138 detached with trypsin and fixed with 4% paraformaldehyde. Infections were
139 analyzed with a FACSAria Flow Cytometer (BD Biosciences).

140 *Mice and in vivo experiments*

141 All mice were kept under controlled conditions and observed for any sign of
142 disease. Experimental work was conducted in agreement with European and
143 national directives for protection of experimental animals and with approval from
144 the Ethics Committee of the University of Zaragoza (approved protocol PI46/12).

145 Intravesical instillations were performed following protocols described
146 previously (14, 15). C57BL/6 female mice were anesthetized with isoflurane, and
147 urine was drained by slight pressure to the lower abdomen. After disinfecting the
148 urethral orifice with iodine, a 24-gauge catheter (BD Insyte Autoguard, Beckton

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149 Dickinson) connected to a 1-ml syringe was carefully inserted through the urethra.
150 The catheter and syringe were maintained during the different procedures.

151 For tumor implantation, mouse bladders were pre-treated with 100 μl of
152 poly-L-Lysine (0.1 mg/ml) for 20 minutes. Then, 100,000 MB49-Luc cells were
153 instilled per mouse and retained for 60 minutes. Three days after bladder tumor
154 induction, mice were randomly divided into three groups (6-9 animals/group) and
155 intravesically treated with 100 μl of BCG Pasteur or MTBVAC in PBS (10^7
156 CFU/animal) for one hour or with the same volume of PBS as a control. The
157 number of bacterial CFUs was determined for all treatments by plating on 7H10
158 ADC. Treatment schedule was based on a previously described protocol (14).
159 Mycobacteria were administered at days 3, 9 and 16 post-tumor implantation. For
160 *in vivo* tumor visualization, mice were inoculated intraperitoneally at days 7 and
161 14 post-inoculation with 200 μl of 15 mg/ml D-luciferin potassium salt (Perkin
162 Elmer) and luminescence was analyzed with an IVIS lumina imaging device
163 (Xenogen). The animals that did not show any luminescence signal and presence of
164 haematuria at the initial stages of the experiment were discarded from the study as
165 we considered that tumor cells had not been successfully implanted in these
166 animals. Absence of tumours in these mice was corroborated by pathological
167 examination of the bladders.

168 Animals were monitored for 60 days and scored three times a week based
169 on weight loss percentage, presence of haematuria, tumor presence and general
170 behaviour. Mice were sacrificed based on pre-established endpoint criteria, and
171 the bladders were harvested and fixed for haematoxylin-eosin staining and tumor
172 presence evaluation.

173 *Confocal microscopy*

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174 For confocal microscopy studies, $4x10^5$ cells of the indicated cell lines were
175 seeded in 24-well plates containing sterile 12 mm-round cover glasses. At the time
176 points indicated, the cells were fixed, and the nuclei were stained with Hoechst
177 33342 (Invitrogen) for 15 minutes at room temperature. In the indicated
178 experiments, cells were incubated with Alexa $Fluor_{594}$-labeled dextran (10,000
179 MW) (Invitrogen) at a final concentration of 0.5 mg/ml and incubated for 30
180 minutes. In another set of experiments, an anti-Mtb antibody (Acris GmbH)
181 (1/500) followed by phycoerythrin (PE)-conjugated anti rabbit IgG (1/500) was
182 added to non-permeabilized cells or cells permeabilized using a Cytofix/Cytoperm
183 kit (BD). Following a washing step with deionized water, microscope preparations
184 were performed by placing the cover glass over a slide with a 3-μl drop of Prolong
185 Gold Antifade reagent (Invitrogen). Images were acquired with a FluoView® FV10i
186 confocal microscope (Olympus).

187 *Cell growth inhibition assay*

188 Cell growth was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-
189 diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma). After culture
190 supernatants were removed, and the cells were washed three times with PBS, MTT
191 1 mg/ml in complete medium was added and incubated with the cells for 3 hours
192 at 37°C. After the medium was removed, water-insoluble dark blue formazan was
193 dissolved in 6N HCl in 1-propanol (acidic isopropanol) for 1 hour at room
194 temperature. Finally, absorbance was measured at 570 nm (MTT) and 690 nm
195 (background) using a plate reader (Biotek Synergy HT).

196 To assess cell death, plasma membrane integrity was evaluated with 7-
197 actinomycinD (7-AAD) (BD Biosciences) staining according to the manufacturer's
198 instructions, and stained cells were analyzed using flow cytometry.

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199 *Statistics*

200 Sample size calculation was performed with the StatsToDo online tool
201 (https://www.statstodo.com/SSizSurvival_Pgm.php). GraphPrism software was
202 used for the rest of statistical analysis. Sample size used in the animal experiments
203 was powered enough to demonstrate statistical differences between MTBVAC-
204 treated and non-treated groups (α=0.05; (1-β)=0.8). The statistical tests chosen for
205 each experiment are indicated in the figure legends. All statistical tests used were
206 two-tailed. Differences were considered significant at $p<0.05$. Analysis of the
207 results of this study was not blinded.

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208 RESULTS

209 **Increased capacity of MTBVAC to infect human bladder tumor cells**

210 The need for close contact between BCG and urothelial tumor cells to
211 trigger an efficient antitumor response is well accepted, and strategies to enhance
212 this contact have been shown to improve treatment efficacy (16). Thus, we first
213 evaluated *in vitro* MTBVAC internalization by bladder tumor cells. We performed
214 these studies using the cell lines T24 and J82, which have been widely used for this
215 type of experiment. We incubated cells with GFP-expressing MTBVAC at a low
216 (10:1) and high (100:1) multiplicity of infection (MOI) and percentage of infected
217 cells was monitored daily by flow cytometry for one week. The differences
218 between both MOIs were substantial (**fig. 1a**). MTBVAC infection at a high MOI
219 reached 80% of the cells before day 3. At MOI 10, the infection kinetics increased
220 progressively until reaching 40% and 80% of J82 and T24 cells, respectively, at day
221 seven. Comparison of MTBVAC and BCG showed dramatic differences between
222 both strains. Even with an MOI of 100:1, our results indicated the limited
223 infectivity of BCG, with an infectivity lower than 20% at seven days post-infection
224 (**fig. 1b**), a value that is in agreement with previous studies (17). MH-S murine
225 macrophage infection results were comparable for both strains, indicating that the
226 BCG used in this study did not present any alterations that led to lower infection
227 capacity (**supp. fig. 1**).

228 Since flow cytometry did not allow for the discernment of whether bacteria
229 were internalized or attached to the plasma membrane, we visualized infected
230 cultures using confocal microscopy. These data confirmed a general cytosolic
231 localization of the bacteria and corroborated the flow cytometry results, showing a
232 substantial qualitative increase in the number of infected cells in the case of

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233 MTBVAC compared with the results for BCG (**fig. 2a**). In addition, we added a
234 phycoerythrin (PE)-conjugated antibody specific for mycobacteria to the culture.
235 Thus, bacteria attached to cells were labelled with the antibody, whereas
236 internalized bacteria were unaffected by the presence of the antibody. This
237 experiment confirmed the predominant intracellular localization of MTBVAC and
238 BCG following infection. (**fig. 2b**).

239 **MTBVAC infection inhibits human bladder cancer cell growth.**

240 Even though it is controversial, some authors have reported that BCG is able
241 to exert cell growth inhibition on tumor cells (12). To evaluate this claim, we used
242 an MTT assay to assess cellular growth of T24 and J82 cultures previously infected
243 with BCG or MTBVAC. Our results showed a strong dose response profile in the
244 case of MTBVAC between the MOI and the rate of inhibition. At low MOI (10:1), the
245 inhibitory effect of MTBVAC was limited, but was close to 80% at an MOI of 100:1.
246 Remarkably, in the case of BCG, we did not detect any inhibitory effect under the
247 conditions tested, even at an MOI of 100 bacteria per cell (**fig. 3a**). Microscopy
248 images confirmed these results showing a lower number of cells following
249 MTBVAC long-term infection (**fig. 3b**). Altogether, these data showed that MTBVAC
250 was able to inhibit cell growth in experimental conditions in which BCG did not
251 exert direct cell growth inhibition.

252 **Macropinocytosis contributes to MTBVAC internalization**

253 With the aim of elucidating the internalization mechanism of MTBVAC, we
254 infected cells in the presence of different inhibitors described to be involved in the
255 mechanism of BCG engulfment (17): Nystatin, a cholesterol-depleting agent that
256 disrupts lipid rafts and endocytosis; Wortmannin, an inhibitor of the PI3K

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257 pathway; IPA-3, an inhibitor of p21-activated kinase (PAK); and finally, ethyl-
258 isopropyl amiloride (EIPA), an inhibitor of Na+/H+ exchangers and a well-known
259 inhibitor of macropinocytosis. We first analyzed the toxicity of these molecules
260 over J82 and T24 cells at different concentrations, with the aim of identifying non-
261 toxic concentrations for subsequent experiments (**supp. fig. 2**).

262 Once determined non-toxic concentrations, we added the inhibitors to
263 MTBVAC-infected cultures. Results showed no effect in the case of IPA-3, Nystatin
264 and Wortmannin, whereas they demonstrated a wide inhibition of MTBVAC entry
265 following incubation with EIPA. suggesting a strong contribution of
266 macropinocytosis to MTBVAC internalization (**fig. 4a**). Inhibition by EIPA was
267 corroborated with confocal microscopy (**fig. 4b**). As a control, we tested the
268 capacity of EIPA to inhibit dextran internalization, a molecule previously described
269 to enter cells through macropinocytosis (18) (**supp. fig. 3**). EIPA also impaired
270 BCG engulfment (**fig. 4c**), demonstrating that macropinocytosis was also involved
271 in BCG engulfment, in accordance with published results (17).

272 In addition, we performed an MTT assay of infected cultures in the presence
273 of EIPA. The results showed a partial reduction in the growth inhibition induced by
274 MTBVAC in the presence of EIPA (**fig. 4d**). These results suggest that bacterial
275 entry into tumor cells is required at least in part to impair cell growth.

276 **Antitumor effect of intravesical treatment with MTBVAC in an**
277 **orthotopic mouse model of BC**

278 Finally, we aimed to compare the *in vivo* efficacy of MTBVAC with that of
279 BCG in a physiological model of BC. To this end, we used a well-accepted murine
280 orthotopic model induced by intravesical instillation of MB49 cells. Prior to carry

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281 out this study, we performed some *in vitro* experiments to confirm that MB49 cells
282 behaviour was comparable with the previously observed in the case of human cells.
283 Both flow cytometry and confocal microscopy experiments indicated a higher
284 infectivity of MTBVAC in MB49 murine cells in comparison to BCG (**fig. 5**).

285 For *in vivo* experiments, we administered three mycobacteria treatments,
286 with the first instillation at three days post tumor implantation (**fig. 6a**). Animals
287 were followed-up and euthanized when required according to pre-defined
288 endpoint criteria. Following tumor cell administration, haematuria events were
289 observed in all mice included in the study in the following days after instillation,
290 indicating that the cells had been implanted successfully in the bladder. In addition,
291 as we used a luciferase-expressing version of MB49 cells, we assessed tumor
292 implantation by visualization of the luminescence produced by tumor cells in the
293 bladder area (**supp. fig. 4**). Animals that did not show any luminescence signal and
294 haematuria at the initial stages of the experiment were discarded from the study as
295 we considered that tumor cells had not been successfully implanted in these mice.

296 The results revealed 60% (7/12) mortality in the PBS-treated group, 40%
297 (7/18) in the BCG-treated group and less than 10% (1/13) in the MTBVAC-treated
298 group (**fig. 6b**). Deceased animals showed bladder positivity for tumor presence in
299 all cases (**supp. fig. 5**). At day 60, the remaining mice were sacrificed and found to
300 be tumor-free in all cases. Statistical analysis indicated a significant effect in the
301 MTBVAC group compared to that in the PBS-treated mice ($p<0.05$). When
302 compared both vaccine treatments, despite the higher survival in the case of
303 MTBVAC, we did not find significant differences between the groups ($p=0.079$).
304 Finally, comparison of BCG- and PBS-treated animals showed no significant
305 difference ($p>0.05$) (**fig. 6b**).

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306 DISCUSSION

307 Live attenuated BCG is the first choice to treat non-muscle invasive BC and
308 is one of the most successful biotherapies for cancer. Nonetheless, there is a wide
309 margin for BCG improvement because this treatment fails in up to 50% of patients,
310 considering recurrence episodes and patients who must withdraw from treatment
311 due to severe adverse events, who represent approximately 10% of BC cases (4).
312 In addition, a high proportion of patients experiences side effects less serious but
313 that affect their lifestyle, with more than 60% and 30% reporting local and
314 systemic effects, respectively (6). Moreover, BCG is not a defined strain but a
315 diverse family of substrains with genetic heterogeneity. As a result, there are
316 phenotypic differences among BCG substrains with respect to antigenicity and
317 reactogenicity (19). In addition, we should consider the periodic shortages of BCG
318 supply, which represent a serious problem that necessitates the use of suboptimal
319 BCG doses or alternative chemotherapeutic treatments with lower efficacy (20).
320 Thus, searching for novel treatments that are more efficient and less toxic than
321 BCG is justified.

322 It is assumed that close contact between BCG and tumor cells is necessary
323 for optimal therapeutic effects (8), and it has been observed that *in vivo* blocking of
324 BCG internalization in mouse models impairs treatment efficacy (21). In this
325 regard, we have demonstrated *in vitro* the superior ability of MTBVAC to colonize
326 bladder tumor cells (human and murine) compared with that of BCG, and found a
327 strong contribution of macropinocytosis in this process (although other
328 mechanisms of internalization can not be excluded to participate). We could
329 speculate that this higher capacity of infection could be a concern in terms of safety.
330 However, according to our data this risk seems unlikely. MTBVAC has been

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331 extensively characterized in different animal models and safety has been evaluated
332 in a human cohort. In all cases, the persistence and reactogenicity of MTBVAC was
333 similar or lower than the observed with BCG, and when we studied vaccine
334 biodistribution in mice, bacteria presence was restricted in all cases to lymph
335 nodes and spleen, and MTBVAC was never detected in non-lymphoid organs (9,
336 11).

337 BCG internalization is mediated by direct binding of bacteria to fibronectin
338 through fibronectin-attachment proteins (FAPs) (7). Two main protein complexes
339 have been described to bind directly to fibronectin: FAP, which corresponds to the
340 secreted antigen MPT32 from *M. tuberculosis* (21), and Ag85A, Ag85B and Ag85C
341 from the Ag85 complex (22). MPT32, Ag85A and Ag85C have been reported to be
342 substrates of the twin-arginine translocation (TAT) system (23, 24). Interestingly,
343 our previous study revealed that deletion of *phoP* in MTBVAC leads to profound
344 downregulation of the non-coding RNA *mcr7*, which is a negative regulator of the
345 TAT system, and therefore, MTBVAC demonstrates a greater capacity to secrete
346 TAT substrates (including Ag85A and Ag85C) (24). In addition, BCG expresses a
347 polymorphism in the *ag85b* gene that drives the expression of an unstable protein
348 (25) that is indeed absent in the extracellular fraction of BCG cultures (26).
349 Together, these processes could result in a higher capacity of MTBVAC to bind
350 fibronectin, with the subsequent attachment to urothelial cells and internalization.

351 The significance of BCG-induced direct cytotoxicity to treatment outcome is
352 controversial (8), with several studies showing different findings that might be
353 attributed to differences in experimental conditions, or the BCG strains used in
354 each work (12, 17, 27, 28) . Under our experimental conditions, we did not detect
355 any antiproliferative effect associated with BCG incubation, even at high MOI

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356 values over seven days. However, we observed significant cellular growth
357 inhibition following MTBVAC infection. Our results indicate that MTBVAC entry
358 into host cells is directly related to this effect and suggest that the antiproliferative
359 effect triggered by MTBVAC could contribute to the antitumor response.

360 MTBVAC has demonstrated in different animal models, as well as in humans,
361 to induce a strong Th1 response (10, 11). The Th1 response is normally associated
362 with tumor immunosurveillance, which could also explain the therapeutic efficacy
363 of MTBVAC in the present study. In addition, incubation of MTBVAC with human
364 dendritic cells leads to increased production of inflammatory cytokines, such as
365 TNFa or IL-6, which are important for triggering the inflammatory cascade
366 responsible for the BCG antitumor effect (29). Interestingly, the higher infectivity
367 of MTBVAC might drive higher presentation of mycobacteria-derived antigenic
368 peptides by MHC molecules on the surface of infected tumor cells, and therefore, T
369 cells specific for mycobacteria could also participate in the antitumor response. In
370 this regard, it has been demonstrated a higher efficacy of BCG as BC therapy in
371 individuals with an immune response specific of mycobacterial antigens (14).

372 In the present study, we have compared the antitumor efficacy of MTBVAC
373 and the BCG substrain Pasteur. However, possible differences between BCG
374 substrains in terms of clinical efficacy have been reported. Concretely, a previous
375 study showed a lower level of recurrence in patients treated with BCG Connaught
376 in comparison to BCG Tice, when maintenance regimen was not administered (30).
377 Thus, even though BCG Pasteur has shown comparable clinical benefits with
378 respect to other BCG (31), we find crucial to assess in the future the MTBVAC
379 antitumor effect in comparison to other BCG substrains licensed as bladder cancer
380 therapies, including the widely used Tice and Connaught.

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381 In the present study, we have demonstrated MTBVAC efficacy in a relevant
382 orthotopic mouse model of BC. In addition, our data indicate substantial
383 differences between MTBVAC and BCG in terms of *in vitro* vaccine interaction with
384 bladder tumor cells. Notably, GMP (Good-Manufacturing Practices) production of
385 MTBVAC as tuberculosis vaccine has been developed, and the vaccine has
386 demonstrated an excellent safety profile and immunogenicity in humans, when
387 delivered by the intradermal route. Altogether, our results support further
388 exploration of MTBVAC as a potential novel bladder cancer immunotherapy.

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501 FIGURE LEGENDS

502 **Figure 1. MTBVAC and BCG infectivity in J82 and T24 cells.** J82 and T24
503 cells were infected with GFP-expressing BCG Pasteur or MTBVAC strains, and the
504 percentage of infection was analyzed using flow cytometry at the indicated time
505 points. **a**, MTBVAC infection kinetics over seven days. **b**, BCG and MTBVAC
506 infectivity comparison at MOI of 20:1. The data in the graphs are presented as the
507 mean±SD. A representative experiment out of two (**a**) and three (**b**) experiments is
508 shown in the figure. Statistical analysis was performed using two-way ANOVA and
509 Bonferroni's post-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

510 **Figure 2. MTBVAC and BCG are internalized by J82 and T24 cells. a**, T24
511 (left) or J82 (right) cells were infected with BCG Pasteur-GFP or MTBVAC-GFP
512 (MOI 50:1) for four days, and bacterial localization was analyzed with confocal
513 microscopy. Phase contrast (PC) images (left) and merged images of Hoechst
514 33342 (blue) and GFP (green) fluorescence signals (right) are shown. **b**, Infected
515 cells (MOI 10:1) were labeled with an anti-Mtb followed by PE-conjugated
516 secondary antibody in the absence of permeabilization (upper panels) or after
517 permeabilization with saponin (lower panels). White arrows indicate PE-labelled
518 bacteria attached to plasma membrane. The images are representatives of two
519 independent experiments. Scale bar, 40 μm.

520 **Figure 3. MTBVAC infection inhibits cell growth. a**, T24 and J82 cells
521 were infected with GFP-expressing BCG Pasteur or MTBVAC strains at the
522 indicated MOI for four days, and cell growth was analyzed with the MTT assay. The
523 data in the graphs represent the mean±SD. A representative experiment of two
524 experiments is shown in the figure. Statistical analysis was performed with one-
525 way ANOVA and Bonferroni's post-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ****

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526 $p<0.0001$. **b**, Representative confocal images of Hoechst 33342-stained T24 cells
527 infected with BCG and MTBVAC at the indicated MOI for four days. A
528 representative experiment of two experiments is shown in the figure.

529 **Figure 4. MTBVAC is internalized by macropinocytosis. a**,**b**, T24 and J82
530 cells were infected with GFP-expressing MTBVAC strain at MOI 10:1 in the
531 presence of EIPA, IPA-3, nystatin or wortmannin at the concentrations indicated,
532 and the percentage of infection was analyzed with flow cytometry or confocal
533 microscopy at four days. **c**, T24 and J82 cells were infected with BCG or MTBVAC at
534 MOI 20:1 in the presence of 10 μM EIPA and the percentage of infection was
535 analyzed by cytometry. **d**, T24 and J82 cell growth inhibition was analyzed with the
536 MTT assay four days post-infection. The data in the graphs are presented as the
537 mean±SD. A representative experiment out of two experiments is shown in the
538 figure. Statistical analysis was performed with one-way ANOVA (**a**) or two-way
539 ANOVA (**c**, **d**) and Bonferroni's post-test. ns: non-significant; * $p<0.05$; ** $p<0.01$;
540 *** $p<0.001$; **** $p<0.0001$.

541 **Figure 5. MTBVAC and BCG infectivity in MB49 cells.** MB49 murine cells
542 were infected with GFP-expressing BCG Pasteur or MTBVAC strains? (MOI 20:1),
543 and the percentage of infected cells was analyzed with flow cytometry (**a**) and
544 confocal microscopy (**b**). The data in the graph represent the mean value±SD of
545 one representative out of 3 independently performed experiments. ****$p<0.0001$,
546 determined by two-way ANOVA and Bonferroni's post-test.

547 **Figure 6. Intravesical treatment with MTBVAC increases the survival**
548 **of tumor-bearing mice. a**, Schedule of *in vivo* experiments. Groups of mice were
549 instilled with 10^5 MB49-luc cells and treated intravesically at 3, 6, and 9 days after
550 tumor induction with PBS or 10^7 CFU of BCG Pasteur or MTBVAC. **b**, Animals were

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551 followed-up for 60 days, and survival was determined according to pre-established
552 endpoint criteria approved by the ethics committee. Pooled data from two
553 independent experiments are plotted in a survival curve. Data represent the
554 percentage of surviving animals ±SD. Death/total events are represented in
555 brackets. Statistical significance was calculated with a log-rank test after
556 Benjamini-Hochberg multiple testing adjustment. ns: non-significant; $*p<0.05$.

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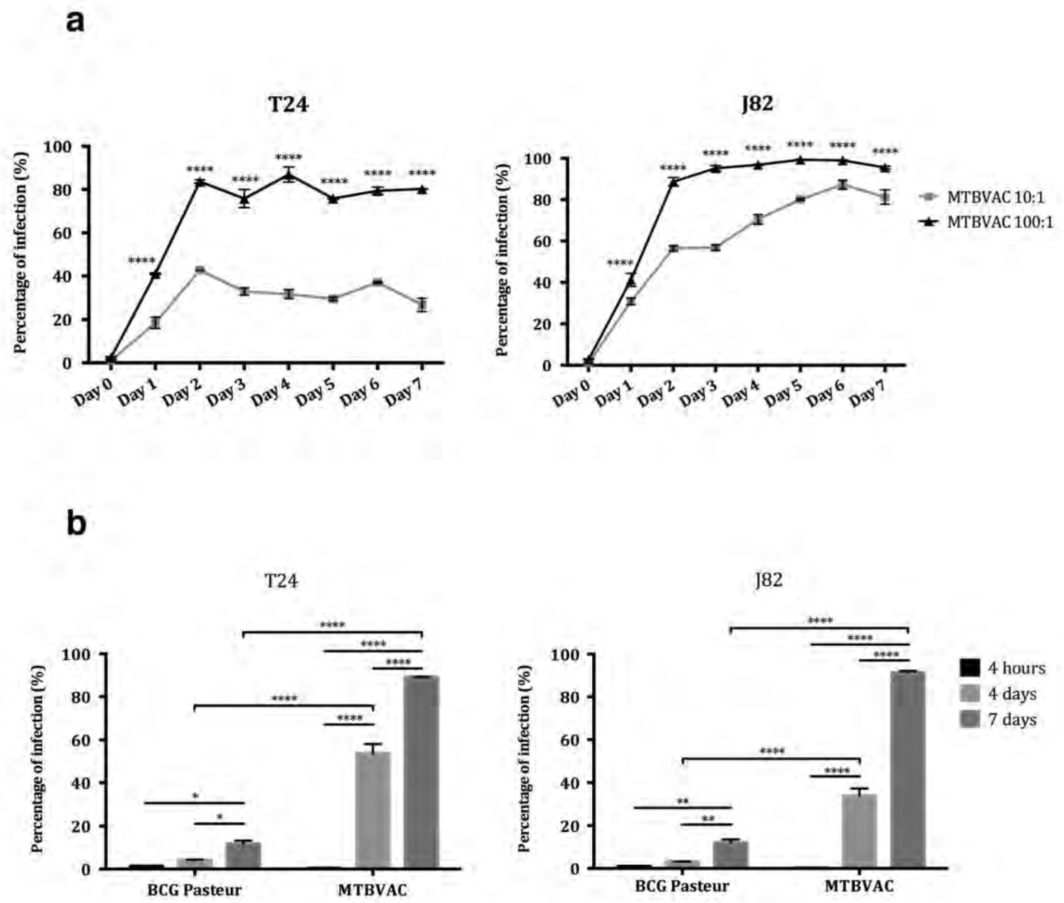

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560 REVISED FIGURE 1.tif

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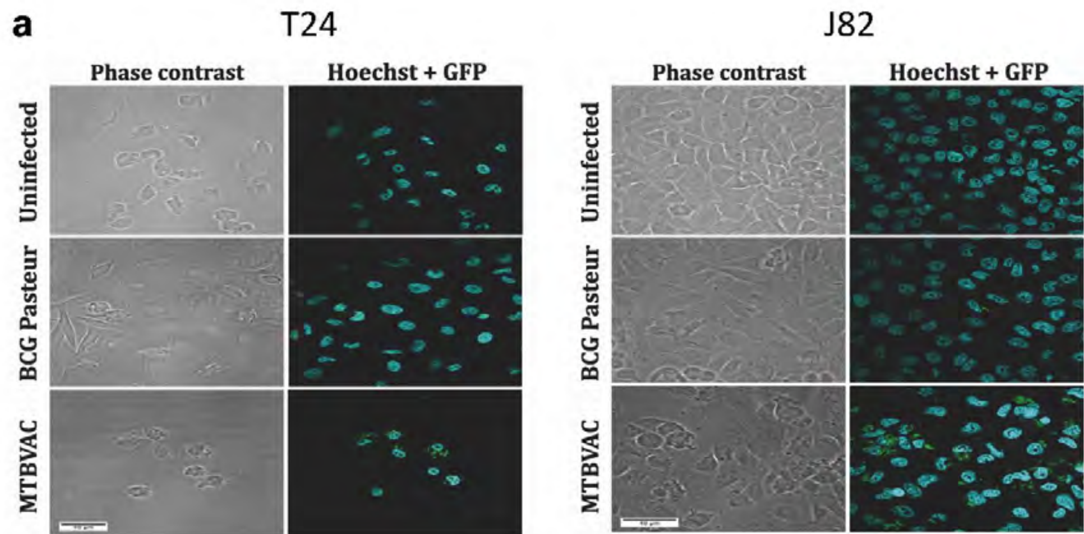

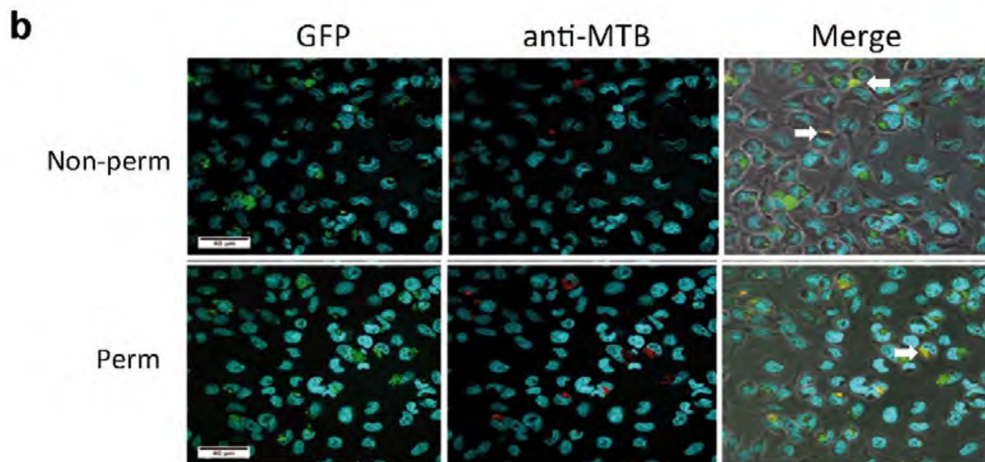

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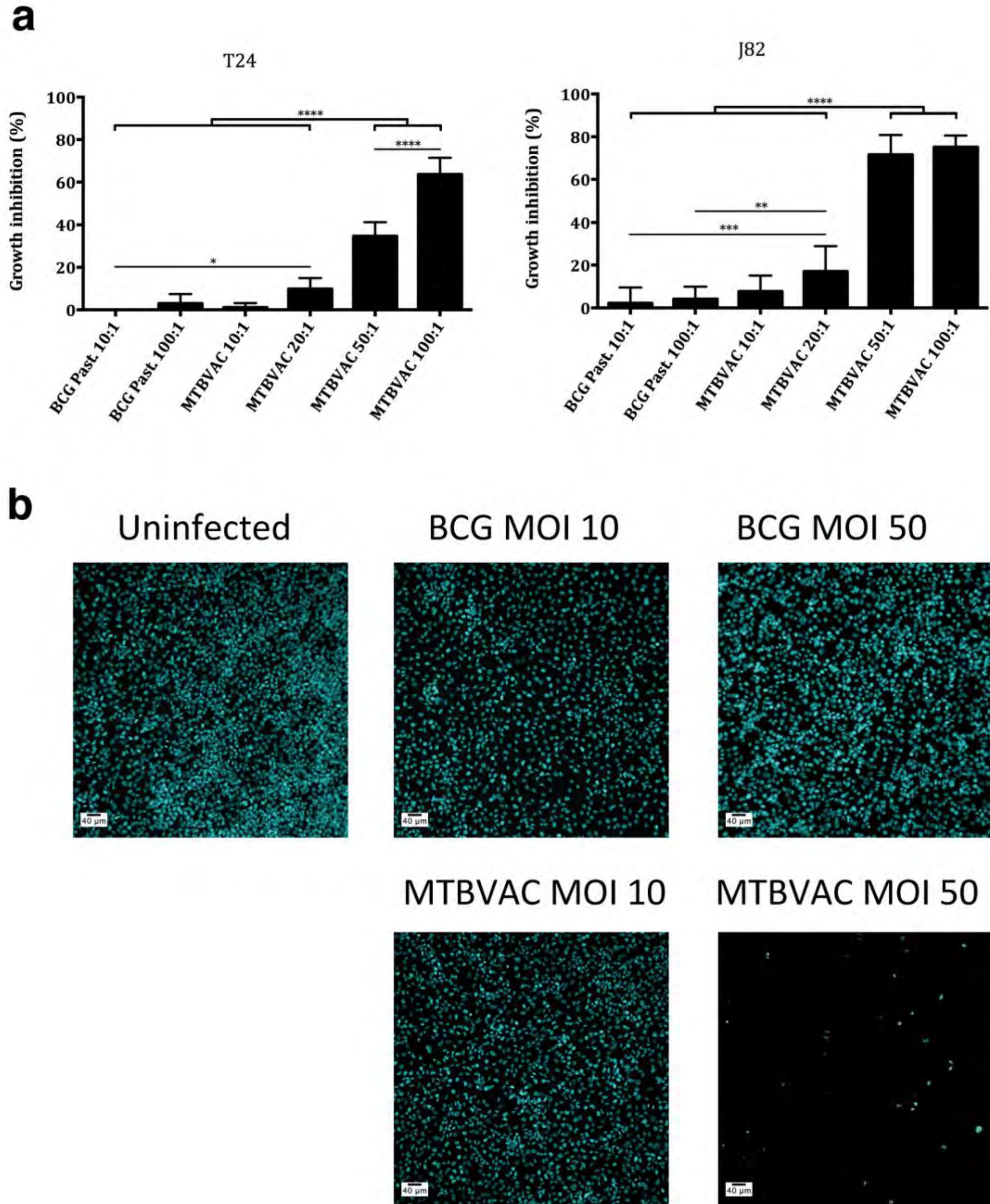

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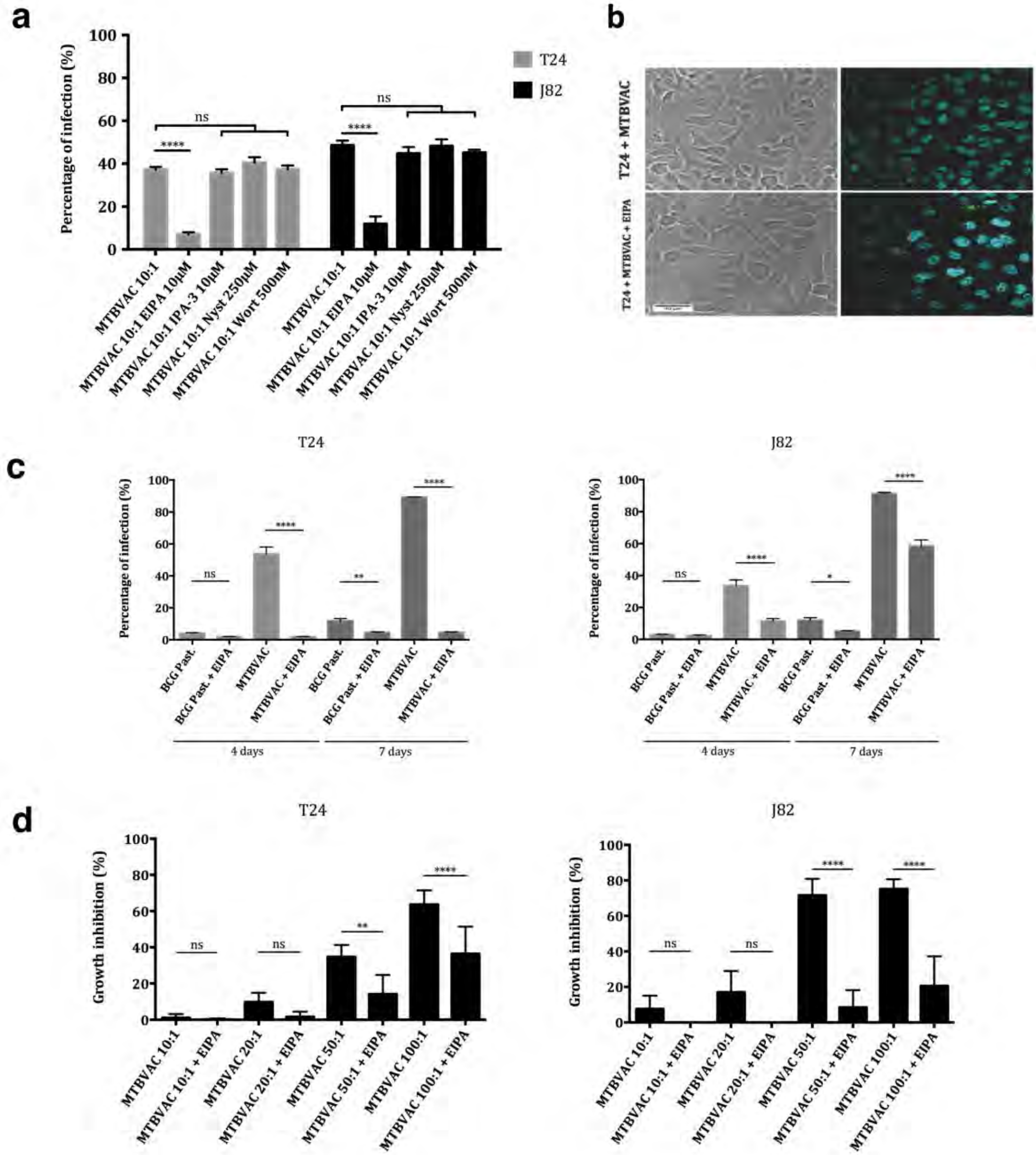

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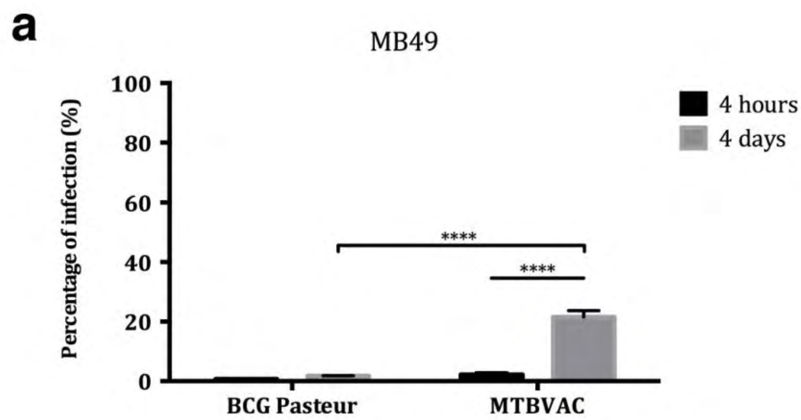

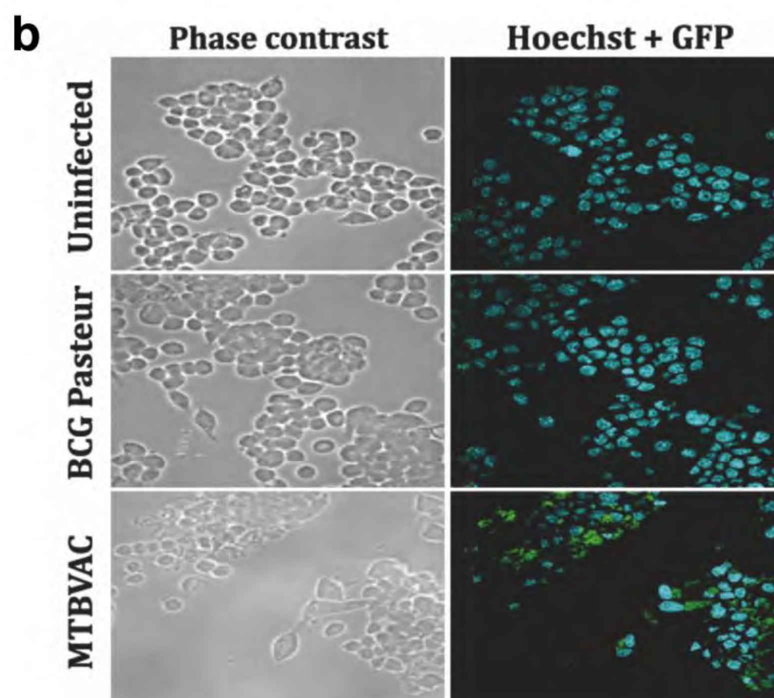

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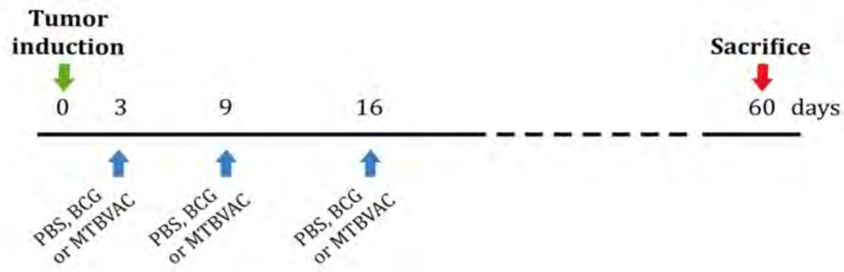

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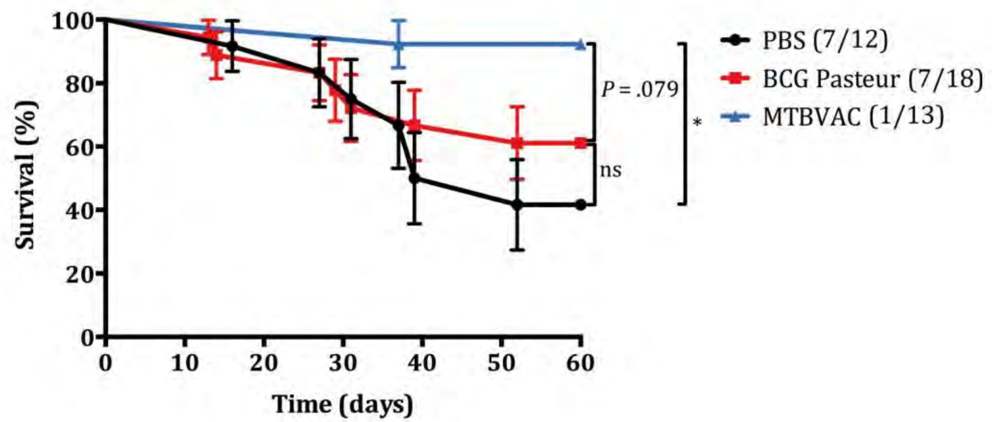

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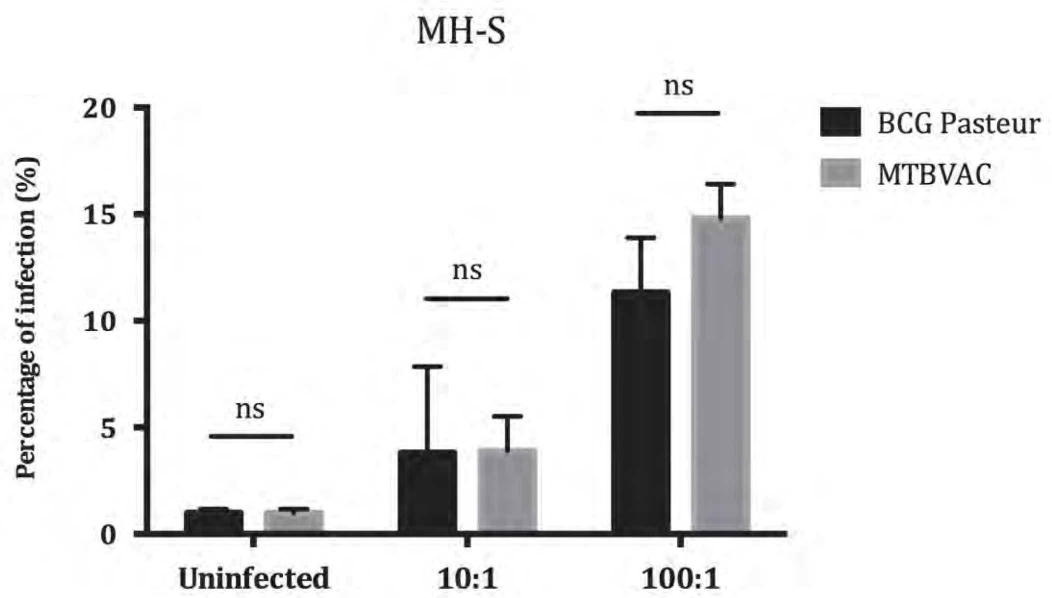

SUP FIG 1

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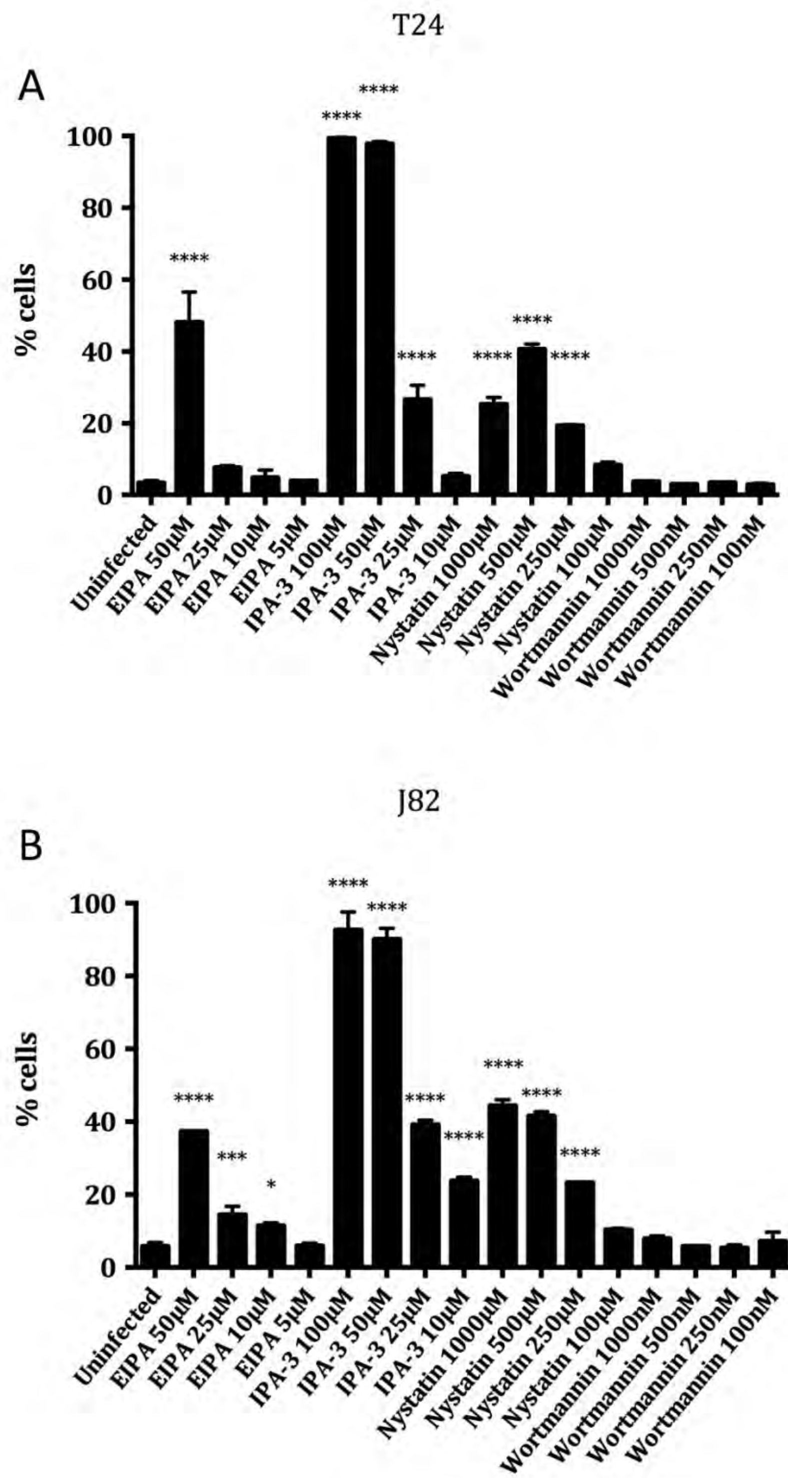

SUP FIG 2

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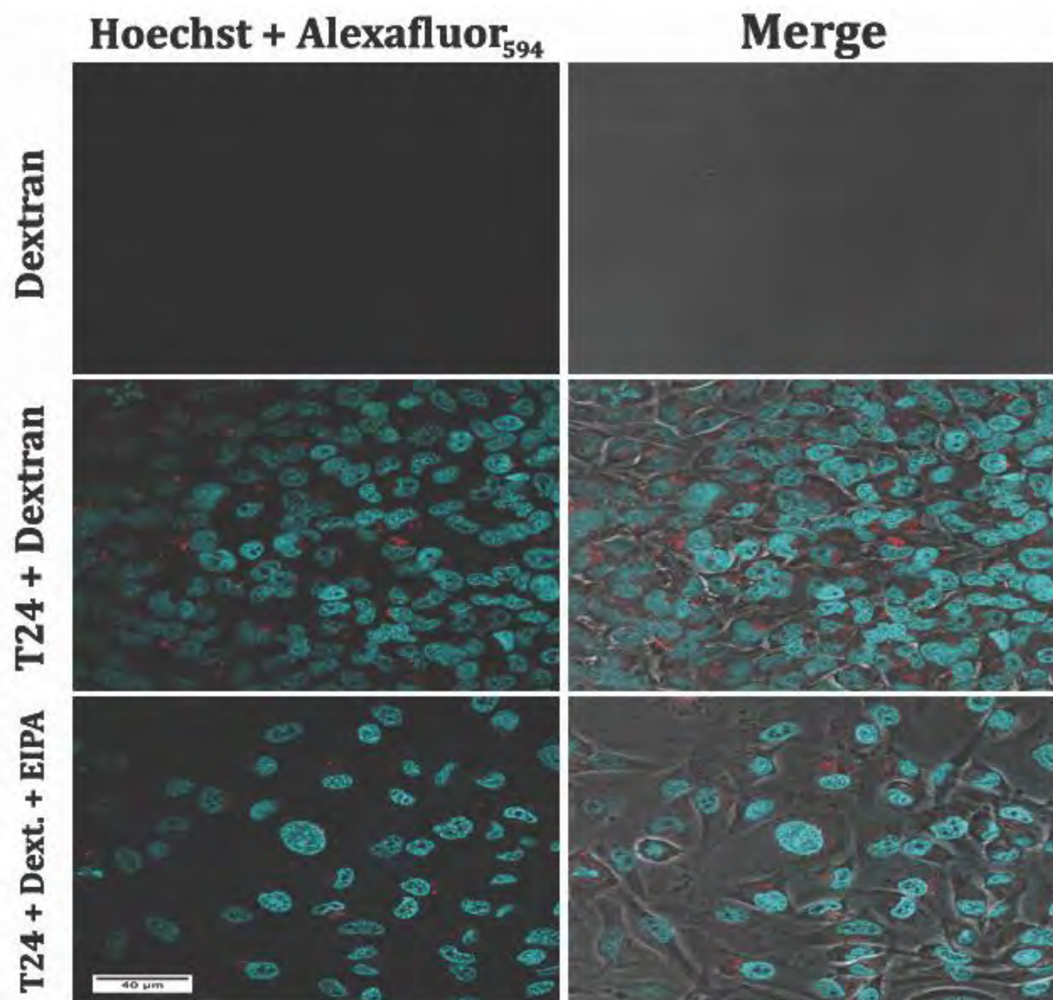

SUP FIG 3

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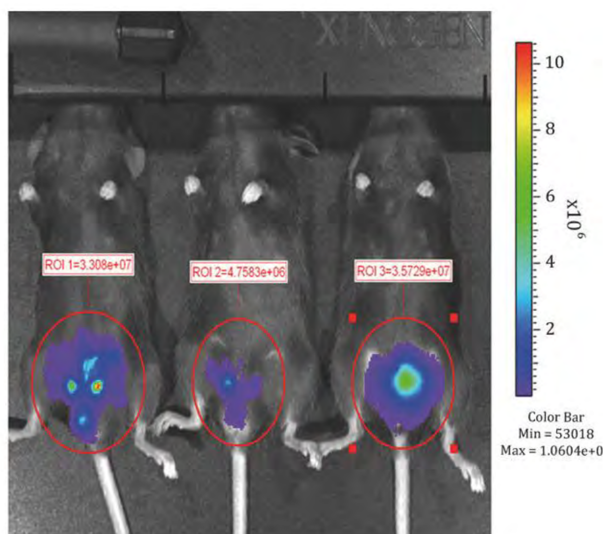

SUP FIG 4

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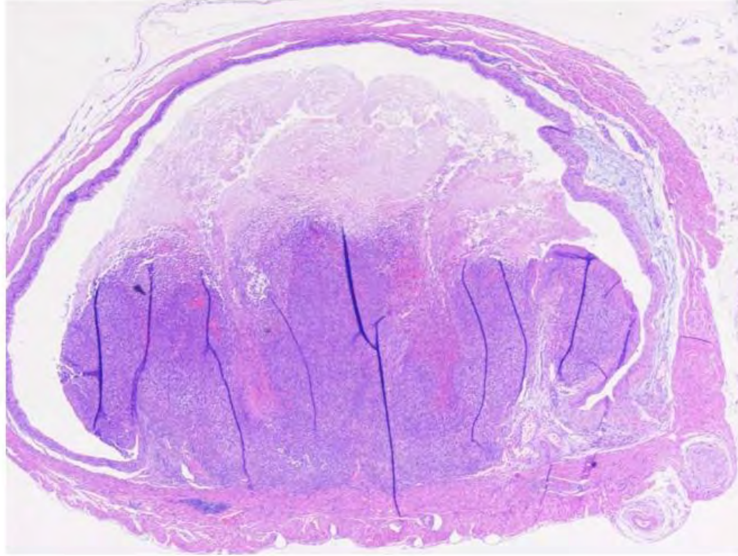

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