

1 **A secreted *Echinococcus multilocularis* activin A homologue promotes**
2 **regulatory T cell expansion**

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15 **Running Title:** Helminth activin expands host regulatory T-cells

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20 **ABSTRACT**

21 **Background:** Alveolar echinococcosis (AE), caused by the metacestode larval stage of
22 the fox-tapeworm *Echinococcus multilocularis*, is a chronic zoonosis associated with
23 significant modulation of the host immune response. A role of regulatory T-cells (Treg) in
24 generating an immunosuppressive environment around the metacestode during chronic
25 disease has been reported, but the molecular mechanisms of Treg induction by *E.*
26 *multilocularis* remain elusive so far.

27 **Methodology/Principal findings:** We herein demonstrate that excretory/secretory (E/S)
28 products of the *E. multilocularis* metacestode promote the formation of Foxp3⁺ Treg from
29 CD4⁺ T-cells *in vitro* in a TGF- β -dependent manner. We also show that host T-cells
30 secrete elevated levels of the immunosuppressive cytokine IL-10 in response to
31 metacestode E/S products. Within the E/S fraction of the metacestode we identified an *E.*
32 *multilocularis* activin A homolog (EmACT) that displays significant similarities to
33 mammalian Transforming Growth Factor- β (TGF- β)/activin subfamily members. EmACT
34 obtained from heterologous expression promoted host TGF- β -driven CD4⁺ Foxp3⁺ Treg
35 conversion *in vitro*. Furthermore, like in the case of metacestode E/S products, EmACT-
36 treated CD4⁺ T-cells secreted higher levels of IL-10. These observations suggest a
37 contribution of EmACT in the *in vitro* expansion of Foxp3⁺ Treg by the *E. multilocularis*
38 metacestode. Using infection experiments we show that intraperitoneally injected
39 metacestode tissue expands host Foxp3⁺ Treg, confirming the expansion of this cell type
40 *in vivo* during parasite establishment.

41 **Conclusions/Significance:** In conclusion, we herein show that *E. multilocularis* larvae
42 secrete a factor with clear structural and functional homologies to mammalian activin A.
43 Like its mammalian homolog, this protein induces the secretion of IL-10 by T-cells and
44 contributes to the expansion of TGF- β -driven Foxp3⁺ Treg, a cell type that has been
45 reported crucial for generating a tolerogenic environment to support parasite
46 establishment and proliferation.

47

48 **AUTHOR SUMMARY**

49 The metacestode larval stage of the tapeworm *E. multilocularis* grows infiltratively, like a
50 malignant tumor, within the organs of its human host, thus causing the lethal disease
51 alveolar echinococcosis (AE). Immunosuppression plays an important role in both survival
52 and proliferation of the metacestode, which mainly depends on factors that are released
53 by the parasite. These parasite-derived molecules are potential targets for developing new
54 anti-echinococcosis drugs and/or improving the effectiveness of current therapies.
55 Additionally, an optimized use of such factors could help minimize pathologies resulting
56 from over-reactive immune responses, like allergies and autoimmune diseases. The
57 authors herein demonstrate that the *E. multilocularis* metacestode releases a protein,
58 EmACT, with significant homology to activin A, a cytokine that might support host TGF- β
59 in its ability to induce the generation of immunosuppressive regulatory T-cells (Treg) in
60 mammals. Like its mammalian counterpart, EmACT was associated with the expansion
61 of TGF- β -induced Treg and stimulated the release of elevated amounts of
62 immunosuppressive IL-10 by CD4⁺ T-cells. The authors also demonstrate that Treg are
63 locally expanded by the metacestode during an infection of mice. These data confirm an

64 important role of Treg for parasite establishment and growth during AE and suggest a
65 potential role of EmACT in the expansion of these immunosuppressive cells around the
66 parasite.

67

68 **INTRODUCTION**

69 The metacestode larval stage of the fox-tapeworm *Echinococcus multilocularis* is
70 the causative agent of alveolar echinococcosis (AE), one of the most dangerous zoonoses
71 world-wide [1,2]. Intermediate hosts (rodents and, occasionally, humans) usually get
72 infected by oral ingestion of infectious eggs that contain the oncosphere larval stage. Upon
73 hatching in the small intestine and penetration of the intestinal wall, the oncosphere gains
74 access to the host organs and, almost exclusively within the liver, develops into the cyst-
75 like metacestode, following a process of stem cell-driven metamorphosis [3,4]. The multi-
76 vesicular *E. multilocularis* metacestode tissue subsequently grows infiltratively, like a
77 malignant tumor, into the surrounding host tissue, eventually leading to organ failure and
78 host death [2]. In later stages of the disease, metastases can be formed in secondary
79 organs, which is probably due to the distribution of parasite stem cells via bloodstream
80 and the lymphatic system [3]. In mice, the initial establishment phase of the parasite (the
81 oncosphere-metacestode transition) is typically accompanied by a potentially
82 parasitocidal, Th1- dominated immune response which, in permissive hosts, is skewed
83 towards a permissive Th2-dominated immune response during the chronic phase of the
84 disease [5]. Current treatment options against AE are very limited and include surgery,
85 which can only be applied in few cases, and/or chemotherapy with benzimidazoles [2].
86 However, due to significant adverse side effects, only parasitostatic doses of these

87 compounds can be applied and, consequently, the drugs often have to be administered
88 lifelong [2]. These limitations in current AE therapy underscore an urgent need for the
89 development of novel anti-parasitic measures.

90 During asexual multiplication, the *E. multilocularis* metacestode tissue persists for
91 prolonged periods of time in close contact to immune effector cells without being expelled
92 by the host immune response [5]. Immune suppressive mechanisms, provoked by
93 parasite surface structures and/or excretory/secretory (E/S) products, have thus been
94 proposed to support long-term persistence of the parasite within the host [5-8].
95 Accordingly, PBMCs of patients with active AE and host cells in the vicinity of parasite
96 liver lesions in mice have been demonstrated to produce elevated levels of the
97 immunosuppressive cytokines TGF- β and IL-10 and are believed to play important roles
98 in the pathophysiology of AE [9–11]. Furthermore, immune effector cells from *E.*
99 *multilocularis*-infected hosts typically display impaired immune reactivity [9,12-16]
100 whereas those from hosts with degenerating parasite tend to recover their immune
101 potential [17]. Moreover, host immune-stimulation during an infection can lead to
102 considerably reduced disease progression [18,19]. Although the molecular and
103 immunological basis for the immune suppression in AE is largely elusive so far, parasitic
104 helminths as a whole have repeatedly been reported to exploit the host immune system's
105 own self-regulatory signaling pathways for successful establishment of an infection and
106 long-term persistence within the host [20].

107 Of particular importance for regulation in mammalian immune responses are
108 signals delivered by TGF- β superfamily members. On the basis of sequence similarities,
109 two cytokine sub-families can be distinguished within this superfamily: the TGF- β /activin

110 sub-family and the Bone Morphogenetic Protein (BMP) sub-family [20]. The former
111 subfamily has gathered considerable interest concerning mechanisms of immune
112 homeostasis maintenance [21,22]. Produced as large pro-forms consisting of an N-
113 terminal signal peptide, followed by a pro-peptide separated by a furin recognition motif
114 from the C-terminal active peptide (~15 kDa), TGF- β /activin ligands are secreted as
115 dimers of their active peptide, following cleavage of the signal sequence and pro-peptides
116 [23]. Two particularly relevant peptides within the TGF- β /activin subfamily, TGF- β 1 and
117 activin A (i.e. inhibin beta A homodimers), have drawn considerable attention in the search
118 for mechanisms that lead to an impairment of immune effector cell functions and,
119 ultimately, to an expansion of tolerogenic cells [21,22]. Both cytokines were reported to
120 impair the function of dendritic cells (DC), NK cells, macrophages, and T-cells, and
121 stimulate the expansion of regulatory DC and T-cells [21,22].

122 During echinococcosis, the impaired host immune response is paralleled by an
123 increased expression of TGF- β signaling components in periparasitic host cells and
124 tissues [10,11,16,24–27] with the expansion of tolerogenic CD4⁺ CD25⁺ Foxp3⁺ Treg cells
125 [6,15,28–30]. *Echinococcus* antigens can stimulate the expression of CD25 by CD4⁺ T
126 helper cells from AE infected patients, contributing to the differentiation into Treg [31].
127 Using a murine system of intraperitoneal AE (secondary echinococcosis), Mejri *et al.* [15]
128 reported increased percentages of CD4⁺CD25⁺ T-cells in the peritoneum of *E.*
129 *multilocularis* infected mice at an advanced (chronic) stage of the disease, when
130 compared to non-infected mice. This group also found Foxp3 gene expression to be
131 elevated in these cells and a higher frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells in the
132 peritoneum and the spleen of *E. multilocularis*-infected mice [15,28]. Subsequent studies

133 then convincingly revealed Foxp3⁺ Treg as key players in the immunoregulatory
134 processes that facilitate the establishment and persistence of *E. multilocularis*
135 metacestode in their mammalian hosts [28-30]. Consistent with such observations, our
136 previous report of the ability of *E. multilocularis* metacestode E/S products to expand host
137 Treg *in vitro* [6], ultimately suggested that Treg expansion during AE, as increasingly
138 reported in the literature, could go beyond a simple homeostatic balancing mechanism.

139 In the present study, we have specifically followed up on these observations to
140 further investigate the ability of the *E. multilocularis* metacestode to increase host Tregs.
141 We report on a parasite TGF- β superfamily ligand, EmACT (*E. multilocularis* Activin),
142 which is released by the metacestode, and which promotes the ability of host TGF-beta
143 to induce Treg conversion and the production of IL-10 by host T-cells. Our data support a
144 role of parasite-derived factors in the impairment of host immune response during AE and
145 identify EmACT as a potent driver of host immune suppression by the *E. multilocularis*
146 metacestode.

147

148 **METHODS**

149 **Animals and Ethics statement**

150 Wild type C57Bl/6 mice and Mongolian jirds were purchased from Charles River
151 and housed at the local animal facilities of the Institute of Hygiene and Microbiology and
152 the Institute for Virology and Immunobiology of the University of Würzburg (Germany) at
153 least 1-2 weeks before experimentation. OT-II mice (TCR transgenic mice where CD4⁺ T
154 cells are specific for I-A^b presentation of OVA₃₂₃₋₃₃₉ peptide) were kindly provided by
155 Francis Carbone, Melbourne, Australia. OT-II mice were crossed with C57Bl/6 Rag-1^{-/-}

156 mice (devoid of mature B and T-cells), a generous gift from Thomas Winkler, Erlangen,
157 Germany. All animal handling, care and subsequent experimentation were performed in
158 compliance with European and German regulations on the protection of animals
159 (*Tierschutzgesetz*). Ethical approval of the study was obtained from the local ethics
160 committee of the government of Lower Franconia (Regierung von Unterfranken, 55.2-
161 2531.01-31/10 and 55.2-2531.01-26/13).

162
163 ***In vitro* maintenance of *E. multilocularis* metacestode and collection of E/S**
164 **products**

165 *E. multilocularis* metacestodes were isolated, separated from host contaminants
166 and axenically cultivated as previously described [6]. For the collection of E/S products,
167 axenically maintained metacestode vesicles were washed thrice in 1 x PBS and
168 resuspended in collection medium DMEM10redox i.e. Dulbecco's Modified Eagle's
169 Medium, 4.5 g glucose/L (DMEM + GlutamaxTM, GIBCO) supplemented with 10% Fetal
170 Bovine Serum Superior (Biochrom AG), 100µg/ml penicillin/streptomycin (PenStrep
171 solution, Biochrom AG), 20 µg/ml Levofloxacin (Tavanic, Sanofi-Aventis, Deutschland
172 GmbH), 143 µM β-mercapthoethanol (Sigma-Aldrich, cat. M6250), 10 µM Bathocuproine
173 disulfonic acid (Sigma, cat. B-1125) and 100 µM L-Cysteine (Sigma, cat. C-1276) under
174 axenic conditions (i.e. sealed in Nitrogen filled Ziploc freezer bag and placed in a 5%CO₂
175 incubator at 37°C). After 48 hours of culture, the supernatants containing the metacestode
176 E/S products were collected and filtered through a 0.2 µm sieve (Filtropur S filter,
177 SARSTEDT). The total amount of E/S product proteins was determined using the
178 bicinchoninic acid assay (Pierce BCA Protein Assay Kit, ThermoScientific, prod # 23228)

179 and the E/S products stored at -80°C until use.

180

181 **Injection of *E. multilocularis* metacestodes and *in vivo* follow-up**

182 *Preparation of parasite material and injections.* For *in vivo* assays, metacestode
183 vesicles were obtained from infected Mongolian jirds (*Meriones unguiculatus*). The
184 recovered parasite homogenates were washed thrice in PBS (1X) then transferred to
185 DMEM10redox for axenic maintenance with medium change twice per week for up to 10
186 days. The complete removal of host contaminants was assessed by PCR as previously
187 described [6]. The host-free parasite homogenates were then washed in PBS (1X),
188 separated in aliquots of 5000 acephalic cysts resuspended in a total volume of 500 µl PBS
189 (1X) to be used for intraperitoneal injections. An equal volume (500 µl) of the carrier
190 solution PBS (1X) was used in parallel for mock injections. Parasite preparations from 5
191 unrelated iird infections were used to include any eventual parasite-related variation in the
192 analysis.

193 *Peritoneal lavage and cell collection.* At defined points within a time frame of 42
194 days post intraperitoneal injection, mice were sacrificed by CO₂ asphyxiation. Ice cold
195 PBS (1x) with 10% heat-inactivated filtered FBS Superior (Biochrom AG) was then used
196 to wash the peritonea and retrieve the peritoneal exudate cells. In each of the five
197 experimental replicates (injections performed using five different isolates), the peritoneal
198 cells were harvested from naïve (a pool of 3 mice) or infected (1 mouse) mice at each
199 time point. The suspensions were filtered through a 70µm nylon cell strainer (BD
200 Biosciences). Red blood cells in the filtrates were lysed with 1.4% NH₄Cl for 5 minutes at
201 37°C. The filtrates were then washed in R10 medium and the total numbers of recovered

202 cells determined using the trypan blue (Biochrom) exclusion test on a bright-line Neubauer
203 counting chamber prior to analysis. At the end of the infection follow up (42 days), the
204 parasite tissues were thoroughly harvested from the peritonea of each infected mice and
205 the masses were determined.

206 *Flow cytometry.* 2×10^5 peritoneal exudates cells were stained with fluorochrome-
207 conjugated antibodies (anti-mouse) directed against the T-cell subset surface marker CD4
208 (Biotin, Miltenyi Biotec), the alpha chain of the IL-2 receptor CD25 (PE, eBioscience)
209 present on activated T-cells and the intracellular master transcription factor of Treg, Foxp3
210 (APC, Miltenyi Biotec). Biotinylated CD4 antibodies were detected by incubation with
211 either FITC- or Pe-Cy5-conjugated streptavidin (BD Biosciences). As isotype control of
212 activated/regulatory T-cells, Rat IgG1 K Isotype (PE, eBioscience) was used. The staining
213 procedure was executed as per the manufacturer instructions (Treg Detection Kit, Miltenyi
214 Biotec). The cells were resuspended in FACS buffer (1x PBS supplemented with 3% heat-
215 inactivated and filtered FCS and 0.1%NaN₃) and acquired on a cytometer (FACSCalibur,
216 Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on
217 FlowJo software (Tree Star, USA).

218

219 ***In vitro* Treg suppression assay**

220 From the spleen of a healthy mouse, and peritoneal exudates cells of mice 7 days
221 post infection (20 mice pooled), CD4⁺ cells were separately isolated using mouse CD4⁺
222 T-cell negative selection protocol (EasySep™ Mouse CD4⁺ T-cell Enrichment Kit, Stem
223 Cell Technologies) to a purity of > 90% according to the manufacturer's instructions.
224 Purified CD4⁺ T-cells were stained with CD4 antibody (Biotin, Miltenyi Biotec) and CD25

225 antibody (PE, eBioscience) followed by incubation with Pe-Cy5-conjugated streptavidin
226 (BD Biosciences). CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were then sorted on a MoFlo high-
227 speed sorter (Cytomation). Sorted CD4⁺CD25⁻ splenic cells (responders) were labeled
228 with 2µM CFSE (CFDA SE, Molecular Probes/Invitrogen) at 37°C for 10 min and washed
229 twice in R10 medium before use. For *in vitro* proliferation of the isolated responder cells,
230 total splenocytes from a healthy mouse, were labeled with a cocktail of non-APC binding
231 antibodies namely the murine T-cell lineage directed anti-Thy-1.2 antibody (BD
232 Pharmingen) and the T-cell subsets recognizing CD4 antibody (eBioscience) and CD8
233 antibody (eBioscience) on Ice for 30 minutes. The cells were then washed in 1x PBS
234 supplemented with 3% heat-inactivated and filtered Fetal Calf Serum (FCS, PAA
235 Laboratories) prior to antibody mediated complement lysis (Rabbit complement,
236 Cedarlane) at 1/10 dilution for 45 minutes under constant agitation at 37°C. Next, the
237 suspension was filtered through a 70 µm nylon cell strainer (BD Biosciences) and the
238 filtrate, representing antigen presenting cells (APC), washed and resuspended in R10
239 medium (RPMI-1640 from GIBCO BRL supplemented with 100U/ml Penicillin (Sigma),
240 100µg/ml Streptomycin (Sigma), 2mM L-glutamin (Sigma), 50 µM β-mercaptoethanol
241 (Sigma) and 10% heat-inactivated and filtered (0.22 µm, Millipore) fetal calf serum (FCS,
242 PAA Laboratories). APC were then irradiated on an X-ray unit (Faxitron, CellRad) with 20
243 Grays and counted using the trypan blue (Biochrom) exclusion test on a bright-line
244 Neubauer counting chamber. A total of 2 x 10⁵ irradiated APCs was then cultured in CD3
245 antibody (1ug/ml, eBioscience) pre-coated 96-well round-bottom plates with 2 x 10⁴
246 CFSE-labelled responders (Splenic CD4⁺CD25⁻ cells) and 1-2 x 10⁴ CD4⁺CD25⁺ cells for
247 5 days. The cells were then harvested, resuspended in FACS buffer (1x PBS
248 supplemented with 3% heat-inactivated and filtered FCS and 0.1% NaN₃) prior to

249 acquisition on a cytometer (FACSCalibur, Beckton Dickinson) equipped with CellQuest
250 software. Results were further analyzed on FlowJo software (Tree Star, USA).

251

252 **Identification, cloning and analysis of the *Emact* cDNA and gene**

253 The full length sequence of the *Schistosoma mansoni* TGF- β /activin subfamily
254 member (SmlnAct, DQ863513) and the human inhibin beta A chain (HsINH β A, P08476)
255 were used to search the *E. multilocularis* genome on Wormbase
256 (https://parasite.wormbase.org/Echinococcus_multilocularis_prjeb122/Info/Index/) using
257 *tblastn* and *blastp* algorithms. A predicted gene with a truncated 5' end
258 (EmuJ_000178100) could be retrieved from the available *E. multilocularis* genome
259 sequence [32]. The full-length coding sequence of the corresponding cDNA was identified
260 by screening of a complementary DNA library [33] and termed *Emact*. Briefly, a consensus
261 sequence between the *E. multilocularis* genome scaffold 6 and Smlnact was used as
262 template for primer design. The following primers were designed and used for retrieval of
263 the 5' (*Emact_5'*: 5'-ACA GTA GTT GGG TTC-3') and 3' (*Emact_3'*: 5'-GAA CCC AAC
264 TAC TGT-3') ends of *Emact*. These primers were used in pairs with primers specifically
265 recognizing the carrier vector part of the cDNA library, pJG4-5 [34]. Once recovered, the
266 5' and 3' ends of the parasite putative *act* reading frame, were used to design primers for
267 the full length amplification of the *Emact* coding sequence, namely *Emact_Dw* (5'-ATG
268 ACC ATT ACT ACC CCC ATG AAG-3') and *Emact_Up* (5'-ACT ACA ACC GCA CTC
269 TAG GAC AAT G-3'). Metacestode RNA was isolated using Trizol reagent (Invitrogen)
270 and 1 μ g of total RNA was reverse transcribed with Omniscript RT kit (Qiagen) according
271 to the manufacturers' instructions. The generated cDNA was used as template for

272 amplification of the *emact* full transcript using the primer pair *Emact_Dw* x *Emact_Up* by
273 high fidelity polymerase chain reaction (Phusion, NEB). Resulting amplicons were sub-
274 cloned into the pDrive cloning vector (QIAGEN) and five clones were picked and
275 sequenced in both directions identically revealing the full coding sequence of *emact*
276 (EmuJ_000178100). Sequence similarities between the deduced amino acid sequence of
277 *Emact* and other members of the TGF- β superfamily were determined through multiple
278 sequence alignments using BIOEDIT (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>),
279 and a neighbor-joining tree was generated from alignments using MEGA [34].

280

281 **EmACT Antibody production**

282 For the production of polyclonal antibodies, EmACT was expressed in the bacterial
283 pBAD/TOPO ThioFusion Expression Kit (Invitrogen). To maximize the recognition of
284 EmACT after processing by the generated antibodies, full *Emact* (without stop codon)
285 amplified using the primer pair *Emact_Dw* / *Emact_Up* coding for the preproprotein
286 EmACT was chosen for immunization and subcloned in pBAD/TOPO ThioFusion
287 expression vector (Invitrogen). The thioredoxin-fusion protein (Thio-EmACT) with histidine
288 tag, expressed in *E.coli* Top10 cells by adding arabinose (2 g/L; 4 hours), was purified on
289 nickel-nitrilotriacetic acid resin (Invitrogen) according to the manufacturer's protocol. The
290 purified Thio-EmACT was then diafiltered on Centrifugal Filter Units (Millipore) against
291 sterile PBS (1X) before quantification using the BCA assay (ThermoScientific). NMRI mice
292 were injected subcutaneously at two different locations with 100 μ g of the recombinant
293 Thio-EmACT resuspended in 100 μ l Freund Incomplete adjuvant (Sigma). The double
294 injections were repeated four weeks later to boost the mice anti- EmACT response. Finally,

295 ten days after the boost, the mice were bled from the heart and the serum collected
296 and stored at -20°C until use. In parallel, naïve mice were also bled and the serum
297 collected and stored as normal mouse serum.

298

299 **Recombinant expression of EmACT in Human Embryonic Kidney cell line**

300 *Emact* (without signal peptide) was subcloned in the eukaryotic pSecTag2
301 expression system (Invitrogen) to generate the pSegTag2-*Emact* vector construct as per
302 the manufacturer instructions. Human embryonic kidney cell-line 293T (HEK 293T) were
303 transfected with the expression vector construct pSegTag2-*Emact* or the empty pSecTag2
304 vector (Invitrogen) as control. Transfections were performed using linear
305 polyethylenimine (25 kDa, Sigma) according to the manufacturer's instructions. All
306 transfections were performed in petri dish (92 x 16 mm [Ø x height], SARSTEDT). HEK
307 cells were seeded 16 hours prior to transfection (3×10^6 cells / dish). 24 hours post-
308 transfection, the supernatants were replaced with fresh DMEM10 medium (i.e. Dulbecco's
309 Modified Eagle's Medium, 4.5g glucose/L (DMEM + Glutamax, GIBCO) supplemented
310 with 10% Fetal Bovine Serum Superior (Biochrom AG), 100µg/ml Penicillin/streptomycin
311 (PenStrep solution, Biochrom AG) and 20µg/ml Levofloxacin (Tavanic, Sanofi-Aventis,
312 Deutschland GmbH).). The supernatants of transfected HEK cells were then collected
313 after 24 hours of incubation, filtered over a bottle top filter (Filtropur BT50, SARSTEDT),
314 normalized for the total protein content (BCA Protein Assay Kit, ThermoScientific) and
315 stored as aliquots at -80°C until use.

316

317 **Immunodetection**

318 To detect EmACT in supernatants of parasite cultures (natural) or HEK 293 T cell
319 cultures (recombinant), 1ml of metacestode vesicle E/S products (MVE/S) or pSecTag2-
320 *Emact*-transfected HEK cell supernatant was resuspended in 9 volumes of 100% ice-cold
321 ethanol. The mixtures were kept at -80° C for at least 2 hours, and then centrifuged for 30
322 min at 14,000 rpm in a refrigerated centrifuge. The supernatants were discarded and the
323 pellets were dried thoroughly at 50°C and resuspended in 50 µl of 2 x STOPP mix (2 ml
324 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4 ml H₂O, 0.4 ml 0.05% (w/v)
325 bromophenol blue, 7 µl β-mercaptoethanol per 100 µl) and boiled for 10 min at 100°C.
326 10µl of each protein sample were separated by SDS-PAGE and transferred to a
327 nitrocellulose membrane for immunodetection with anti-EmACT antiserum or with mouse
328 pre-immune serum.

329

330 **Generation of murine bone marrow-derived dendritic cells (BMDC)**

331 Dendritic cells were obtained by GM-CSF-driven differentiation of mice bone
332 marrow precursor cells as previously described [35]. Briefly, C57Bl/6 mice (Charles
333 River/Wiga, Sulzfeld, Germany) aged 6-14 weeks and bred within the animal facility of the
334 Institute of Virology and Immunobiology, University of Würzburg, under specific pathogen-
335 free conditions were sacrificed using CO₂ asphyxiation. Femurs and tibiae were removed
336 and purified from the surrounding muscle tissue. Thereafter the marrow was flushed with
337 PBS (1X), resuspended by gently pipetting and washed once in medium. The medium
338 used here was R10 medium composed of RPMI-1640 (GIBCO BRL) supplemented with

339 100 U/ml Penicillin (Sigma), 100 µg/ml Streptomycin (Sigma), 2 mM L-glutamine (Sigma),
340 50 µM β-mercaptoethanol (Sigma) and 10% heat-inactivated and filtered (0.22 µm,
341 Millipore) fetal calf serum (FCS, PAA Laboratories) as previously defined. Once
342 resuspended, the bone marrow precursor cells were counted using the trypan blue
343 (Biochrom) exclusion test on a bright-line Neubauer counting chamber. 2×10^6 precursor
344 cells were cultured in R10 medium supplemented with 10% GMCSF-containing
345 supernatant as previously defined [35]. At day 8, non-adherent cells representing at a high
346 frequency newly differentiated dendritic cells (70-89% CD11c⁺) were harvested, washed
347 once in R10 medium prior to subsequent assays.

348

349

350 **Isolation of splenocytes and lymph node cells**

351 Single cell suspensions were obtained from the spleen and lymph nodes of C57Bl/6
352 mice by mechanically squeezing the tissues with glass slides in cold PBS and filtered
353 through a 70 µm nylon cell strainer. Red blood cells in the spleen filtrate were lysed with
354 1,4% NH₄Cl for 5 min at 37°C, and the splenocytes were washed in R10 medium, that is
355 RPMI 1640 (GIBCO BRL) supplemented with penicillin (100 U/ml, Sigma, Deisenhofen,
356 Germany), streptomycin (100 µg/ml, Sigma), L-glutamin (2 mM, Sigma), 2-
357 mercaptoethanol (50 µM, Sigma and 10% heat-inactivated fetal calf serum (FCS, PAA
358 Laboratories, Parsching, Austria). Cell counts were subsequently determined for
359 splenocytes and lymph node cells using the trypan blue (No.26323, Biochrom, Berlin,
360 Germany) exclusion test on a bright-lined Neubauer counting chamber.

361

362 **Treg conversion assays**

363 CD4⁺ T-cells were isolated from murine splenocytes and lymph node cells using
364 a T-cell negative selection kit (Easy Sep mouse T-cell enrichment kit, Stem Cell
365 Technologies) to a purity >90% as per the manufacturer's instructions. CD4⁺ T-cells were
366 further enriched for CD25⁻ cells using Miltenyi Biotec's LD columns with a suitable MACS
367 separator achieving > 90% purity as per the manufacturer's instructions. Murine BMDCs
368 at day 8 of cultivation were incubated with 3-fold higher numbers of CD4⁺ CD25⁻ T-cells
369 (OT-II or OT-II.RAG-1^{-/-}) and 200 ng/ml OVA peptide (323-339, grade V, Sigma)
370 supplemented or not with our different stimuli. In some assays, the cultures were
371 supplemented with 20 µg/ml of a pan-vertebrate anti-TGF-β blocking antibody 1D11 (R&D
372 Systems), alongside stimuli addition. In others, isolated naïve T-cells were pre-incubated
373 for 30 minutes with 5 µM of an inhibitor of TGF-β superfamily type I activin receptor-like
374 kinase (ALK) receptors ALK4, ALK5, and ALK7 (SB431542 [36]) before cultivation with
375 BMDC.

376 Alternatively, CD4⁺ T-cells were purified from wild type C57Bl/6 mice and
377 subsequently activated on plate-bound CD3 (1 µg/ml) and CD28 (0.5 µg/ml) antibodies in
378 the absence or presence of our different stimuli. Recombinant human TGF-β1 (R&D
379 Systems) was used as positive control. After 5 days of incubation, the cells were
380 harvested and stained using the Treg detection kit (Miltenyi Biotec), resuspended in FACS
381 buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN₃)
382 prior to acquisition on a cytometer (FACSCalibur, Beckton Dickinson) equipped with
383 CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

384

385 **CD4⁺ T-cells stimulation assay**

386 CD4⁺ T-cells were isolated from splenocytes and lymph node cells of wild type
387 C57Bl/6 mice (6-8weeks old) using a T-cell negative selection kit (Easy Sep CD4⁺ T-cell
388 enrichment kit, Stem Cell Technologies) to > 90% purity according to the manufacturer's
389 instructions. The CD25⁻ fraction was further enriched using Miltenyi Biotec LD columns
390 with a suitable MACS separator achieving > 90% purity for CD4⁺CD25⁻ T-cells. Next, 2 x
391 10⁵ CD4⁺CD25⁻ T-cells were seeded in a 24-well tissue culture plate (Flat bottom,
392 SARSTEDT) that had been coated with CD3 antibody (0.1µg/ml, eBioscience) overnight
393 at 4°C in R10 medium. The cells suspension was supplemented with 5µg/ml of CD28
394 antibody (eBioscience) and different stimuli were subsequently added. After 72h, T-cells
395 supernatants were probed for IL-10 release by ELISA as per the manufacturer's
396 instructions (BD OptEIA - Mouse IL-10 ELISA Set - BD Biosciences with a detection limit
397 of 19pg/ml).

398

399 **Statistical Analyses**

400 All results were expressed as mean ± standard deviation (SD). Differences
401 observed between groups were evaluated using the Wilcoxon/Mann-Whitney U test, a
402 nonparametric test that does not assume normality of the measurements (it compares
403 medians instead of means). Values of p<0.05 were considered statistically significant.
404 Statistical analyses were performed with a statistical software analyzing package
405 (GraphPad Software).

406

407

408 **Accession numbers**

409 The complete *Emact* cDNA sequence reported in this paper was deposited in the
410 GenBank database under the accession number HF912278. All GenBank accession
411 numbers of genes and sequences used in this study are listed in S1 Tab.

412

413

414

415 **RESULTS**

416 ***E. multilocularis* metacystode tissue drives Foxp3⁺ Treg expansion in**
417 **experimentally infected mice.**

418 In previous reports, it has been shown that Treg are expanded during chronic
419 secondary AE 6-12 weeks post infection [15,28]. It has not been elucidated, however,
420 whether this expansion resulted from an inherent host protective mechanism in the course
421 of a chronic infection in order to minimize tissue damage, or whether Treg expansion was
422 actively driven by the parasite. Since chronic AE, other than early AE, is associated with
423 severe depletion of T-cells after 6 weeks of infection [37], we investigated the dynamics
424 of host CD4⁺ T-cell responses during experimental secondary AE for up to 7 weeks (42
425 days) post infection; i.e. up to the chronic phase of the disease. To this end, we injected
426 mice intraperitoneally with 5000 *E. multilocularis* accephalic cysts (metacystode),
427 axenically sub-cultured for up to 10 days to remove host cells (as confirmed by species-
428 specific PCR, see Fig 1A), and analyzed the peritoneal exudate cells over a 7-weeks
429 period (42 days).

430

431 **Fig 1: Injected *E. multilocularis* metacestode tissue proliferates in the face of**
432 **accumulating CD4⁺ T-cells. (A)** Parasite cysts were harvested from infected jirds and
433 kept under axenic conditions for 10 days. The presence of host contaminants was
434 assessed by organism-specific PCR. Chromosomal DNA was isolated, the host (Jird)-
435 specific β -tubulin or the parasite-specific *e/p* genes were separately amplified. Jird tissue
436 was used as a negative control for the parasite-specific gene *e/p*. **(B)** Peritoneal exudate
437 cells were collected, counted and analyzed by flow cytometry for CD4 expression.
438 Parasite-driven accumulation of total **(B up)** or CD4⁺ T-cells **(B down)** is shown for D3-42
439 post injection. **(C)** Masses of parasitic tissue injected and recovered after 42 days.
440 Horizontal bars stand for mean levels. Data represent means \pm SD from groups of five
441 mice for each time point assayed individually (Infected). Naive mice were clustered in sub-
442 groups of 3 mice pooled as one per assay (15 mice for each time point).*, $p < 0.05$.

443
444 We observed a significant increase of peritoneal exudate total and CD4⁺ T-cells
445 over time in *E. multilocularis* infected mice as compared to mock (PBS)-injected controls
446 (Fig 1B). Interestingly, despite the anti-AE role of host cellular immunity in general and
447 CD4⁺ T-cell mediated effector functions in particular [38], the CD4⁺ T-cell expansion in
448 infected mice was paralleled by an increase in parasite mass during the study period
449 (Figure 1C).

450 We then examined the subsets of CD4⁺ T-cells expanded in infected mice by
451 expression levels of CD25 and Foxp3. A separation into CD25⁺Foxp3⁻ CD4⁺ activated
452 effector T-cells (Teffs) and CD25⁺Foxp3⁺ CD4⁺ T-cells as Tregs was applied (Fig 2A).
453 Although we noted a general increase of Foxp3⁺ Treg numbers in infected mice when
454 compared to naïve mice throughout the study period (Fig 2B), a transient but significant

455 increase of the proportion of Tregs was uniquely detectable at 7 days post-infection within
456 the peritoneal exudates of mice (Fig 2C). The Tregs induced by the parasite 7 days post
457 intraperitoneal inoculation were able to repress proliferative responses of CFSE-labeled
458 conventional CD4⁺CD25⁻ T-cells (Fig 3), indicating that they were functionally
459 suppressive.

460
461 **Fig 2: Initial CD4⁺ T-cell responses to *E. multilocularis* metacestodes are biased**
462 **towards Foxp3⁺ Treg.** The effects of intraperitoneal injection of *E. multilocularis* cysts in
463 C57Bl/6 mice were followed by analysis of peritoneal exudate CD4⁺ cells harvested at
464 days 3, 7, 14 and 42 post-injection, respectively. The cells were analyzed by flow
465 cytometric analysis for CD25 and Foxp3 expression. **(A)** The CD25⁺ population was
466 clustered into Teffs or Tregs with regards to Foxp3 expression. **(B)** The kinetics of Foxp3⁺
467 Treg numbers was monitored. A Mann Withney U test was performed separately at each
468 time point to compare naive and infected mice. **(C)** Kinetics of Treg/Teff ratio over time as
469 a measure of the bias of parasite-associated CD4⁺ T-cell response. Each ratio for infected
470 mice was subtracted of the corresponding naive mice ratio. Data represent means +/- SD
471 from groups of five mice for each time point assayed individually (Infected). Naive mice
472 were clustered in sub-groups of 3 mice pooled as one per assay (15 mice for each time
473 point).*, $p < 0.05$.

474
475 **Fig 3: *E. multilocularis* metacestode-induced Treg are functionally suppressive *in***
476 ***vitro*.** Peritoneal exudate cells from mice infected for 7 days with 5000 acephalic *E.*
477 *multilocularis* cysts, and naive splenocytes from control mice, were prepared by CD4⁺ T-
478 cell magnetic selection, then FACS-sorted into CD4⁺CD25⁺ and CD4⁺CD25⁻ populations.

479 Splenic naive CD4⁺CD25⁻ cells (responders) were then polyclonally stimulated in the
480 presence or absence of CD4⁺CD25⁺ cells from either control or *E. multilocularis*-infected
481 mice. **(A)** Representative plots of CFSE-labelled responder cells proliferation with
482 increasing amounts of CD4⁺ CD25⁺ Treg. **(B)** Proportions of labeled live CD4⁺ cells in
483 each generation of assay conducted at 1:1 ratio, as gated by CFSE dilution. A
484 representative experiment out of two with similar results is displayed.

485
486 Taken together these analyses showed that *E. multilocularis* metacestodes can
487 grow in these mice and raise a CD4⁺ T-cell response but with a transient overproportional
488 expansion of suppressive Tregs.

489
490 **E/S products of the *E. multilocularis* metacestode induce Foxp3 expression and IL-**
491 **10 production by host T-cells *in vitro*.**

492 We previously demonstrated Foxp3⁺ Treg expansion *in vitro* from OT-II naïve CD4⁺
493 T-cells activated with OVA-loaded DC in the presence of *E. multilocularis* metacestode E/S
494 products [6], suggesting either a direct induction of Treg conversion by the parasite
495 products or a mitogenic effect of these products on pre-existing OT-II Treg. To further
496 examine these alternatives, we isolated naïve OT-II.RAG-1^{-/-} CD4⁺T-cells from spleens
497 and lymph nodes of naïve animals, genetically devoid of Foxp3⁺ Tregs (Fig 4A). The cells
498 were activated *in vitro* with OVA-loaded DCs in the presence of *E. multilocularis*
499 metacestode E/S products (MVE/S) as previously described [6]. MVE/S failed to activate
500 BMDC cultures beyond the baseline level obtained with medium, arguing against a
501 potential contamination of the harvested parasite products with endotoxins [6]. Notably,
502 Foxp3⁺ Treg frequencies were considerably enhanced in cultures supplemented with

503 MVE/S, similar to TGF- β (Fig 4B), suggesting that *E. multilocularis* metacestode E/S
504 products can induce *de novo* Foxp3⁺ Treg conversion from naive CD4⁺ T cells *in vitro*. We
505 also measured the production of the immunosuppressive cytokine IL-10 in DC-T-cell co-
506 cultures in the presence or absence of the parasite products. We noted a significantly
507 increased production of IL-10 in cultures supplemented with *E. multilocularis* metacestode
508 products (Fig 4C) indicating that the parasite products can both expand host Foxp3⁺ Treg,
509 and also trigger an elevated production of IL-10 by host immune cells.

510
511 **Fig 4: E/S products of *E. multilocularis* metacestode promote the *de novo* Foxp3⁺**
512 **Treg conversion *in vitro*.** (A) Staining of CD4⁺ Foxp3⁺ T-cell within the bulk of spleen
513 and lymph node cells from wild type C57Bl/6 or C57Bl/6 OT-II.RAG-1^{-/-} mice over a
514 C57Bl/6 background. (B) MVE/S promote *de novo* CD4⁺CD25⁺Foxp3⁺ Treg conversion *in*
515 *vitro*. Freshly generated DCs (Day 8) were co-cultured with naïve CD4⁺CD25⁻ T-cells
516 from OT-II.RAG-1^{-/-} mice at a DC:T-cell ratio of 1:3 in R10 medium supplemented with
517 OVA peptide (200ng/ml). E/S-free medium (DMEM10redox) or MVE/S-containing medium
518 was added to the cultures prior to incubation. Different doses of recombinant human TGF-
519 β 1 were used as positive controls. 5 days later, cells were harvested and stained for CD4,
520 CD25 and Foxp3 prior to flow cytometry analysis. (C) Additionally, culture supernatants
521 were collected and probed for IL-10 by ELISA. (B, C) Summarized in the graph are the
522 percentages of CD25⁺ Foxp3⁺ cells within the CD4⁺ T-cell population and the production
523 of IL-10 measured after exposure to the indicated stimuli. Data represent mean \pm SD from
524 two independent experiments with products from two different parasite isolates. (D)
525 Foxp3⁺ Treg frequencies in CD4⁺ Tcells cultured for 5 days on CD3/CD28 antibody-coated

526 plates in the presence of E/S-free medium (DMEM10redox) or MVE/S-containing medium.
527 Bars represent the mean \pm SD of results obtained with E/S products from 4 different
528 parasite isolates. *, $p < 0.05$. **(E)** Naïve CD4⁺ CD25⁻ T-cells freshly isolated from C57Bl/6
529 mice were stimulated at 2×10^5 /ml with CD3/CD28 antibodies in the presence of parasite
530 E/S-free cultivation medium (DMEM10redox) or MVE/S-containing medium. After 72
531 hours, the T-cells supernatants were collected and probed for IL-10 concentration by
532 Elisa. Horizontal bars represent the mean from experiments conducted with E/S products
533 from 4 different parasite isolates. *, $p < 0.05$; **, $p < 0.005$.

534
535 Next, to investigate the role of the DC population in T-cell modulation by *E.*
536 *multilocularis* products, naïve CD4⁺ T-cells from spleens of C57Bl/6 mice activated with
537 plate-bound anti-CD3 and anti-CD28 antibodies (instead of DC-based activation) in the
538 presence of *E. multilocularis* metacestode E/S products were both analyzed for Foxp3
539 expression and IL-10 production (see material and methods for experimental set-ups). We
540 observed an increased rate of Foxp3⁺ Treg (Fig 4D), and a significantly elevated
541 production of IL-10 (Fig 4E) in host T-cell cultures indicating that *E. multilocularis*
542 metacestode products can induce Foxp3⁺ Treg conversion and trigger IL-10 release by
543 naïve T-cells in a DC-independent manner.

544
545 **Host TGF- β and TGF- β signaling are essential for Treg conversion driven by**
546 **metacestode E/S products.**

547 It has previously been shown that the conversion of CD4⁺ T-cells to Treg requires
548 TGF- β [39] and we cannot exclude that the complex, serum-containing media required for
549 parasite cultivation do contain this cytokine to a certain amount. To analyze whether

550 metacestode E/S products require host TGF- β activity to promote Treg conversion, anti-
551 TGF- β neutralizing antibodies were used. The performed assay showed a clear inhibition
552 of Foxp3⁺ Treg conversion by metacestode E/S products when TGF- β was neutralized
553 (Fig 5). To additionally confirm an important role of TGF- β in the ability of metacestode
554 products to expand host Treg, the TGF- β signaling inhibitor SB431542 [36] was used.
555 Again, we observed a drastic reduction of the rate of Foxp3⁺ Treg induced by metacestode
556 E/S products (Fig 5). Taken together, we conclude from these studies that metacestode
557 E/S products can induce the conversion of naive CD4⁺ T-cells into Foxp3⁺ Treg *in vitro*
558 and that this activity depended on the presence of host TGF- β and functional TGF- β
559 signaling in host cells.

560

561 **Fig 5: Blocking TGF- β signalling or host TGF- β alone abrogates *E. multilocularis*-**
562 **driven Treg conversion *in vitro*.** (A) Representative plots of CD25 versus Foxp3
563 expression, gated on CD4⁺ T-cells, from OT-II naïve CD4⁺ T-cells cultivated with freshly
564 generated DC (Day 8) at a DC:T-cell ratio of 1:3 in R10 medium supplemented with OVA
565 peptide (200ng/ml) in the presence of MVE/S-containing medium alone (supplemented
566 with DMSO in one out of two experiments), combination of MVE/S-containing medium
567 with TGF- β antibody or combination of MVE/S-containing medium with SB431542
568 (resuspended in DMSO). Flow cytometry was performed 5 days later. (B) Mean
569 percentages of Foxp3⁺ Treg within the CD4⁺ T-cell population of DC/T-cell cultures
570 supplemented with the indicated stimuli. Bars represent mean \pm SD from two independent
571 experiments with cells from individual mice and products from two different parasite
572 isolates. *, $p < 0.05$.

573

574 ***E. multilocularis* expresses an activin A – like cytokine.**

575 By literature search for molecules that could exert activities as observed above for
576 metacestode E/S products, we found striking similarities to the mammalian TGF- β -like
577 cytokine activin A. Like metacestode E/S products, activin A can induce Treg conversion
578 *in vitro*, which depends on host TGF- β and functional TGF- β signaling [40,41], and it can
579 induce the secretion of IL-10 by CD4⁺ T-cells [41]. Interestingly, the expression of activin-
580 like cytokines, SmlnAct [42] and FhTLM [43], have previously been reported for the related
581 flatworm parasites *Schistosoma mansoni* and *Fasciola hepatica*, respectively. Both of
582 these molecules influence parasite development and immunoregulatory functions have
583 also been demonstrated for the latter [44]. We therefore hypothesized that *E. multilocularis*
584 might also express an activin A-like molecule and performed extensive BLASTP analyses
585 on the published genome sequence [32], using mammalian inhibin beta A (activin A
586 monomer) and SmlnAct as queries. These analyses revealed the presence of one single-
587 copy gene, EmuJ_000178100, encoding a protein with significant homologies to both
588 query sequences. Since further genome mining did not yield indications for the presence
589 of additional inhibin/activin-encoding genes, implying that the cytokine encoded by
590 EmuJ_000178100 can only form homo- but not heterodimers, the respective gene was
591 designated *Emact* (for *E. multilocularis* activin).

592 The full length cDNA of *Emact* was cloned and sequenced and comprised 1536 bp
593 that encoded a 507 aa protein, EmACT, with a hydrophobic region at the N-terminus,
594 indicating the presence of an export-directing signal peptide (Fig 6). Structurally, EmACT
595 displayed several conserved features of the TGF- β cytokine superfamily such as a C-
596 terminal, cysteine-rich active domain, separated by a tetrabasic RTRR cleavage motif

597 from a large N-terminal and less well conserved pre-protein sequence. Within the C-
598 terminal active domain of all TGF- β superfamily members (activins and BMPs) are seven
599 invariant cysteine residues, six of which form a rigid, heat stable “cysteine knot” [23].
600 Accordingly, the C-terminal domain (130 aa) of EmACT contained all these invariant
601 cysteines (Fig 6, Fig 7), as well as two additional cysteines (Fig 6, Fig 7) that are
602 characteristic of TGF- β /activin subfamily members, but that are not present in BMP
603 subfamily members (Fig 7).

604
605 **Fig 6: Nucleotide and deduced amino acid sequence of the *Echinococcus***
606 ***multilocularis act* cDNA.** The 5' signal sequence is underlined. The potential N-
607 glycosylation sites (NRT, NLT and NSS) are shown in solid boxes. The paired dibasic
608 cleavage motif is shown within an open box followed by a TGF- β superfamily active
609 domain located at the carboxyl end highlighted in grey. Nine cysteine residues found at
610 invariant positions in TGF- β active domains are circled.

611
612 **Fig 7: Alignment of the C-terminal amino acid sequences of EmACT and seven other**
613 **representatives of the TGF- β superfamily.** The paired dibasic cleavage motif is shown
614 within a red open box. Residues that are identical are highlighted in black, similarities in
615 grey. Gaps introduced to maximize the alignment are represented by dashes. Two
616 conserved cysteines found only in TGF- β /activin subfamily are shown with asterisks.
617 Numbers at the start and finish of each line correspond to the amino acid numbers in each
618 respective sequence. Accession numbers for the sequences shown are listed in S2 Tab.

619

620

621 Sequence comparisons to several TGF- β superfamily members (Fig 7) and
622 BLASTP analyses of the conserved C-terminal portion of EmACT against protein
623 databases confirmed that its closest relatives are inhibin beta A chains. Highest
624 homologies were detected to SmlnAct (49% identical amino acid residues) and human
625 inhibin beta A (34%) (Fig 7). To further confirm that EmACT is an activin/inhibin ortholog,
626 we carried out phylogenetic analyses. The putatively bioactive C-terminal domain of
627 EmACT was aligned to those of several TGF- β superfamily members and the degree of
628 homology was represented on a phylogenetic tree. EmACT clearly clustered with TGF-
629 β /activin subfamily members but not with the BMP subfamily (Fig 8) and, again, showed
630 highest similarity to SmlnAct. Taken together, all structural analyses clearly indicated that
631 EmACT is a member of the TGF- β /activin subfamily of TGF- β like cytokines.

632
633 **Fig 8: Phylogenetic clustering of EmACT with TGF- β /activin subfamily members.** A
634 non-redundant set of TGF- β superfamily members sequences were aligned and an
635 unrooted neighbor-joining tree was computed by MEGA. EmACT is shown clustering with
636 members of the TGF- β /activin subfamily (pink box), but not with members of the
637 BMP/growth differentiation factor subfamily (blue box). Conserved residues in the C-
638 terminal region of each homolog (final 94–106 amino acids) were used in the analysis.
639 Percentages at branch points are based on 1,000 bootstrap runs.

640
641 Finally, by using the EmACT sequence as a query in BLASTP analyses against the
642 recently determined genome sequences of other cestodes [32], we identified *Emact*
643 orthologs in *E. granulosus* (EgrG_000178100), *Taenia solium* (TsM_000011500), and
644 *Hymenolepis microstoma* (HmN_000204000), which encoded proteins with 99, 95 and

645 66% amino acid sequence identity to EmACT, respectively. Hence, the presence of activin
646 A – encoding genes appears to be a common feature of tapeworm genomes.

647

648 **Expression of EmACT**

649 Preliminary deep sequencing transcriptome data collected during the *E.*
650 *multilocularis* whole genome sequencing project [32] indicated that *Emact* is actively
651 transcribed in the *E. multilocularis* metacestode. To closely investigate the formation of
652 the gene product, EmACT, an anti-EmACT antiserum was raised in mice by subcutaneous
653 injection of tag-fused EmACT. This polyclonal serum was used to specifically assess
654 whether EmACT is secreted by *E. multilocularis*. The supernatant of *in vitro* cultivated
655 metacestode vesicles was probed with the anti-EmACT antiserum. We clearly detected
656 reactive proteins of 15- to 25-kDa in the supernatant (Figure 9A), indicating that EmACT
657 has a complex processing and is secreted as different variants of the active protein (~130
658 amino acids) by *E. multilocularis* metacestodes.

659 For functional characterization of EmACT, the entire protein-coding region was
660 recombinantly expressed in HEK 293 cells under the control of the cytomegalovirus
661 promoter using a mammalian expression system. As shown by Western blotting using the
662 anti-EmACT antiserum, recombinant EmACT (rEmACT) was secreted to the medium by
663 transfected HEK 293 cells as 15- to 25-kDa variants (Fig 9B), which was in agreement
664 with the observed secretion pattern of mature EmACT by the *E. multilocularis*
665 metacestode (Fig 9A). Complementarily, HEK 293 cells were transfected with EmACT
666 containing a Myc tag within the coding sequence, after the furin cleavage site and prior to
667 the mature peptide (S2 Fig) to conceptually enable the secretion of a N-tagged mature
668 EmACT in culture to validate that the assumed complex processing typical of TGF- β

669 superfamily is relevant in EmACT. Indeed, the secretion of c-myc-N-tagged mature
670 EmACT was assessed by immunoprecipitation of the transfected HEK cell supernatant
671 using bead-bound anti-c-myc antibodies and probing the beads' eluate with anti-c-myc
672 antibodies, revealing specific bands by around 15- to 25-kDa (S2 Fig).

673

674 **Fig 9: Immunodetection of EmACT.** Expression and purification of EmACT fusion
675 protein. EmACT was cloned into the bacterial expression vector pBADThio/TOPO.
676 Competent *E.coli* (Top 10) bacteria were transformed with the Thio-Emact plasmid and
677 induced to express the fusion Thio-EmACT protein under arabinose control. A C-terminal
678 histidine repeats fused to the expressed Thio-EmACT fusion protein by the
679 pBADThio/TOPO expression vector was used as target tag for protein purification over
680 Nickel-supplemented beads. Lysates of *E.coli* transformed with pBADThio/TOPO-Emact
681 construct before and after arabinose-driven protein expression as well as purified Thio-
682 EmACT were separated by SDS PAGE, blotted over a nitrocellulose membrane and the
683 proteins revealed by Ponceau S staining. The arrow indicates the recombinant Tagged-
684 EmACT. **(A)** Secretion of EmACT by *Echinococcus multilocularis* metacystode vesicles
685 in culture. Shown is a western blotting of ethanol-precipitated MVE/S probed with normal
686 mouse serum or mouse anti-EmACT Immunserum followed by ECL detection and
687 autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown
688 on the left. The bracket indicates the position of EmACT variants. **(B)** Secretion of
689 recombinant EmACT by pSecTag2-*emact*-transfected HEK cells. Shown is a western
690 blotting of the Ethanol-precipitated supernatant of pSecTag2-*emact*- transfected 293T
691 HEK cells probed with either normal mouse serum (or mouse anti-EmACT immune serum
692 followed by ECL detection and autoradiography. The positions of the molecular mass

693 markers (in kilodaltons) are shown on the left. The bracket delimitates the location of
694 recombinant EmACT variants.

695
696 Collectively these results showed that EmACT is secreted by *E. multilocularis*
697 metacestodes as differentially processed variants, which could also be efficiently
698 produced by recombinant expression of EmACT in HEK cells.

699
700 **rEmACT induces Treg conversion *in vitro*.**

701 Similar to our previous assays using metacestode E/S products, we then
702 investigated whether rEmACT has activin A-like activities. Again, purified naïve
703 CD4⁺CD25⁻ T-cells from spleens and lymph nodes of OT-II.Rag-1^{-/-} mice were isolated
704 and co-cultured with OVA-pulsed DCs. The supernatants of *Emact*-transfected HEK cells
705 (rEmACT) or vector-transfected HEK 293 cells (control) were added to the DC-T-cell co-
706 cultures and the rate of Foxp3⁺ Treg conversion was measured 5 days later by flow
707 cytometry. When compared to the supernatant of vector-transfected HEK 293 cells,
708 rEmACT-containing HEK cell supernatant alone failed to expand Foxp3⁺ Treg but could
709 considerably promote TGF- β -driven Foxp3⁺ Treg conversion (Fig 10). These data
710 indicated that EmACT is unable to induce the *de novo* Foxp3⁺ Treg conversion alone, but
711 synergizes with TGF- β .

712
713 **Fig 10: EmACT promotes host TGF- β -dependent Foxp3⁺ Treg conversion *in vitro*.**

714 Freshly generated BMDCs (Day 8) were co-cultured with naïve (CD25⁻) OT-II.RAG-1^{-/-}
715 CD4⁺ T-cells at a DC:T-cell ratio of 1:3 in R10 medium supplemented with OVA peptide

716 (200ng/ml) in the presence of supernatant from pSecTag2-transfected HEK (Control) or
717 pSecTag2-*emact*-transfected HEK (rEmACT) supplemented or not with rhTGF- β 1 (1
718 ng/ml). After 5 days of incubation, cells were harvested and stained for CD4, CD25 and
719 Foxp3 prior to flow cytometry analysis. (A) Representative plots of two independently
720 performed Treg conversion assays with supernatant from 2 batches of transfected HEK
721 cells summarized in (B). The bars represent the mean \pm SD.

722

723

724 **rEmACT induces the secretion of IL-10 by CD4⁺ T-cells *in vitro*.**

725 Finally, we also investigated whether rEmACT, like mammalian activin A, is able to
726 stimulate the secretion of IL-10 by CD4⁺ T-cells. Naïve CD4⁺ CD25⁻ T-cells from spleens
727 of C57Bl/6 mice were activated with plate-bound anti-CD3 and anti-CD28 antibodies and
728 the supernatants of *Emact*- or vector-transfected HEK 293 cells were added as test and
729 control, respectively. We noted a considerably higher production of IL-10 in T-cell cultures
730 supplemented with rEmACT-containing HEK supernatant when compared to the control
731 (Fig 11) suggesting that rEmACT can trigger IL-10 release by CD4⁺ T-cells *in vitro*.

732

733 **Fig 11: EmACT promotes IL-10 release by CD4⁺ T-cells *in vitro*.** CD4⁺CD25⁻ T-cells
734 freshly isolated from C57BL/6 mice were stimulated with CD3/CD28 antibodies in the
735 presence of supernatants from pSecTag2-transfected (Control) or pSecTag2-*emact*-
736 transfected HEK cells (rEmACT). After 72 hours, the T-cells supernatants were collected
737 and probed for IL-10 concentration by Elisa. Horizontal bars represent the mean from two
738 independent experiments with T-cells from two different isolations individually activated in
739 the presence of HEK supernatant batches from two different transfections.

740

741 **DISCUSSION**

742 AE is a chronic disease characterized by continuous and infiltrative (tumor-like) growth of
743 the *E. multilocularis* metacestode stage over years or even decades within the organs of
744 the intermediate host [1,2,5]. Previous work established that this is associated with
745 considerable immune suppression, provoked by parasite surface structures and
746 metabolites that are released by the actively growing metacestode [1,5,6,8,9,13–
747 17,31,28,37,45]. In other long-lasting helminth infections, regulatory T-cells have been
748 identified as a major contributor to parasite-induced immune suppression [20,46–48] and,
749 very recently, evidence for the expansion of this cell type during secondary AE has been
750 obtained [15,28] with a critical role demonstrated for their immunosuppressive functions
751 in the mitigation of host anti-AE immune response [28]. However, it was not clear from
752 these studies whether Tregs were actively induced by the parasite *in vivo*. Evidence for a
753 certain capacity of *Echinococcus* E/S products to induce Tregs was recently obtained by
754 us in a DC-based Treg expansion assay [6]. This study did not discriminate, however,
755 between mitogenic effects on pre-existing Tregs and *de novo* Treg conversion.
756 Furthermore, the precise mechanism of Treg expansion by *E. multilocularis* remained
757 elusive.

758 Our present results clearly indicate an active induction of Treg by the parasite as follows:

759 i) Using a well established *in vivo* infection model for secondary AE [8,14–16,37,38,45],
760 we observed a significant expansion of Foxp3+ Treg over effector T-cells in the
761 peritoneum of mice in a time window around 7 days post infection. ii) We demonstrate that
762 Treg formed at this time point are functionally suppressive. iii) Rather than inducing the

763 proliferation of pre-existing Treg, metacestode E/S products can efficiently promote the
764 *de novo* conversion of host Treg *in vitro*, in a TGF- β -dependent manner. iv) In addition to
765 inducing tolerogenic phenotypes in T-cell-priming DC [6], metacestode E/S products can
766 also promote host Treg conversion in a direct, DC-independent manner. Taken together,
767 these data clearly indicate that Foxp3⁺ Tregs can be actively induced by the *E.*
768 *multilocularis* metacestode to drive host immune suppression during AE.

769 Interestingly, we also found that metacestode E/S products induce IL-10 release by CD4⁺
770 T-cells. Whether IL-10 in our assays is produced by Foxp3⁻ or Foxp3⁺ CD4⁺ T-cells is not
771 fully clear at this point. However, since the CD4⁺ T-cell-dependent production of IL-10 in
772 response to parasite E/S products clearly preceded the expansion of Foxp3⁺ Treg (3 days
773 vs. 5 days), Foxp3⁻ cells are likely to be the main source of CD4⁺ T-cell-derived IL-10 in
774 our assays; this would therefore suggest that the parasite-driven Treg expansion and the
775 induction of IL-10 producing T-cells occur largely independently from each other during
776 AE. This is consistent with previous studies on products of the related trematodes
777 *Schistosoma mansoni* [49] and *Fasciola hepatica* [50] for which also Treg expansion and
778 elevated CD4⁺ T-cell dependent IL-10 production had been observed. Hence, such an
779 independent induction of IL-10 producing T-cells would add to the immunosuppression by
780 Foxp3⁺ Treg and could further contribute to parasite establishment. It also provides for
781 the first time a mechanistic explanation for the elevated IL-10 levels observed in tissues
782 and body fluids of AE patients [9,51,52].

783 Within the *E. multilocularis* metacestode E/S fraction we identified a component,
784 EmACT, that most likely contributes to the Treg expansion and the induction of IL-10
785 secretion by T-cells. Like metacestode E/S products, solutions containing recombinantly

786 expressed EmACT promoted Treg conversion *in vitro* and required host TGF- β to do so.
787 Furthermore, rEmACT-containing solutions also triggered the release of IL-10 by host T-
788 cells. We cannot completely rule out at the moment that the metacestode E/S fraction also
789 contains additional factors that could contribute to the observed induction of IL-10 by T-
790 cells and/or to Treg conversion. In fact, this is rather certain given the inability of
791 recombinant EmACT to directly and independently induce the conversion of Foxp3 Treg
792 in our assays. The dependency on host TGF- β suggests an accessory, rather than central,
793 role of this factor in the observed ability of *E. multilocularis* metacestode to expand host
794 Treg. Clearly, other unidentified *E. multilocularis* factors might possess the Treg inducing
795 ability herein reported and in so doing, possibly act in concert with EmACT to promote
796 immunoregulation. In this regard, the *E. multilocularis* genome [32] does, for example,
797 encode homologs of the schistosome ribonuclease omega-1 [53,54] or mammalian BMPs,
798 which have the ability to induce Treg conversion in a TGF- β -dependent manner [55,56].
799 However, unlike EmACT, these factors have not been reported to induce IL-10 production
800 in T-cells. To further investigate this aspect, we already tried to block EmACT activities in
801 the E/S fraction by using the available anti-EmACT antiserum. Unfortunately, several
802 attempts to immunoprecipitate native EmACT from E/S products using our generated
803 serum failed, indicating that the available antibodies might only recognize the mature
804 protein in its denatured form. To investigate whether additional metacestode E/S
805 components are capable of inducing Treg conversion and/or IL-10 production by T-cells
806 the availability of neutralizing antibodies that recognize native EmACT would thus be
807 necessary. Nevertheless, even if additional parasite components could contribute to the
808 immunosuppressive activities of the metacestode E/S fraction, our experiments on

809 recombinantly expressed EmACT strongly suggest that it is a major component of the
810 cascade of events that promote a Treg and IL-10 rich environment during AE.

811 In an important previous contribution, Grainger *et al.* [47] demonstrated that E/S
812 products of the nematode *Heligmosomoides polygyrus* can induce Treg *de novo* and
813 suggested a 'TGF- β mimic' as the major E/S component to mediate these effects.
814 Although the precise molecule has now been identified in this study as a non-TGF- β
815 superfamily member [57], these authors demonstrated that their molecule acted via the
816 host TGF- β signaling cascade to mediate its effect. In fact, identified nematode TGF- β
817 orthologs also have the capacity to bind to mammalian TGF- β receptors [58]. We now
818 show that a helminth-derived TGF- β -superfamily member can indeed promote Treg (TGF-
819 β dependent) and, at least concerning immune cells, displays clear functional homologies
820 to activin A such as the induction of IL-10 in T-cells [40,41]. Interestingly, our *in silico*
821 analyses could identify similar activin-like molecules in the genomes of other cestodes.
822 Notably, *E. granulosus*, *Taenia solium* and *Hymenolepis sp.* which are pathogens
823 reported to expand Foxp3⁺ Treg and elevated IL-10 production in their hosts [59–63], do
824 all harbor *Emact* orthologs. An implication of this family of molecules in the modulation of
825 the host immune response by these related helminths is therefore possible and merits
826 closer examination.

827 The fact that E/S products from *E. multilocularis* metacestodes can induce IL-10-
828 secreting and Foxp3⁺ T-cells, which themselves might produce or convey to other immune
829 cells the ability to produce immunosuppressive cytokines like TGF- β and IL-10 [64,65],
830 could explain the high doses of these cytokines found in parasite vicinity during AE
831 infections [10,11,51]. This tightly reconciles with the reported expansion of CD4⁺ Tregs

832 within the periparasitic environment during AE [15,28] and the debilitating role of this cell
833 type on the host ability to control the infection [28]. Since these granuloma also contain
834 CD8⁺ T-cells [10,11,51] we cannot exclude that immunosuppressory mechanisms
835 associated with suppressive CD8⁺ T-cells [15] are also at work. However, since it has
836 been shown that the CD4⁺ fraction is highly important for parasite clearance [38], we think
837 that CD4⁺ Tregs, as expanded by EmACT, are major actors in the impairment of host
838 immunity during AE. Experiments to further verify this have now been performed [28–30]
839 supporting a critical role of this parasite-driven modulation of cell-mediated immunity by
840 Tregs during AE.

841 Due to the relatively close phylogenetic relationship between helminths and
842 mammalian hosts, it is now clear that they can communicate via evolutionarily conserved
843 signaling systems [66]. Examples are the induction of Epidermal Growth Factor (EGF)
844 signalling in trematodes and cestodes by host derived EGF that binds to evolutionarily
845 conserved EGF receptor kinases [67,68]. We previously demonstrated that also host
846 insulin can stimulate *Echinococcus* development by acting on evolutionarily conserved
847 insulin signaling systems [69]. This apparently also extends to cytokines of the FGF family
848 [70] and the TGF- β /BMP family and respective parasite receptors since host BMP2 has
849 been shown to stimulate a TGF- β family receptor kinase of *E. multilocularis* [71] and
850 similar evidence has also been obtained for schistosomes [72]. It is thus reasonable to
851 assume that parasite-derived cytokines of this family can also functionally interact with
852 TGF- β /BMP receptors of the host. Although we have not yet identified the precise receptor
853 system that is stimulated in T-cells by EmACT, we propose that it acts directly on the
854 Activin receptor-like kinase (Alk) system that is involved in Treg conversion [39,47,73,74].

855 Further investigations as to which mammalian TGF- β /BMP receptor systems are activated
856 by cestode TGF- β family ligands such as EmACT are clearly necessary.

857 Although the induction of Treg might be beneficial to *Echinococcus* from the very
858 beginning of the infection, we herein mostly focused on E/S products of the metacestode
859 since we previously showed that E/S products of *Echinococcus* primary cells, which
860 functionally resemble the oncosphere-metacestode transition state [6], did not induce
861 Treg conversion [6] and failed to trigger IL-10 release by T-cells [7]. The reason for these
862 differences might be different composition of the E/S fractions from metacestode vesicles
863 and early primary cells. Indeed in transcriptome data collected during the genome project
864 [32], we already observed clear differences between primary cells and metacestodes in
865 the expression of potentially secreted proteins. Furthermore, we also observed that
866 primary cells secrete a factor EmTIP which induces IFN- γ in T-cells and which is not
867 secreted by the metacestode [7]. Hence, different stages of the parasite (i.e. less
868 protected (primary cells) and well protected (metacestode)) might act differently on T-cells
869 due to a differential E/S profile, and might use different mechanisms for establishing a
870 protective environment. In the case of primary cells, this could include the induction of
871 apoptosis and tolerogenicity in DCs, because they are the first actors at the site of infection
872 [6]. In the case of the metacestode, this could, in addition, involve the formation of Tregs
873 in order to not only contain the host response against the actively growing larva, but most
874 probably also to limit extensive tissue damage in the host.

875 It has been shown that in addition to immunosuppression, chronic AE is also
876 associated with a Th2 immune response [5]. This could, in part, be a result of a dominant
877 Th2 differentiation of Foxp3⁺ Tregs upon loss of Foxp3 expression observed after the
878 parasite-driven transient expansion of Foxp3⁺ Tregs after 7 days of infection in our assay.

879 This hypothesis is supported by the reported preferential Th2 differentiation of Treg
880 following Foxp3 loss in human T-cells [75]. On the other hand, a more likely contribution
881 of EmACT in the Th2 response reported during chronic AE might come as a result of its
882 conserved functionalities with mammalian activin A which has been shown to promote, in
883 a context-dependent manner, Th2 effector functions [76,77]. Clearly, more experiments
884 are necessary to address these questions.

885 Taken together, we herein introduce a parasite TGF- β superfamily ligand
886 homologous to activin A, EmACT, which is secreted by the metacestode larva of the
887 tapeworm *E. multilocularis*, and able to promote immunosuppressive features in host T-
888 cells. Moreover, its implicit role in the host immunomodulation by *E. multilocularis* products
889 places EmACT as a therapeutic target for novel anti-*Echinococcus* strategies and a novel
890 tool in the therapeutic regulation of host inflammatory responses.

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1140

1141 **Supporting information captions**

1142 **S1 Tab: List and corresponding accession numbers of gene sequences used**

1143 **S2 Fig: N-term c-Myc tagged EmACT secretion pattern in transfected HEK cells.** The
1144 *Emact*-Psectag2 vector construct was modified by site-directed mutagenesis to
1145 incorporate a c-Myc tag N-terminal of the EmACT mature peptide sequence and after the
1146 furin consensus cleavage motif RTRR. HEK 293 cells were transfected with this construct
1147 and kept in culture for collection of supernatant over time (72H). The collected supernatant
1148 was processed using the c-Myc tagged protein MILD PURIFICATION KIT Ver.2 (MBL) as
1149 per the manufacturer instructions. Briefly, the supernatant was supplemented with anti-c-
1150 myc beads for capture of c-myc EmACT mature protein. The incubated beads were eluted
1151 with c-myc-containing solutions and the eluate probed with anti-c-myc for myc-tagged
1152 proteins.

1153

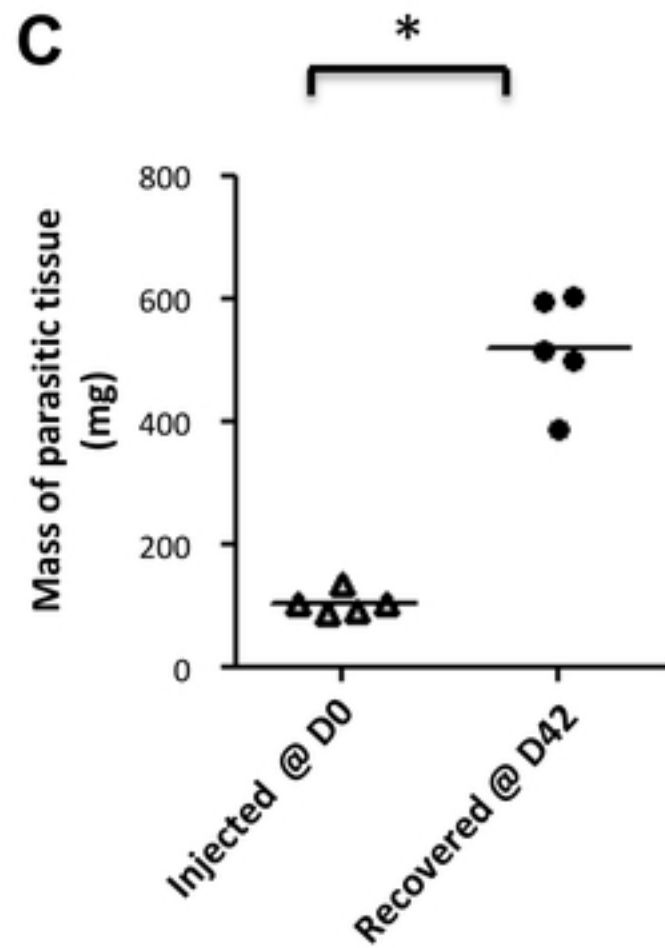
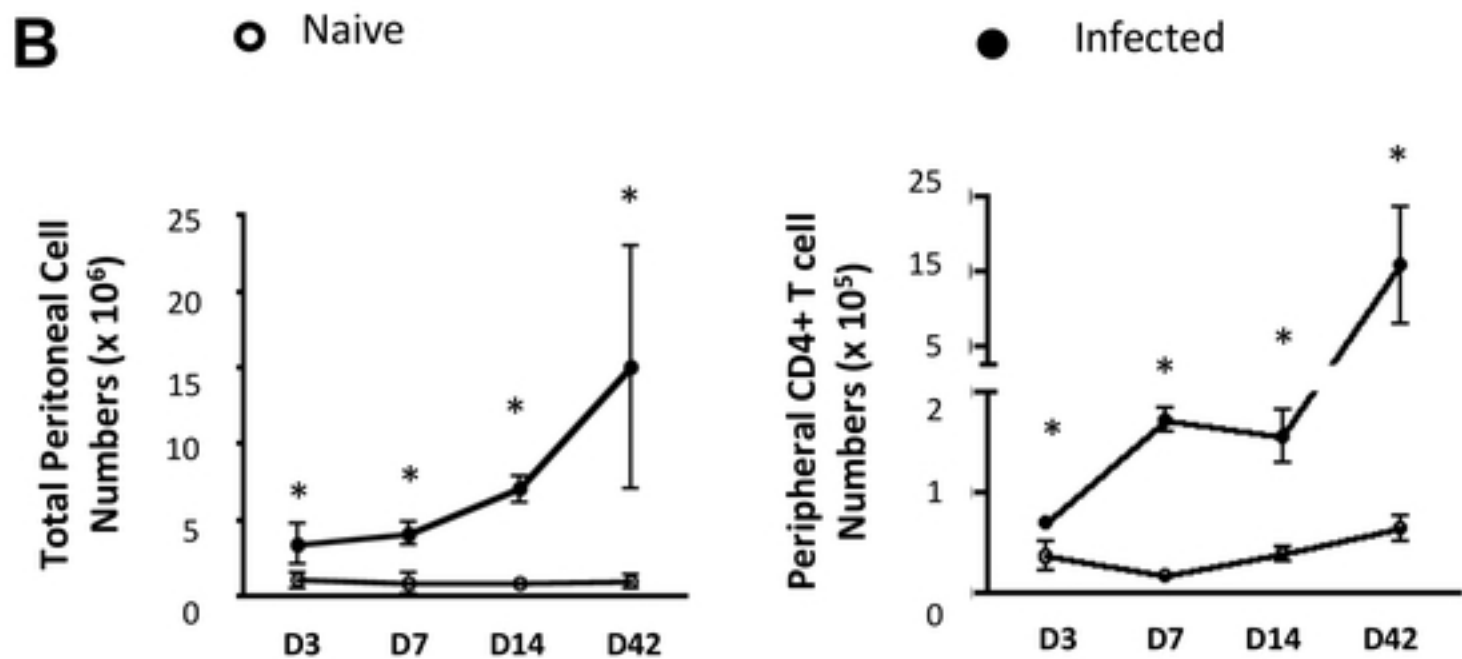
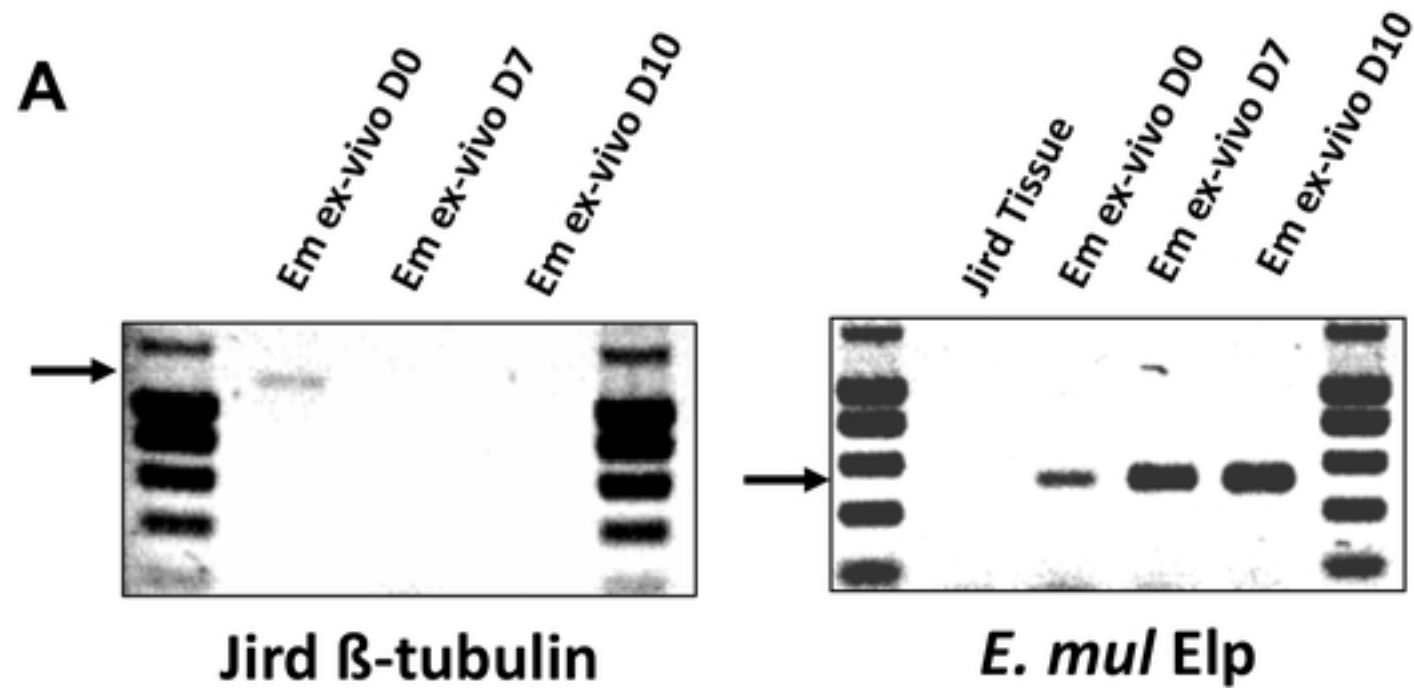


Figure 1

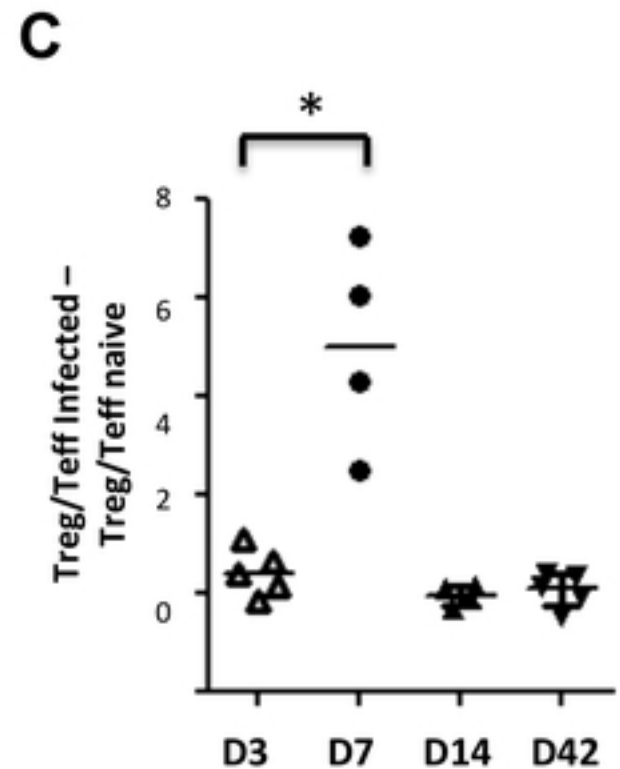
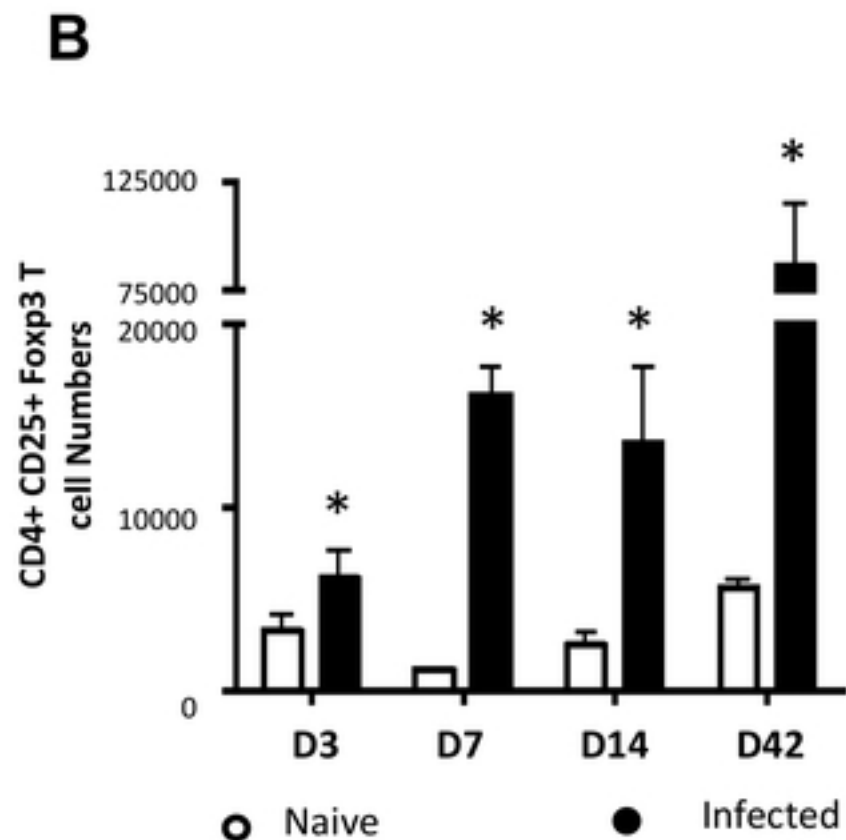
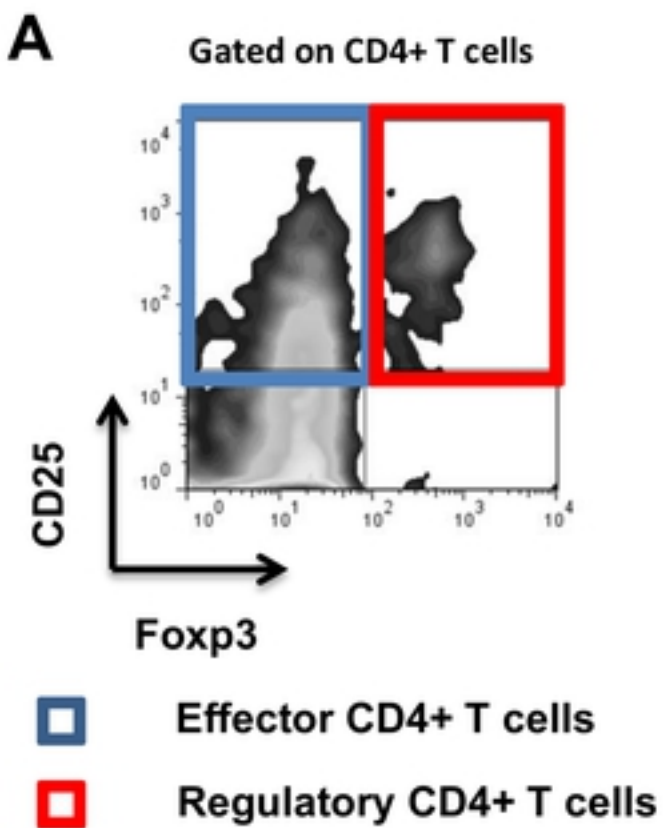


Figure 2

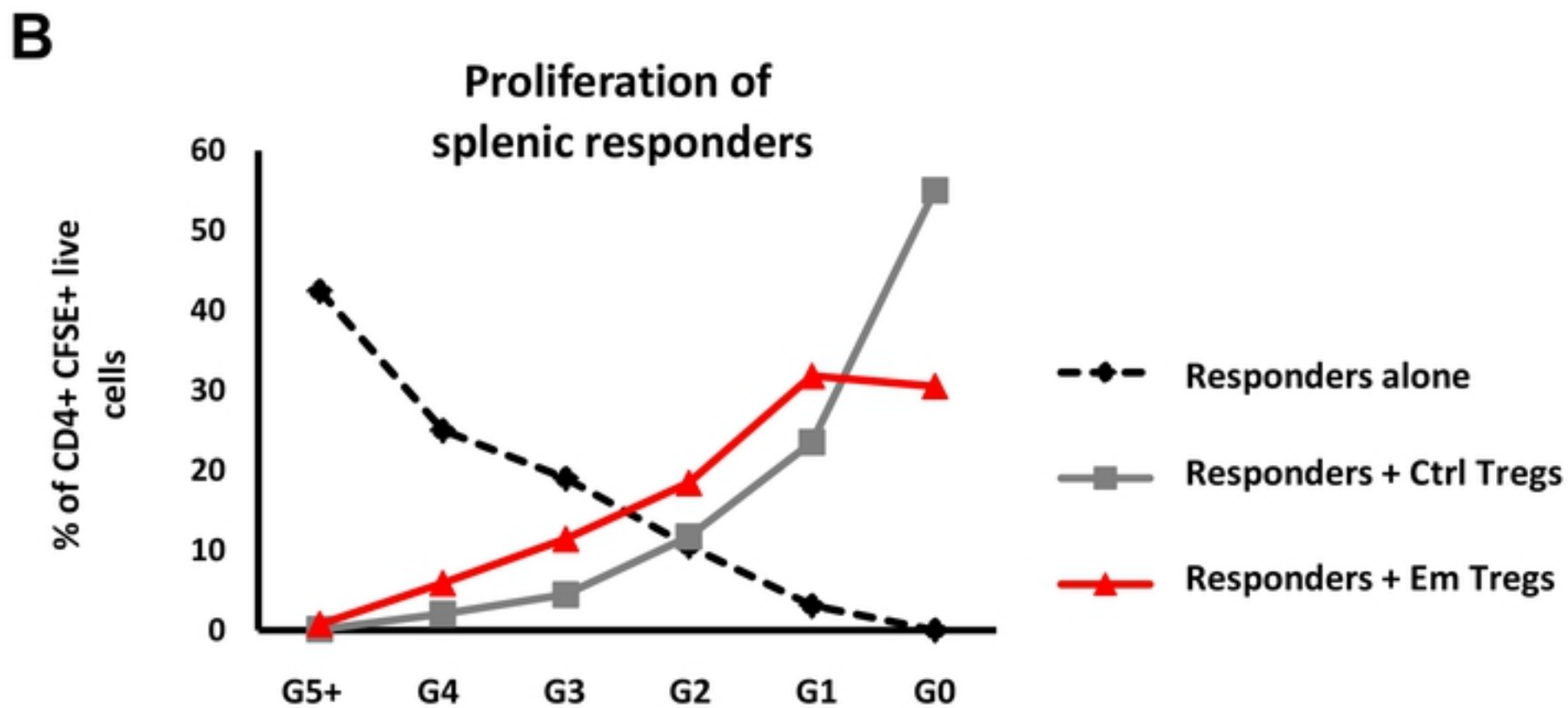
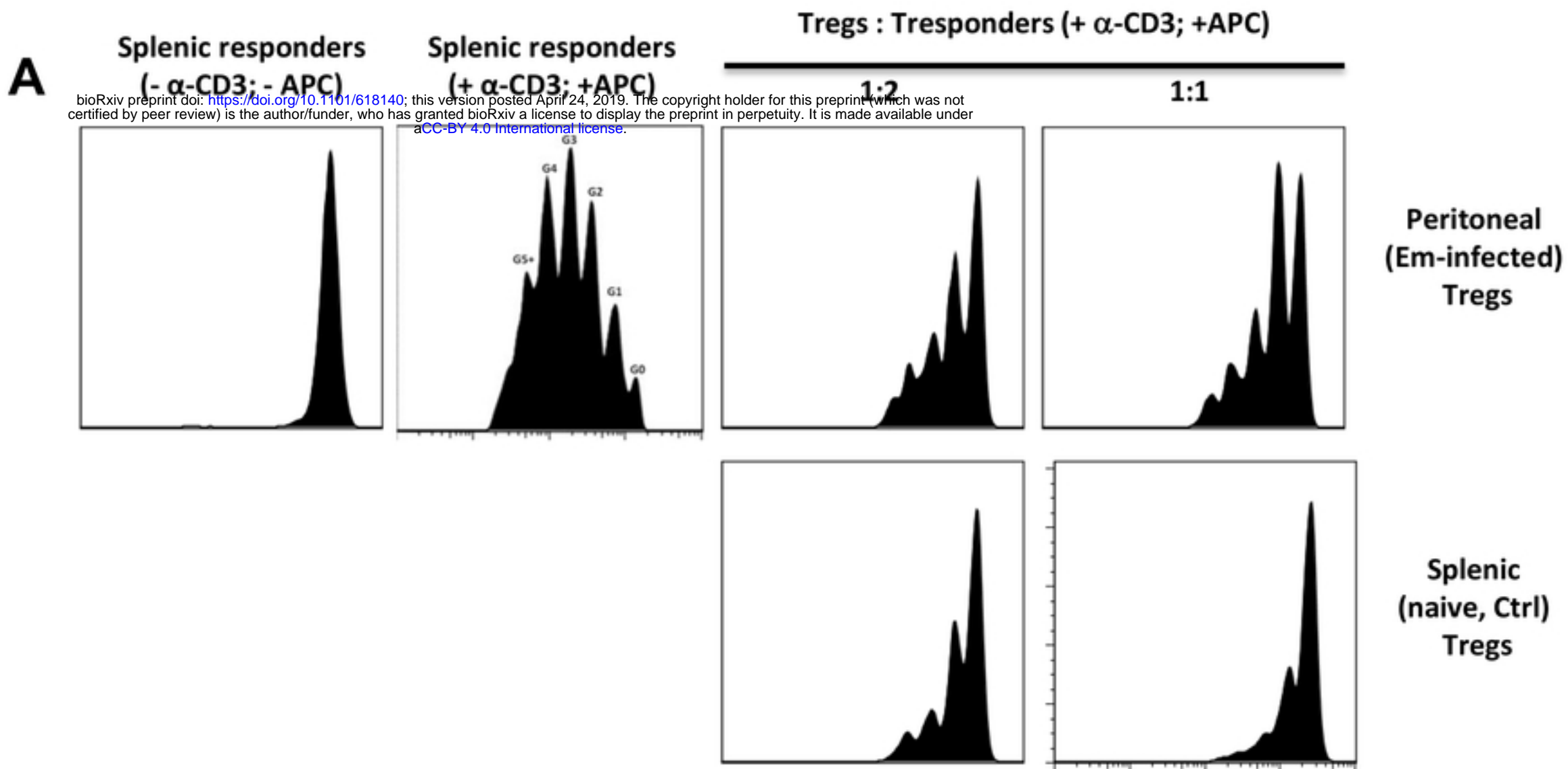


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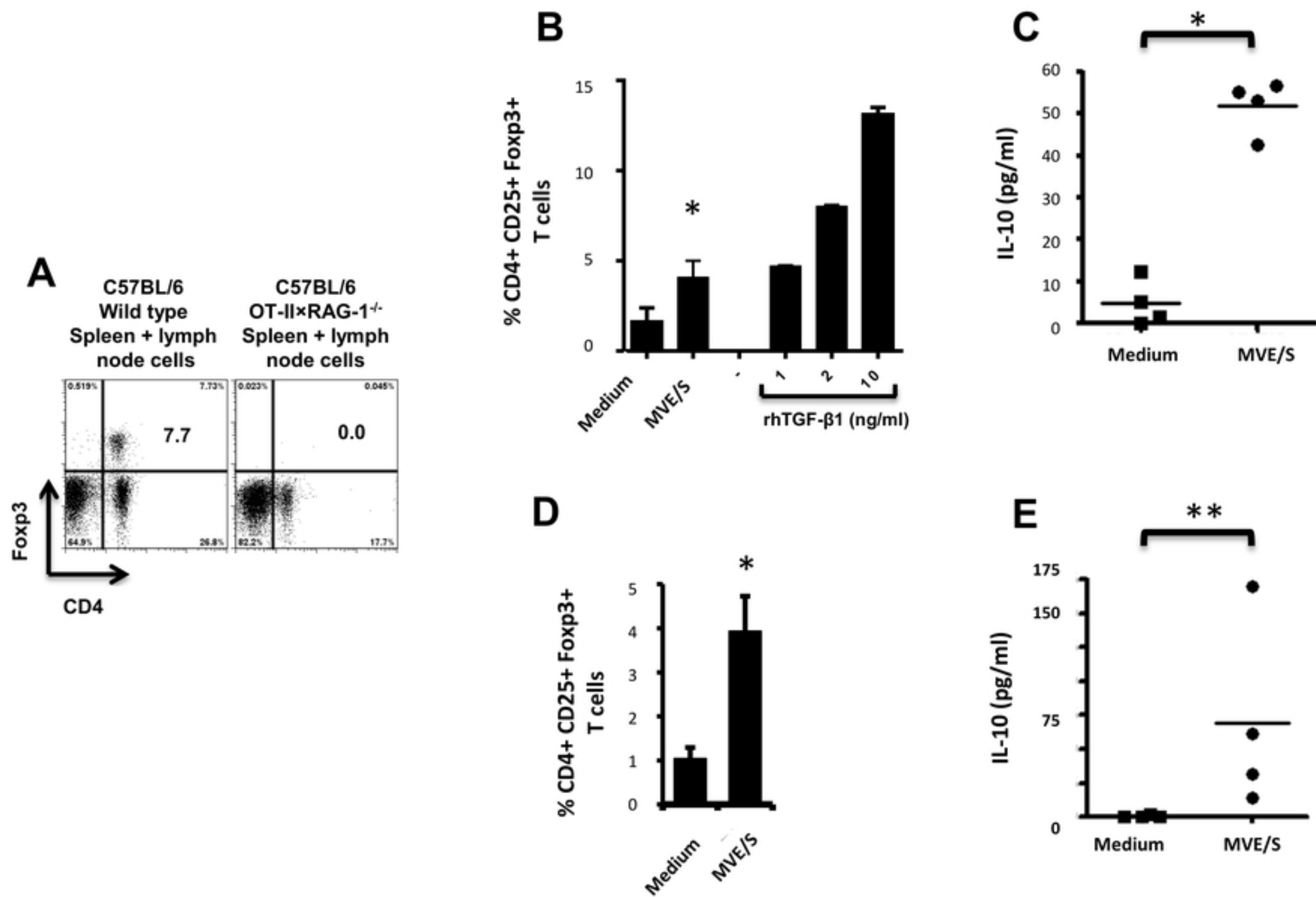


Figure 4

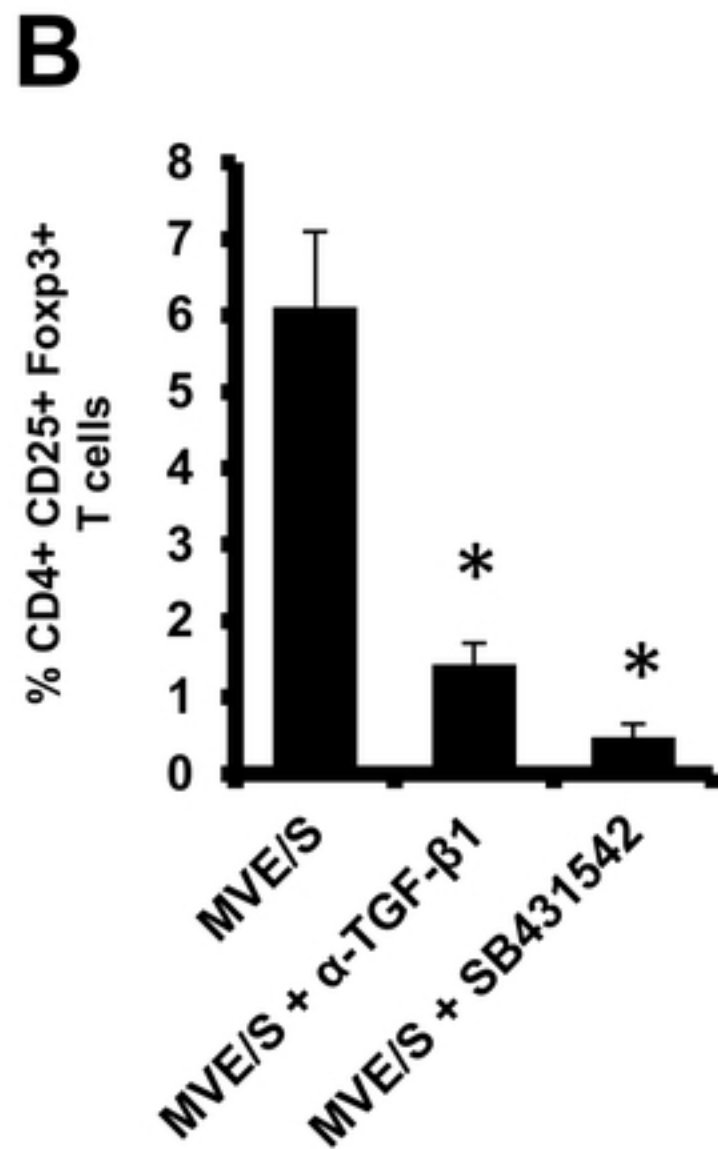
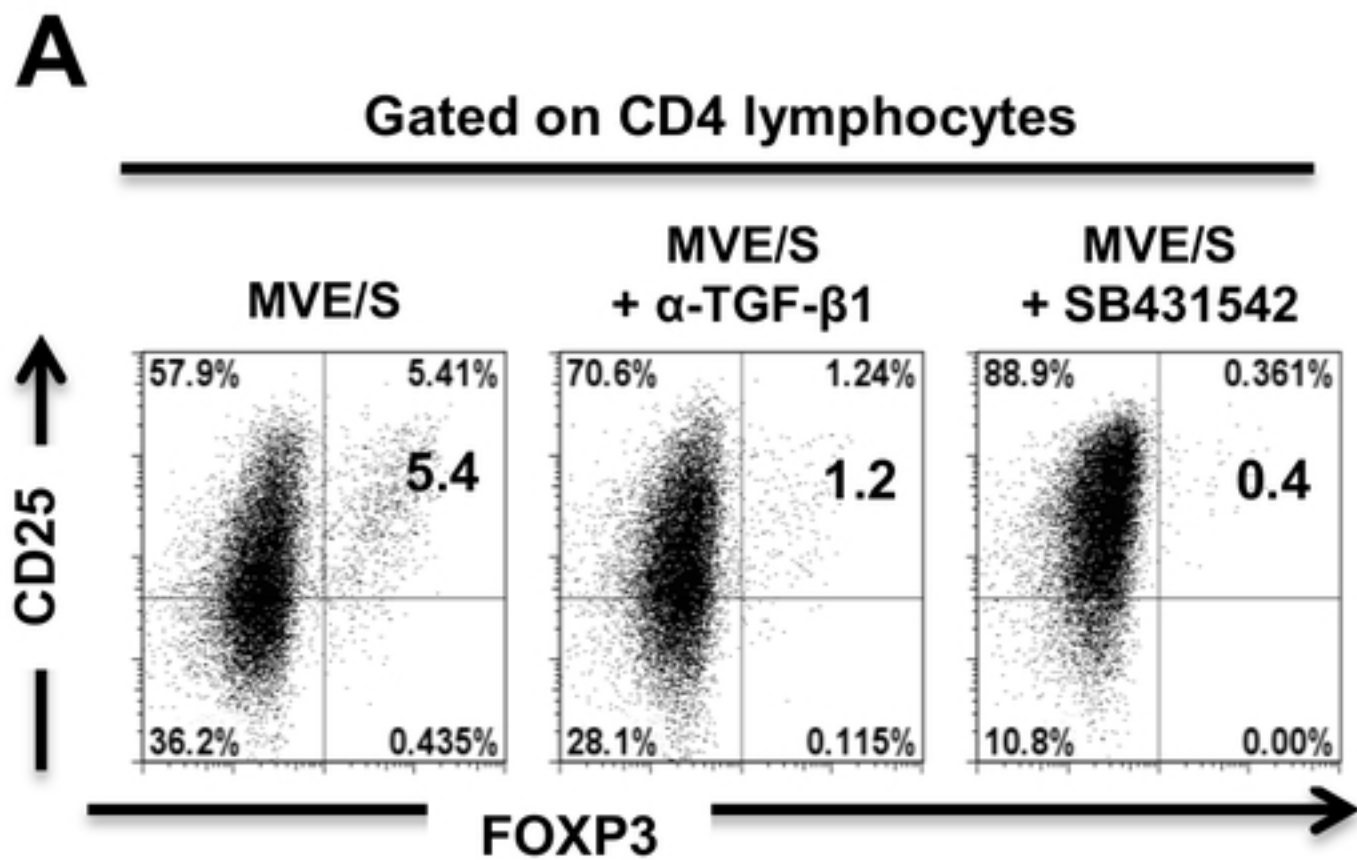


Figure 5

1 M T I T T P M K C G I V L V A L A L I M L G S C P L 26
1 ACCATGACCATTACTACCCCATGAAGTGTGGAATCGTCCTAGTTGCGCTTGCCTTGATAATGCTAGGCAGCTGTCCCCTC 81

27 I H A L F R Q P A I M D G T L L E V P D Q E E E E R M 53
82 ATCCATGCTCTCTTTAGACAGCCTGCAATAATGGATGGAACACTGCTTGAAGTTCCGGACCAGGAAGAGGAGGAGGATG 162

54 W V D P T P K I E D N D G N D V D D V G T K R K L E E 80
163 TGGGTGGACCCAACCCCAAAATCGAAGATAACGATGGCAACGACGTGGACGATGTTGGGACCAAACGAAAGCTGGAGGAG 243

81 T E E R E R E A K R R A D E E E E E F E R L I H I E 107
244 ACGGAGGAGCGAGAGAGGGAAGCAAACGAAGGGCTGACGAGGAGGAGGAGGAGGAGTTTGAACGACTGATCCATATTGAA 324

108 K F K R T L L K R L H L T S P P D F S H H S G M A **N R** 134
325 AAATTCAAAGGACTTTGTTGAAACGTCTCCACTTGACCTCCCCTCTGACTTCAGCCATCACAGTGGTATGGCCAACCGA 405

135 **T** H G R R V L R S L P L A L Q G R L L N Q M R A E D G 161
406 ACACATGGAAGACGTGTGCTTCGATCCCTTCCATTGGCTCTTCAAGGACGCCTCTTAAACCAGATGCGTGCCGAAGATGGA 486

162 M A E P P P D R T D E R E T L I L L K H L H W K L P K 188
487 ATGGCAGAGCCACCACCGGATAGAACGGACGAGAGGGAAACTCTTATCCTTCTCAAACACTTGCACTGGAAGCTACCAAAA 567

189 V A S A T F G I E M A R D I D P S R I Q S A F L R F E 215
568 GTGGCTCTGCTACTTTGGTAAATGAAATGACATCGACCCCTCGAGAAATTCAGTCGGCTTTTCTCCGATTTGAA 648

216 T K N P M L K G Q H V E V W E V F M T P S E E E G K M 242
649 ACGAAGAACCCGATGTTAAAAGGCCAGCACGTTGAGGTTTGGGAGGTCTTCATGACTCCGAGTGAGGAGGAAGGGAAAATG 729

243 T N A A V D Q P S L E Q Y N **N L T** W M F E R Q P T K Y 269
730 ACGAACGCAGCGGTAGACCAACCGTCTTTGGAACAATAACAATAACCTGACGTGGATGTTTGAACGACAACCCACCAAATAC 810

270 T S L P A P T V I R R S L G S P V E R R R T G S I R I 296
811 ACAAGCCTTCCAGCACCCACAGTTATTCGTCGATCCTTAGGGTCCCAGTGGAACGCCGTCGAACTGGTTCCATCCGCATT 891

297 R P G R L A E T F V P S C P G L V Q V T F E I S G S F 323
892 CGTCCGGGTCGTCTGGCGGAGACCTTTGTGCCGAGTTGTCCGGGTTTGGTTCAAGTCACCTTTGAAATCAGTGGCTCCTTC 972

324 A Q W M S H R R R M P L M R K L V R S L I V V C P D C 350
973 GCCCAGTGGATGTCACATAGGCGGCGAATGCCACTGATGCGAAAACACTAGTCCGCTCTCTCATTGTTGTCTGTCCGGATTGC 1053

351 S S H V D P V D V N K G I L E I H H R N V V R **R T R R** 377
1054 AGCAGTCATGTAGATCCTGTTGATGTTAACAAGGGCATCCTTCAAATTCATCACCGCAATGTCGTTTCGACGAACACGGAGG 1134

378 **S L D T N S S** Q H V P I G N P **C** S P K G H K F S **C C** T 404
1135 TCGCTTGACACCAACAGCTCTCAGCATGTCCCATCGGGAATCCTTGCAGCCCGAAGGGACACAAGTTCAGCTGCTGTACG 1215

405 **Q P F S L N L E D V G W N N W I L H P K T V E P N Y C** 431
1216 CAGCCCTTCTCGTTGAACTTAGAGGATGTTGGTTGGAATAACTGGATTCTTCATCCAAAACACTGTTGAACCAACTACTGT 1296

432 **H G S C** Q A D G I Q K T P H S D L M H L Y R S Q N Y D 458
1297 CACGGTTCGTGCCAAGCTGACGGGATTCAGAAGACCCCGCATTCTGACCTGATGCACTTGTACCGGAGTCAAATACGAC 1377

459 **R L S E V Q R E A M L S C C** H P V K M A S T S V L Y V 485
1378 CGCCTCTCAGAAGTTCAGCGGGAGGCGATGCTCTCCTGTTGTCACCCCGTCAAGATGGCAAGCACCAGTGTGCTCTACGTG 1458

486 **D P D N E L H M D T L H N I I V L E C G C S** * 507
1459 GATCCCGACAATGAGTTGCACATGGACACCCTTCAACATCATTGTCCTAGAGTGCGGTTGTAGTTGAGGCGTTATC 1536

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Figure 6

| | | |
|------------------|-----|--|
| EmACT | 374 | RTRRSLDTN SSQHV-----PIGN PCSPK GHKFS SCCT QP 406 |
| SmINH/ACT | 32 | RQRRSLTKG DE-----TIYN VCRSNG HHS SCCT QA 61 |
| HsINHβA | 307 | RRRRGLE -----CDG KVN -- ICCK KQ 325 |
| HsTGF-β1 | 275 | RHRRALD -----TNY CFS STEK- NCCV RQ 297 |
| DmACT | 561 | RVRRAVD -----CGG ALNG - QCC KES 581 |
| CeDAF-7 | 231 | RKRSSHAK -----PVC NAEA QSKG CC LYD 254 |
| HsBMP-2 | 245 | RISFSLHQ DEHS WSQIRP LLVT FGH DG -----K GHPL HKRE KRQAK HKQ RKR -LKSS CKR RHP 300 |
| DmDPP | 422 | RLRRSADEA HER WQHKQP LLFT YTD DGR HKARS IRDV SGGE GGK GGRN KRQ PRR PTRR KNHDD TCRR HS 491 |

| | | |
|------------------|-----|---|
| EmACT | 407 | FSLNLE - DVGWNNWIL HPKT VEPNY CHG SCQAD GIQ KTPHS DL MHL YRSQ NYDRL SE VQRE AML SCCH PV 475 |
| SmINH/ACT | 62 | LSVKFS - DIGW DN WI IHP KSFEP NY CRG SC KVT - STKSL H YDVM DLL RKN LS QFGN V QRDE V QSCCH PT 129 |
| HsINHβA | 326 | FFVSFK - DIGW ND WI IAP SGYH ANY CEGEC PS H IAG TS GS SLSF H STVIN H YMR GH SPFAN L KS CC VP PT 394 |
| HsTGF-β1 | 298 | LYIDFRK DLGWK - WI HE PKGY HAN FCLG PC PYI W SLD TQ Y -----SK VLAL Y NQH NP- GASA AP CC VP Q 359 |
| DmACT | 582 | FYVSFK - ALGW DD WI IAP RGYF ANY CRGD CT GSFR - TPDT F QTF HA HF IE EYR K MGL MN-- GMR PC CA PI 647 |
| CeDAF-7 | 255 | LEIEFE - KIGW D- WIV AP PRY NA YMC RG D CH YN --- AH FN LAET GH SKIM RA AHK V SNPE I GY CC H PT 318 |
| HsBMP-2 | 301 | LYVDFS - DVGW ND WIV AP PGYH AF YCH GE CP F LAD HL NS --- TN HA IVQ T LVNS VN-- SKI PK ACC VP T 364 |
| DmDPP | 492 | LYVDFS - DVGW DD WIV AP LG Y DAY CH GK CP F PL AD H FNS --- TN HA VVQ T LVN N M NP- GK VP KACC VP T 556 |

| | | | |
|------------------|-----|---|--------------------------|
| EmACT | 476 | KMA ST SVLY VD PDNEL HMD TLHNI IV LECG CS 507 | Identity to EmACT |
| SmINH/ACT | 130 | QLT S MSVLY L DSN REL QMHTLHNL IV LGC ACS 161 | 49 % |
| HsINHβA | 395 | KLR P MSMLY Y DDG QNI IKKDI Q NMIV EE CG CS 426 | 34 % |
| HsTGF-β1 | 360 | A LE PLPIV Y Y - VGR K PKVEQ LS NMIV RS CK CS 390 | 24 % |
| DmACT | 648 | K F S S MSLI Y YG DD G - I IK RDL PK MV VE CG CP 678 | 25 % |
| CeDAF-7 | 319 | EYD Y IKLI Y VNR D GRV SI ANV NG MI AK K CG CS 350 | 24 % |
| HsBMP-2 | 365 | E LS AI S MLYL D ENE K VVL KN YQ DM V VE G CG CR 396 | 21 % |
| DmDPP | 557 | QLD S VAMLY L NDQ ST VVL KN YQ EM T V V CG CR 588 | 19 % |

Figure 7

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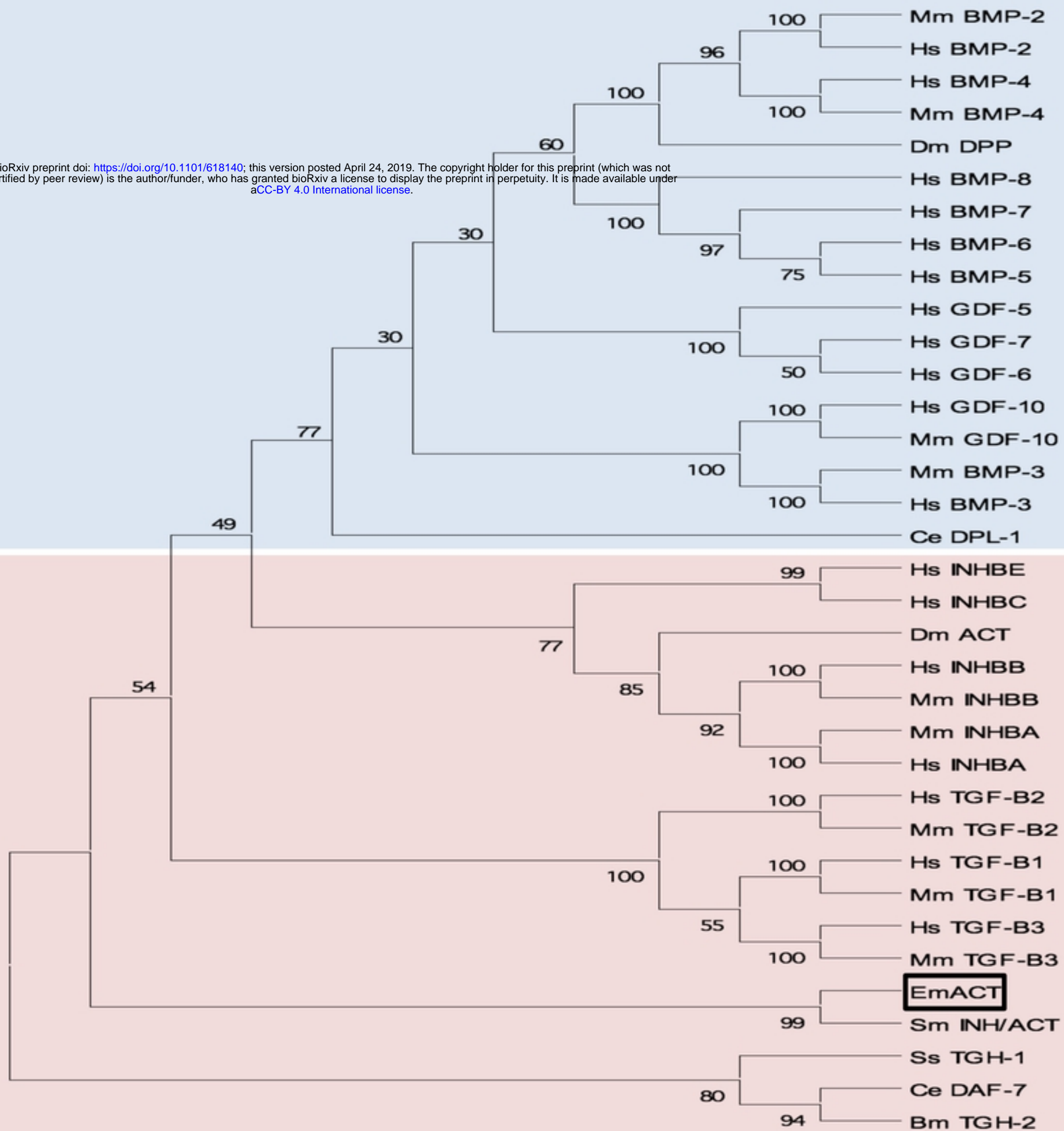


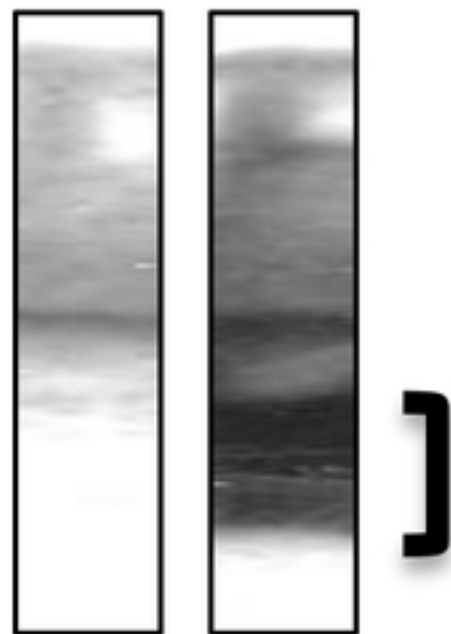
Figure 8

A

kDa

Pre-immune Serum
Anti-EmACT Serum

80 —
60 —
40 —
30 —
25 —
15 —

**B**

kDa

Pre-immune Serum
Anti-EmACT Serum

80 —
60 —
40 —
30 —
25 —
15 —

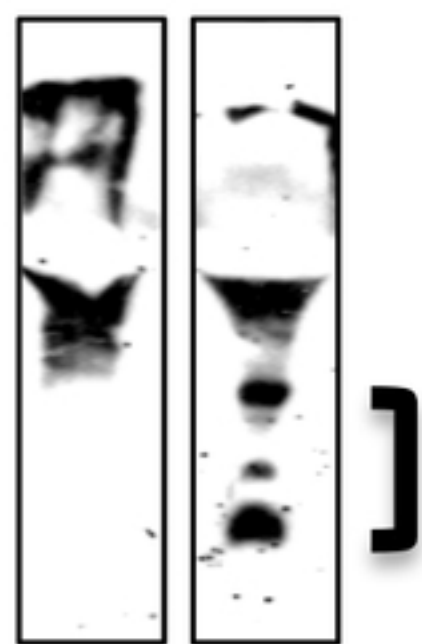


Figure 9

Gated on CD4 lymphocytes

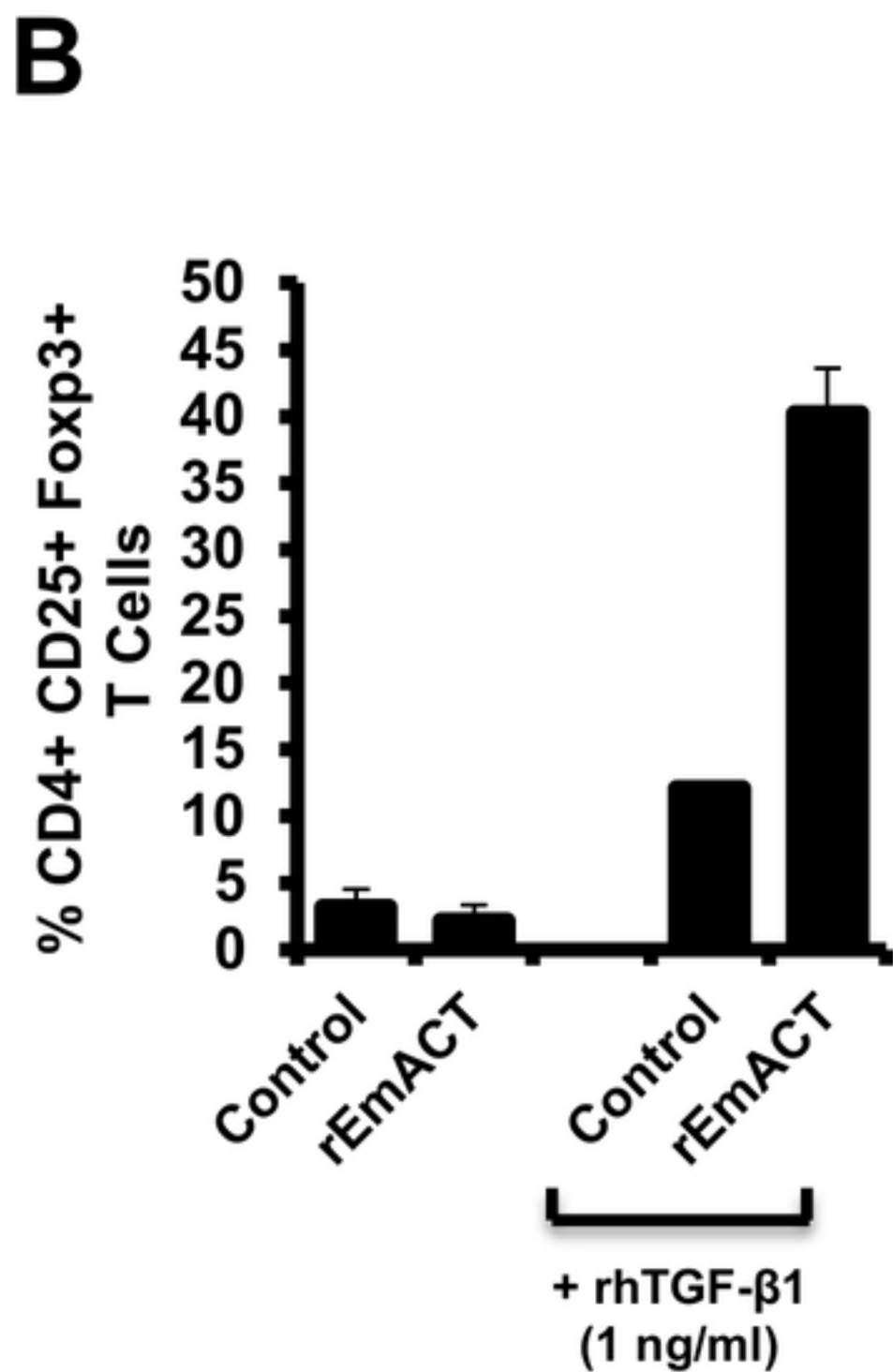
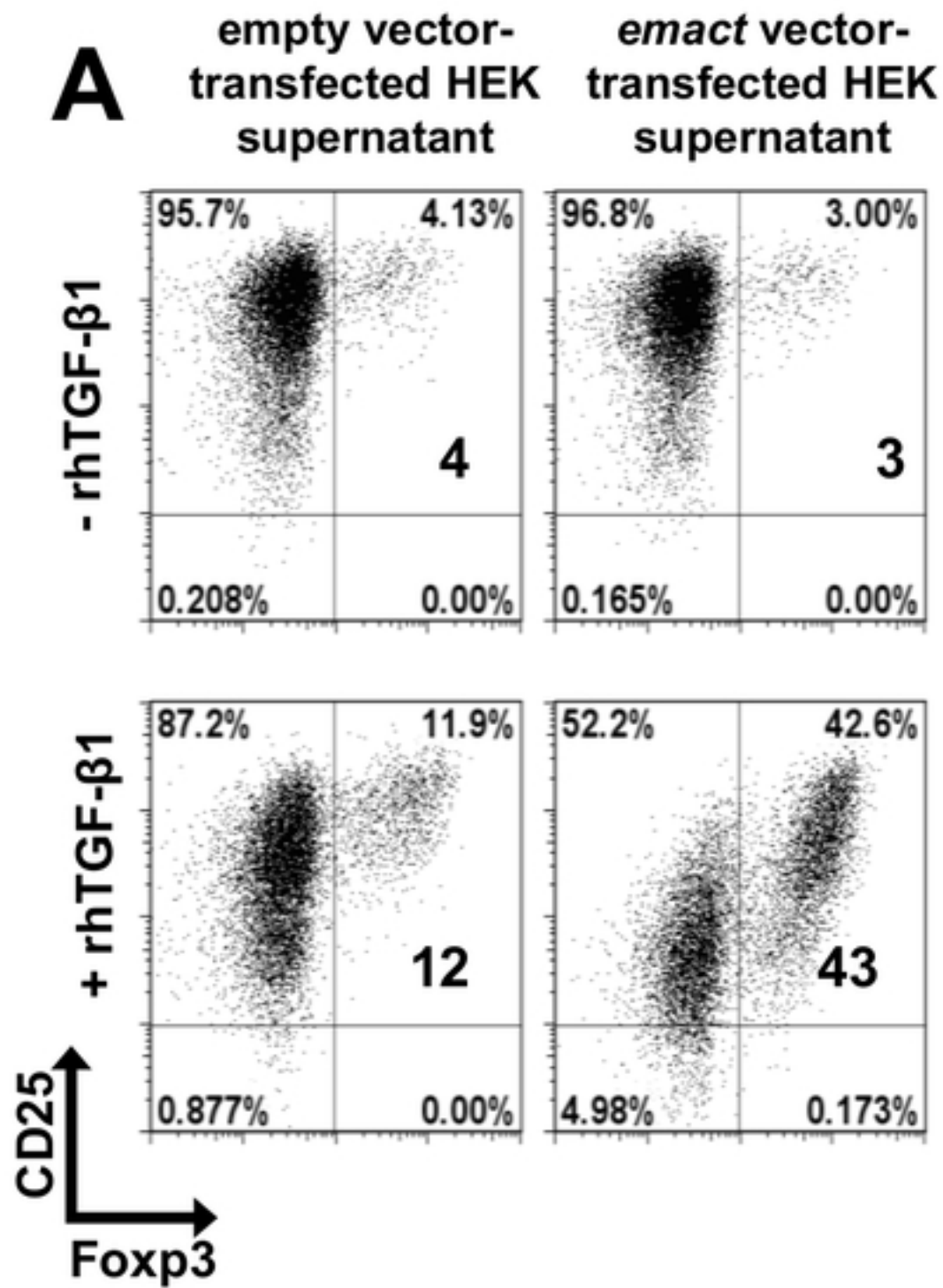


Figure 10

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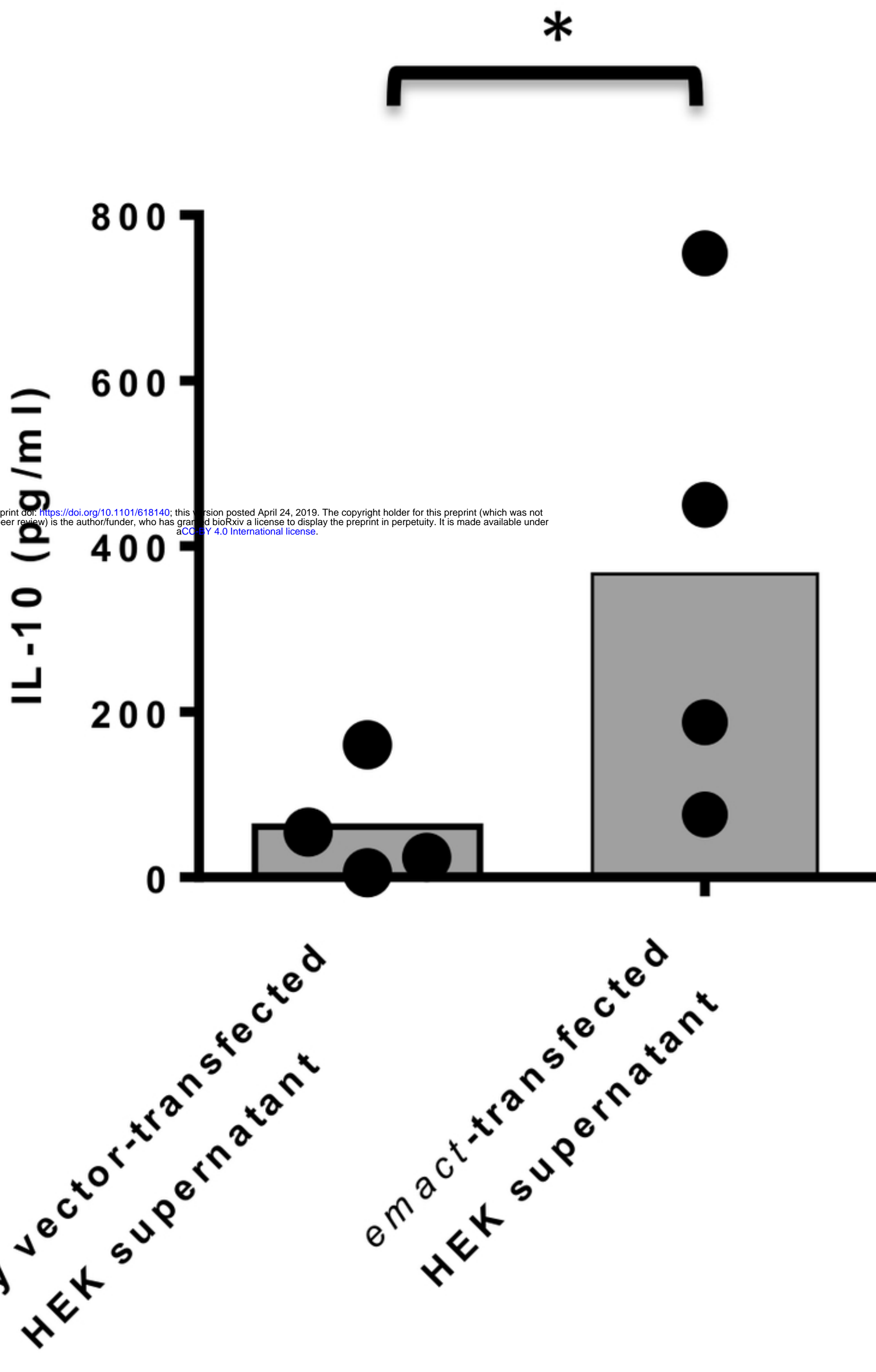


Figure 11