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► To cite this version:

Rita Vicente, Julie Quentin, Anne-Laure Mausset-Bonnefont, Paul Chuchana, Delphine Martire, et al.. Non-classical CD4 + CD49b + Regulatory T Cells as a Better Alternative to Conventional CD4 + CD25 + T Cells To Dampen Arthritis Severity. *Journal of Immunology*, 2015, 196 (1), pp.298 - 309. <10.4049/jimmunol.1501069>. <hal-01834236>

HAL Id: hal-01834236

<https://hal.umontpellier.fr/hal-01834236v1>

Submitted on 18 Dec 2019

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1 **Non-classical CD4⁺CD49b⁺ regulatory T cells as a better alternative to**
2 **conventional CD4⁺CD25⁺ T cells to dampen arthritis severity**

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21
22 Running title: CD49b⁺ Treg cells express multiple canonical Treg markers

25 Footnote:

26 This work was supported in part by research funding from the European Union
27 project Innovative Medicine Initiative 6 (“BeTheCure”; contract number 115142–2 to
28 C.J.) and by institutional fundings. J.Q. was supported by the Arthritis Foundation.

29

30 Abstract

31 Promising immunotherapeutic strategies are emerging to restore tolerance in
32 autoimmune diseases by triggering an increase in the number and/or the function of
33 endogenous regulatory T (Treg) cells, which actively control pathological immune
34 responses. Evidence suggests a remarkable heterogeneity in peripheral Treg cells
35 that warrants their better characterization in terms of phenotype and suppressive
36 function, to determine which subset may be optimally suitable for a given clinical
37 situation.

38 We found that repetitive injections of immature dendritic cells (DCs) expanded
39 FoxP3-negative CD49b⁺ Treg cells that displayed an effector memory phenotype.
40 These expanded Treg cells were isolated *ex-vivo* for transcriptome analysis and
41 found to contain multiple transcripts of the canonical Treg signature shared mainly by
42 CD25⁺ but also by other sub-phenotypes. We characterized the CD49b⁺ Treg cell
43 phenotype, underscoring its similarities with the CD25⁺ Treg cell phenotype and
44 highlighting some differential expression patterns for several markers, including LAG-
45 3, KLRG1, CD103, ICOS, CTLA-4 and Granzyme B. Comparison of the CD25⁺ and
46 CD49b⁺ Treg cells' suppressive mechanisms, *in vitro* and *in vivo*, revealed the latter's
47 potent suppressive activity, which was partly dependent on IL-10 secretion.
48 Altogether our results strongly suggest that expression of several canonical Treg cell
49 markers and suppressive function could be FoxP3-independent, and underscore the
50 therapeutic potential of IL-10 secreting CD49b⁺ Treg cells in arthritis.

51

52 **Introduction**

53 Regulatory T (Treg) cells actively suppress pathological and physiological
54 immune responses, thereby contributing to the maintenance of immunological self-
55 tolerance and immune homeostasis. Their development occurs in the thymus as a
56 result of high-avidity TCR interactions with self-Ags (1), and are called thymus-
57 derived Treg (tTreg) cells. These Treg cell subset are characterized by a stable
58 expression of the transcription factor forkhead box P3 (FoxP3) (2) and constitutive
59 high-level expression of CD25 (IL-2 receptor α chain) and thus denoted as
60 CD4⁺CD25⁺FoxP3⁺ Treg cells. The severity of the autoimmune syndromes caused by
61 deficiencies in FoxP3 - scurfy in mice and IPEX in humans - highlights its central role
62 (reviewed in (3)). Treg cells also differentiate extrathymically from conventional T
63 cells and this differentiation is strongly modulated by cytokines such as IL-2 and
64 TGF- β (4-6). These Treg cells have been termed peripheral Treg (pTreg) and several
65 Ag-induced pTreg cell populations, with IL-10 based regulatory activity, appear to
66 have critical *in vivo* functions (7-9). Several experimental tolerogenic settings have
67 been shown to drive or increase expansion/differentiation of pTreg cells *in vivo*; these
68 include chronic activation and sub-immunogenic Ag presentation (10-12), exposure
69 to orally administered agonist peptides (13, 14), lymphopenia-driven homeostatic
70 expansion (15-17) and use of small molecular weight compounds such as retinoic
71 acid and histone deacetylase inhibitors (18, 19).

72 Alternative strategies to promote *in vivo* generation of stable pTreg cells use
73 the tolerogenic properties of immature dendritic cells (DCs). Indeed, DC-based
74 therapy has been proposed to restore tolerance in the context of several autoimmune
75 diseases (20-22). The two main strategies developed are the direct targeting of
76 antigens to DEC-205⁺ steady state DCs (11, 23-26) and the repetitive injection of

77 tolerogenic DCs (27-29). We have previously demonstrated that repetitive injection of
78 immature and semi-mature DCs can prevent adverse clinical outcome and protect
79 mice from experimental collagen-induced arthritis (CIA) (30, 31). This protection was
80 associated with the expansion of a particular FoxP3-negative CD4⁺ Treg cell
81 population characterized by the expression of CD49b (the alpha2 subunit of the
82 adhesion molecule VLA-2) which specifically binds to collagens I, II and X (30).
83 These induced CD49b⁺ Treg cells, which secrete high levels of IL-4 and IL-10,
84 displayed strong immunosuppressive properties *in vivo*, improving established CIA
85 and attenuating delayed type hypersensitivity reactions (32, 33). Similarly, Benoist
86 and Mathis' group demonstrated that CD4⁺CD49b⁺ Treg cells, present in naïve mice,
87 were more efficient in suppressing the onset of diabetes than CD4⁺CD25⁺ Treg cells
88 (34). As with the cell population we described, these cells' effect was IL-4 and IL-10
89 dependent. Recently, Gagliani *et al.* showed that CD49b and the lymphocyte
90 activation gene 3 (LAG-3) define the IL-10-producing FoxP3-negative T regulatory
91 type 1 cells (35).

92 Altogether these data reveal a remarkable heterogeneity in pTreg cell
93 populations and define the CD49b molecule as a relevant marker for specific Treg
94 cell subsets. Interestingly, recent studies challenged the notion that FoxP3
95 expression is uniquely responsible for all aspects of the transcriptional signature of
96 CD4⁺CD25⁺ Treg cells and showed that FoxP3-independent epigenetic changes are
97 required for Treg cell function (36, 37). These results underscore the need to better
98 characterize the non-classical CD49b⁺ induced Treg cells, which are mainly FoxP3-
99 negative. We therefore investigated their suppressive mechanism *in vivo* and
100 compared it with that of CD25⁺ Treg cells in order to determine their respective
101 therapeutic capacities.

102 **Materials and Methods**

103 **Mice**

104 DBA/1 mice were obtained from Harlan Laboratories and were bred in our own
105 animal facility. Transgenic mice carrying the rearranged V α 11.1 and V β 8.2 TCR
106 chain genes isolated from a collagen-type II (Col II)-specific T cell hybridoma were
107 kindly provided by R. Toes (LUMC, Leiden) with the approval of W. Ladiges. C57BL/6
108 wild type and C57BL/6 IL10^{-/-} knockout mice (KO) were obtained from Janvier
109 (B6.129P2-IL10^{tm1Cgn}/J) and were maintained in our animal facility under specific
110 pathogen free conditions in isolated ventilated cages. Experimental groups were
111 obtained by crossing heterozygous mice to obtain IL10-KO and wild type littermates
112 with the same genetic background. Experiments were performed in accordance with
113 national guidelines and approved by the Ethics committee for Animal Research of
114 Languedoc-Roussillon (CEEA-LR-1067) and French Health Authorities (C34-172-36).

115

116 **DC generation and injections**

117 DCs were generated as previously described (30). Briefly, bone marrow cells were
118 harvested from the femur and tibiae of mice and washed in RPMI following red blood
119 cells lysis. T and B cells were depleted using mouse pan T and pan B Dynabeads®
120 (Dyna) and monocytes were removed by 4h plate adhesion. The remaining cells
121 were cultured in complete medium (RPMI 1640 supplemented with 5% FCS, 2mM L-
122 glutamine, 5 x 10⁻⁵ M β -mercaptoethanol, 100U/ml penicillin, 100 μ g/ml streptomycin,
123 essential amino acids and 1 mM sodium pyruvate) with 1,000 IU/ml of rmGM-CSF
124 (R&D Systems) and 1,000 IU/ml of rmlL-4 (R&D Systems) at 5 x 10⁵ cells/ml in 24-
125 well plates. Culture medium was renewed at days 2 and 4. For *in vivo* experiments,
126 DCs were harvested at day 7. Syngeneic DBA/1, IL-10 KO or wild type littermates

127 were injected i.p. with 0.5×10^6 DCs in 100 μ l PBS, 7, 5 and 3 days before
128 euthanasia for splenic T cell purification.

129

130 **Antibodies and FACS analysis**

131 Spleens were harvested and single-cell suspensions were obtained by gentle
132 passage through 70 μ m nylon mesh filters (BD Biosciences). Following red blood
133 cells lysis using ACK buffer, suspensions were pre-blocked using purified anti-
134 CD16/32 Ab (2.4.G2) for 10 min. For intracellular cytokine staining, cells were
135 stimulated during 48h at 37°C with anti-CD3/anti-CD28 antibody-coated Dynabeads
136 (DynaL Biotech ASA, Oslo, Norway). During the last 4 hours of stimulation, 50 ng/ml
137 of phorbol 12-myristate 13-acetate (PMA), 1 μ g/ml of ionomycin and 10 μ g/ml
138 brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. Subsequently,
139 cells were stained with surface antibodies (20 min, on ice). Cells were fixed using the
140 eBioscience permeabilization kit according to the manufacturer's procedure and
141 subsequently stained for intracellular markers. Data acquisition was performed on a
142 Canto II or LSR Fortessa flow cytometer (BD Biosciences, Mountain View, CA) and
143 analyses were performed using FlowJo software.

144

145 **Treg cell isolation and adoptive cell transfer experiments**

146 Splenocytes from DC-vaccinated mice were recovered by filtration on cell strainer,
147 washed and then CD4⁺ T cells purified by negative selection using Dynabeads. CD4
148 T cells were stained with anti-CD4, anti-CD49b and anti-CD25 conjugated antibodies
149 and cell sorting was performed on FACS Aria (MRI platform Montpellier, Fig.S1).
150 FACS-sorted CD4⁺CD25⁺CD49b⁺ T cells (purity $>95 \pm 2\%$), CD4⁺CD25⁺CD49b⁻
151 (purity $>96 \pm 1\%$) or CD4⁺CD25⁻CD49b⁻ T cells (purity $>96 \pm 1\%$), herein called

152 CD49b⁺, CD25⁺ and CD4⁺ cells, respectively, were washed and 1.5 x 10⁵ cells were
153 injected i.v. in the tail vein of CIA mice or were used for subsequent analyses.

154

155 **Gene Chip hybridization and data analysis.**

156 Total RNA from CD4⁺, CD25⁺ and CD49b⁺ T cells isolated from DC-injected mice and
157 non-injected mice (CD4⁺ only) were prepared using QIAGEN RNeasy Mini kit
158 (QIAGEN). To reduce variability, we pooled cells from multiple mice (n>10) for cell-
159 sorting, and three replicates were generated for CD25⁺ and CD49b⁺ cell groups as
160 well as two replicates for CD4⁺ cells isolated from DC-injected and non-injected mice.
161 All gene-expression profiles were obtained from highly purified FACS-sorted T cell
162 populations (MRI platform Montpellier). RNA was amplified, labeled, and hybridized
163 (IVT Express, Affymetrix) to Affymetrix M430 PM Array Strips that cover almost all
164 known murine genes. Affymetrix microarrays were processed at the Microarray Core
165 Facility located at the IRMB institute. All chip data were uploaded to NCBI Gene
166 Expression Omnibus (accession number is GSE68621,
167 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68621>) and are publicly
168 available. Microarray data were analyzed according to a previously described
169 procedure (38), to define the baseline average signal using the transcriptional profile
170 of CD4⁺ cells isolated from non-injected mice, and to calculate the differential
171 expression variation using the transcriptional profiles of CD4⁺, CD25⁺ and CD49b⁺ T
172 cells isolated from DC-injected mice. To perform a robust analysis of the differentially
173 expressed genes, we used the distribution of the number of differentially expressed
174 transcripts to determine the optimal threshold for both the co-occurrence rate and the
175 transcript expression variation (expression variation ≥ 1.15 and occurrence of 4/4 for
176 CD4⁺ and 6/6 for CD25⁺ and CD49b⁺) (39).

177

178 ***In vitro* suppressive experiments**

179 CFSE-labeled CD4 effector T cells (10^5) were cultured with titrated numbers of either
180 FACS-sorted Treg cells or unlabeled-T effector cells in the presence of irradiated
181 allogenic splenocytes (5×10^5) and 2-5 $\mu\text{g/ml}$ of anti-CD3 ϵ mAb (145-2C11). After 4
182 days of culture, proliferation of effector T cells was assessed by FACS. Data were
183 analyzed using FlowJo software.

184

185 **Collagen-induced arthritis (CIA) induction and evaluation**

186 Male 9-12 week-old mice were immunized at the base of the tail with 100 μg of
187 bovine or chicken Collagen type II (Col II) (MD biosciences) emulsified in CFA
188 (Pierce, complemented to 4mg/ml with Mycobacterium tuberculosis H37RA) for
189 DBA/1 or C57BL/6, respectively. To boost immunization, C57BL/6 mice received an
190 i.v. injection of one million chicken Col II (2 $\mu\text{g/ml}$)-loaded mature DCs on day 0. On
191 day 21, DBA/1 or C57BL/6 mice received a booster immunization at the base of the
192 tail with 100 μg of bovine Col II emulsified in IFA or chicken Col II emulsified in CFA,
193 respectively. Mice were i.v. injected with the FACS-sorted Treg cells on day 28. From
194 day 21, the thickness of each hind paw was measured 3 times a week with a caliper,
195 and the severity of arthritis was graded according to the clinical scale previously
196 described (40) with some modifications. Ankylosis was graded (score 5) and number
197 of inflamed digits was also added to obtain a maximal score of 10 per paw and 40 per
198 mouse. Clinical scores are represented as means \pm SEM on a given day.

199

200 **Cytokine secretion profile**

201 Supernatants of FACS-sorted T cells (100,000 cells/well) were harvested 48 hours
202 following *in vitro* stimulation and stored at -20°C until tested for the presence of
203 murine IFN- γ , IL-10, IL-4, IL-5, and IL-13. All these cytokines were quantified by
204 ELISA kits according to the manufacturer recommendations (R&D Systems).

205

206 **Th1 or Th2 polarization of T cells**

207 For *in vitro* differentiation, naïve OVA-specific CD4⁺ T cells from DO11.10 transgenic
208 mice were cultured during 3 days in Th1 (rIL-12, 10 ng/ml + anti IL-4 antibody, 5 μ g/
209 ml) or Th2 (rIL-4, 10 ng/ml + anti-IFN- γ antibody, 1.25 μ g/ml) polarizing conditions
210 with irradiated splenocytes in the presence of OVA peptide (1 μ g/ml) kindly provided
211 by A. Chavanieu.

212

213 **Statistics**

214 Data are presented as mean \pm SEM and significance was determined using
215 GraphPad Prism software (GraphPad Software). Depending on the distribution of the
216 data, parametric or non-parametric tests with appropriate comparisons were used to
217 compare groups. A one-way or repeated two-way ANOVA with a post hoc multiple
218 comparison test were used when more than two groups were compared.

219

220 **Results**

221 **DC-induced CD49b cells display an effector memory phenotype**

222 As we previously published (30, 32) and as clearly shown in figure S1,
223 repetitive injections of immature DCs significantly induced CD4⁺CD49b⁺ cells (from 5
224 ± 0.2% to 9 ± 0.4%, p<0.0001) without modifying CD4⁺CD25⁺ cell frequencies (12 ±
225 0.2% to 12 ± 0.1%, NS; Fig. S1B). In naïve mice the CD4⁺CD49b⁺ cells are a
226 heterogeneous population containing FoxP3⁺ cells (57 ± 2%) and activated
227 CD25⁺FoxP3^{neg} cells (7 ± 0.4%). After repeated DC injection, the expanded
228 CD4⁺CD49b⁺ population showed a significant decrease in the percentage of FoxP3
229 expressing cells (24 ± 1%, p<0.0001; Fig. S1B) and a significant increase in the
230 percentage of CD25^{neg}FoxP3^{neg} cells (69 ± 0.9%, p<0.0001). These results
231 demonstrate that the induced CD49b⁺ cells were mostly CD25^{neg} and FoxP3^{neg}.
232 Although the frequency of CD4⁺CD25⁺ cells did not significantly change after
233 repeated DC injection, we observed a slight increase in the frequency of these cells
234 expressing FoxP3 (67 ± 1% to 76 ± 2%, p<0.0001). As shown in figure S1A, the
235 analyzed populations were gated as CD4⁺CD49b⁺CD25^{+/-}, CD4⁺CD25⁺ and
236 CD4⁺CD25^{neg}CD49b^{neg} cells and hereafter referred to as gated CD49b, CD25 and
237 CD4 cells, respectively. The same gating strategy was used to sort the three
238 populations.

239 To better characterize the DC-induced CD49b⁺ cells, we compared their cell
240 surface phenotype with those of CD25⁺ and CD4⁺ cells. We first compared the
241 frequency of naïve T cells (defined as CD44^{low}CD62L^{high}) and effector memory T cells
242 (CD44^{high}CD62L^{low}) within the gated CD4, CD25 and CD49b cell populations in non-
243 injected and DC-injected mice (Fig. 1A lower right and upper left quadrant,
244 respectively). The percentage of naïve T cells (Fig. 1B, top panels) was considerably

245 lower both in the CD25⁺ (44 ± 1%) and CD49b⁺ (26 ± 1%) cell populations, than in the
246 CD4⁺ cell population (65 ± 1%) of non-injected mice. Concomitantly, the percentages
247 of effector memory T cells (Fig. 1B, bottom panels) were found to be higher in the
248 CD49b⁺ cell population (48 ± 0.5%) than in the CD25⁺ and CD4⁺ cell populations (25
249 ± 1% and 16%± 1%, respectively) of non-injected mice. Following DC-vaccination, we
250 observed a slight but significant decrease in the percentage of cells with a naïve
251 phenotype within the CD25⁺ cell population (44 ± 1% and 36 ± 1%, p<0.05) and more
252 importantly within the CD49b⁺ cell population (26 ± 1% to 10 ± 1%, p<0.0001). These
253 significant decreases in cells with naïve phenotype were associated with significant
254 increases in cells with effector memory phenotype in the CD49b⁺ (48 ± 0.5% to 66 ±
255 2%, p<0.0001) and to a lesser extent the CD25⁺ (25 ± 1% to 33 ± 1%, p<0.05) cell
256 populations. These data demonstrate that the CD49b⁺ T cell population induced by
257 DC vaccination clearly displayed an effector memory phenotype whereas the CD25⁺
258 T cells phenotype was less impacted.

259

260 **The transcriptional profiles of CD49b⁺ T cells contain multiple transcripts of the**
261 **canonical Treg cell signature shared either by CD25⁺ or other Treg sub-**
262 **phenotypes**

263 To identify the genes differentially expressed by CD25⁺ and CD49b⁺, defined
264 as prototypical Treg transcripts, we compared the gene expression patterns of highly
265 purified T cells. The gating strategy and purity of FACS-sorted CD49b⁺, CD25⁺ and
266 CD4⁺ populations are given in Supplemental Figure 1. We determined the differential
267 transcriptional profiles associated with the DC-vaccination protocol by comparative
268 analysis of the FACS-sorted CD4⁺ cells isolated from non-injected and DC-injected
269 mice. The transcriptional profiles of DC-induced CD25⁺ and CD49b⁺ included both

270 the transcriptional profile associated with the CD4⁺ cell subset and the DC-
271 vaccination induced transcripts. To focus our analysis only on CD49b⁺ and CD25⁺
272 specific transcripts, we removed the transcripts associated with DC vaccination found
273 in CD4⁺. We were therefore able to compare these CD25⁺ and CD49b⁺ differential
274 gene expression profiles with the canonical Treg cell expression signature consisting
275 of 603 probe sets (16, 26, 37). These 603 probe sets defined by Hill *et al.*,
276 correspond to 431 transcripts (138 down-regulated and 293 up-regulated) that
277 revealed a mean probe set redundancy of 1.4 in their study. In our study, the precise
278 and robust analysis of the differentially expressed transcripts is underscored by the
279 mean global score for probe redundancy of 2.0. We found 79 differentially expressed
280 transcripts in the CD49b⁺ cells (18 down-regulated and 61 up-regulated) and 128
281 differentially expressed transcripts in the CD25⁺ cells (28 down-regulated and 100
282 up-regulated) all in common with the canonical Treg signature, with similar
283 modulation described by Hill *et al.* (Fig. 2A). Interestingly the CD25⁺ and CD49b⁺ cell
284 populations shared 59 differentially expressed transcripts (11 down-regulated and 48
285 up-regulated) (Fig 2B), corresponding to 74.6% of the differentially expressed
286 transcripts found in CD49b⁺, therefore underscoring the similarities between CD49b⁺
287 and CD25⁺ Treg cells. Similar transcriptional expression variations were observed
288 between CD49b⁺ and CD25⁺ Treg cells with similar modulations to those described
289 by Hill *et al.* The common transcriptional pattern between CD49b⁺ and CD25⁺
290 contained several prototypical Treg transcripts, including *Itgae*, *Klrg1*, *Nrp1*, *Gzmb*,
291 *Ebi3*, *Entpd1*, *Dusp4*, *Socs2*, *Ahr*, *Swap70*.

292 We also found that each cell population uniquely expressed several canonical
293 Treg cell signature transcripts: 69 for CD25⁺ (17 down-regulated and 52 up-
294 regulated) and 20 for CD49b⁺ (7 down-regulated and 13 up-regulated) (Fig. 2A and

295 Fig. S2). Interestingly among the transcripts specific for CD49b⁺, we found *AcoT7*,
296 *LXN*, *5830474E16Rik*, *Gpr34*, *Pros1* and *Ndr1*. These transcripts have previously
297 been described as differentially expressed in conventional Treg cells isolated from
298 spleen, and highly expressed in CD103⁺ and KLRG1⁺ Treg cells (26). Altogether, our
299 results demonstrate that the CD49b⁺ transcriptional signature contains prototypical
300 Treg cell transcripts shared by either CD25⁺ or other Treg cell sub-phenotypes.

301

302 **CD49b⁺ Treg cells express several canonical markers of CD25⁺FoxP3⁺ Treg**
303 **cells.**

304 To further characterize and compare the phenotypes of the DC-induced
305 CD49b⁺ and CD25⁺ cells isolated from the same DC-vaccinated mice, we performed
306 6-10 colors cytometric analyses. We showed that, despite weakly expressing CD25
307 and the master regulator transcription factor FoxP3 (Fig. S1), CD49b⁺ cells express
308 markers commonly used to characterize CD25⁺FoxP3⁺ Treg cells, including CD103,
309 KLRG1, CTLA-4, latency associated peptide (LAP) and glucocorticoid-induced TNFR
310 family related gene (GITR) (Fig. 3). Interestingly, CD49b⁺ cells also expressed
311 programmed cell death-1 (PD1), shown to play an important role in pTreg cell
312 induction and function (41), although at a lower level than that in CD25⁺ cells (Fig.
313 3A). Moreover, expression of LAG-3, KLRG1 and CD103 molecules was in contrast
314 significantly higher in CD49b⁺ than in CD25⁺ cells (Fig. 3A). Within the CD49b⁺ cell
315 population, we noted that LAG-3 expression was mostly restricted to the FoxP3^{neg}
316 cells, whereas KLRG1 and CD103 expressions were found in both FoxP3⁺ and
317 FoxP3^{neg} cells.

318 Several molecules sustaining the Treg cell suppressive function are known to
319 be highly expressed following activation. As CD25 and CD49b expressions are also

320 modulated following activation, we first purified the T cells from DC-vaccinated mice
321 by FACS-sorting them (Fig. S1) and the three resulting populations were analyzed 48
322 hours following *in vitro* stimulation. Phenotypic analysis of activated T cells clearly
323 showed that all T cells acquired CD25 expression, and that half of the CD25⁺ Treg
324 cells were FoxP3⁺ compared to less than 4% of the CD49b⁺ Treg cells (Fig. 3B).
325 Interestingly, compared to the CD25⁺ T cell population, that of the CD49b⁺ T cells
326 displayed higher percentages or mean fluorescence intensity (MFI) of several
327 markers commonly expressed by Treg cells. These markers included Granzyme B
328 (GrB), GITR, inducible T-cell costimulator (ICOS), LAP and IL-10 in terms of
329 percentages, and CTLA-4 for MFI. We narrowed our focus down to effector
330 mechanisms by comparing the phenotype of IL-10 secreting T cells (gated within the
331 CD49b⁺ cell population) with that of FoxP3⁺ cells (gated within the CD25⁺ cell
332 population) (Fig. S3). Interestingly, the FoxP3⁺ cell sub-population displayed a higher
333 percentage of cells expressing LAP than did the IL-10 secreting sub-population (31 ±
334 8% versus 10 ± 0.5% respectively, p=0.008). Conversely, GITR (96 ± 0.5% versus 78
335 ± 3%, p= 0.026), CTLA-4 (97 ± 0.1% versus 84 ± 5%, p= 0.02) and GrB (31 ± 8%
336 versus 2.5 ± 0.3%, p=0.010) were more frequently expressed among the IL-10
337 secreting CD49b⁺ Treg cells than in the FoxP3⁺CD25⁺ cell sub-population. These
338 results suggest that, besides IL-10, these three molecules could play an important
339 role in the CD49b⁺ suppressive function (Fig. S3).

340

341 **Peripherally induced CD49b⁺ cells express Neuropilin-1 without co-expressing** 342 **Helios.**

343 Neuropilin-1 (Nrp-1) was proposed as a Treg cell surface marker in 2004 (42)
344 and its coordinated expression along with Helios, an Ikaros family transcription factor,

345 was more recently suggested for use in distinguishing thymic derived from inducible
346 Foxp3⁺CD25⁺ Treg cells (43-46). Indeed, pTreg cell populations generated *in vivo*
347 displayed reduced Nrp-1 expression compared with tTreg cells, indicating Nrp-1 as a
348 tTreg specific marker (26). As previously published for NOD and C57BL/6 mice (43),
349 we showed in DBA/1 mice that the majority of CD25⁺ cells express concurrently Nrp-
350 1 and Helios (56 ± 3%) (Fig. 4). Interestingly, Nrp-1 expression was high (58 ± 1%)
351 but Helios expression was significantly lower (15 ± 1%) in CD49b⁺ cells. Moreover,
352 we observed that CD49b⁺Helios⁺ cells co-expressed Nrp-1⁺ and FoxP3⁺ suggesting
353 that among the CD49b⁺ cell population, almost 20% of cells could be considered as
354 natural tTreg cells based on the concomitant expression of Helios, Nrp-1 and FoxP3
355 (Fig.4). Altogether, our results show that induced FoxP3^{neg}CD49b⁺ Treg cells are
356 positive for Nrp-1 but do not co-express Helios, as expected for induced pTreg cells.

357

358 **Peripheral induced CD49b cells express Th1- and Th2-specific transcriptional** 359 **factors and cytokines**

360 Recent evidence suggests that the capacity of Treg cells to control polarized
361 settings can be associated with the expression of specific transcription factors, such
362 as T-bet, interferon regulatory factor 4 (IRF4) and STAT3 to control Th1, Th2 and
363 Th17 responses respectively (47-49). Treg cells expressing these transcription
364 factors can partially mimic the phenotype of the effector T cells, providing them with
365 particular homing, survival, or functional properties (50). It has been demonstrated
366 that 25% of FoxP3⁺ compared to only 5% of FoxP3^{neg} Treg cells isolated from spleen
367 express the canonical Th2 transcription factor Gata3 (51). These authors showed
368 that the expression of Gata3 controlled unbalanced polarization and inflammatory
369 cytokine production in Treg cells, and that it was required for the maintenance of

370 FoxP3 high level expression and promoted the accumulation of Treg cells at inflamed
371 sites (51). In our study in DBA/1 mice, we observed Gata3 expression in $8 \pm 1\%$ of
372 the CD25⁺ cells and in $47 \pm 2\%$ of the CD49b⁺ cell population (Fig. 5A). Furthermore,
373 we observed that only the DC-induced CD49b⁺ cells displayed a considerable
374 proportion of double positive staining for T-bet and Gata3 ($25 \pm 1.5\%$), in contrast
375 with the CD4⁺ and CD25⁺ cells ($2 \pm 0.2\%$ for both populations) (Fig. 5A, right panel).
376 This DC-induced increase in the number of CD49b⁺ cells expressing both T-bet and
377 Gata3 was statistically significant ($25 \pm 1.5\%$ versus $2 \pm 0.2\%$, $p < 0.0001$).

378 C-Maf was the first Th2-specific transcription factor identified and has been
379 shown to play a critical role in trans-activating IL-4 and IL-10 expression during Th17
380 polarization. The ligand-activated transcription factor aryl hydrocarbon receptor
381 (AhR), like the proto-oncogene Maf, was shown to be strongly induced during Tr1 cell
382 differentiation with similarly high levels of expression found in both Tr1 and Th17 cells
383 (52). We thus evaluated the expression of c-Maf and AhR in CD49b⁺ cells and found
384 them in $53 \pm 2\%$ and $82 \pm 1\%$ respectively, compared to in only $38 \pm 1\%$ and $39 \pm 2\%$
385 respectively of the CD25⁺ population (Fig. 5B).

386 To further characterize the cytokine secretion profile we quantified the level of
387 cytokine secretion in the supernatant of highly purified FACS-sorted cells following
388 their *in vitro* activation. Besides the high level of IL-10 secretion (19 ± 7 ng/ml), we
389 measured significantly elevated secretion levels of other type 2 cytokines, including
390 IL-4 (10 ± 2 ng/ml), IL-5 (18 ± 2 ng/ml) and IL-13 (31 ± 0.5 ng/ml), as well as a
391 relatively high amount of IFN- γ (3 ± 1 ng/ml) in the supernatant of the CD49b⁺ T cell
392 population. These results revealed an obvious type 2 dominant cytokine profile for
393 the CD49b⁺ Treg cells and underscored their dissimilarity with Tr1 cells, which
394 secrete high levels of IL-10 without concomitant secretion of IL-4 (40, 53).

395

396 **Polyclonal and Ag-specific CD49b⁺ Treg cells have potent *in vitro* and *in vivo***
397 **suppressive capacities**

398 We compared the *in vitro* potential of CD49b⁺ and CD25⁺ Treg cells to
399 functionally suppress the proliferation of CD4⁺ T cells by co-cultivating Treg and
400 responder cell populations stimulated by a polyclonal T cell receptor stimulator (anti-
401 CD3 mAb) and in the presence of antigen-presenting cells. Addition of CD49b⁺ or
402 CD25⁺ Treg cells reduced the proliferation, as measured by the CFSE dilution, in a
403 dose-dependent manner thus confirming their potent *in vitro* suppressive capacities
404 (Fig 6A).

405 We previously demonstrated the *in vivo* therapeutic potential of CD49b⁺ Treg
406 cells to protect against (30) as well as to improve the condition of established arthritis
407 (32). To further investigate the therapeutic potential of CD49b⁺ Treg cells, we
408 compared their protective effect with that of CD25⁺ Treg cells isolated from the same
409 DBA/1 mice and with CD49b⁺ Treg cells isolated from Col II-specific T cell transgenic
410 mice (TBC). We repeatedly injected syngeneic mice intraperitoneally with 0.5×10^6
411 DCs the week before their euthanasia. CD4⁺ T cells were pre-purified and the Treg
412 cells were FACS-sorted to obtain >98% pure population. The FACS-sorted
413 populations were adoptively transferred intravenously into collagen-induced arthritic
414 (CIA) mice on day 28, at the onset of the clinical signs. In this experimental setting
415 that mimics the clinical situation, we observed a similar decrease of arthritis severity
416 in mice injected with either of the polyclonal Treg cells, CD49b⁺ or CD25⁺, isolated
417 from the same DBA/1 mouse, or with the antigen-specific CD49b⁺ Treg cells (CD49b
418 TBC, Fig. 6B). Similar results were obtained in several independent experiments, and
419 we performed robust statistical analyses using relative arthritic scores calculated

420 using the mean of the PBS-treated mice as 100% disease severity for each
421 experiment. We included in these experiments a control group of mice, which were
422 injected with the CD4⁺ cell population. As shown in figure 6C, injection of polyclonal
423 CD49b⁺ Treg cells markedly and significantly decreased the disease severity
424 compared with PBS-treated or CD4⁺-treated mice. We observed a tendency towards
425 decreased disease severity after injection of the CD25⁺ Treg cells or of the antigen-
426 specific CD49b⁺ Treg cells isolated from TBC mice, however these decreases were
427 not significant. These results in the CIA experimental model suggest that the use of
428 CD49b⁺ Treg cells may represent the best therapeutic strategy, over the use of
429 natural CD25⁺ Treg cells.

430 Finally, to further investigate the *in vivo* suppressive mechanism, we
431 performed similar adoptive transfer experiments with CD49b⁺ Treg cells isolated from
432 IL-10 KO mice. In this experimental setting, CD49b⁺ Treg cells were isolated from IL-
433 10 KO or wild-type littermates and injected on day 28 into arthritis-induced C57BL/6
434 mice. As clearly shown in figure 6D, injection of CD49b⁺ isolated from wild-type
435 littermates significantly protected mice from arthritis whereas IL-10 deficient CD49b⁺
436 Treg cells were less protective. These results underscore the partial involvement of
437 IL-10 secretion in the CD49b⁺ Treg protective effect and suggest that alternative
438 mechanisms might be important for their *in vivo* suppressive function.

439

440 **Discussion**

441 The discovery that Treg cells can control autoimmune inflammatory responses
442 has led to great enthusiasm for their clinical application in autoimmune diseases such
443 as rheumatoid arthritis (RA). The hope is that the impaired Treg cell differentiation
444 may be corrected by adoptive transfer of *in vitro*-generated autologous Treg cells or
445 by immunotherapeutic strategies triggering an increase in the number and/or an
446 improved functioning of endogenous Treg cells.

447 *In vitro* generation of autologous Treg cells could be a treatment option for
448 multiple autoimmune diseases, including experimental autoimmune
449 encephalomyelitis, diabetes, colitis, and lupus (54-56). However, this approach is
450 quite challenging because it is difficult to generate and/or expand Treg cells with
451 specific Ag specificity, especially when the immunodominant epitopes are
452 uncharacterized, such as in RA. Nevertheless, *in vitro* expansion of Col II-specific Tr1
453 cells isolated from RA patients was recently demonstrated (57). Pre-clinical proof-of-
454 concept concerning the therapeutic potential of *in vitro* generated Col II-specific Tr1
455 cells has also been recently validated in two experimental models of arthritis (40).
456 Altogether these results support the therapeutic use of ex-vivo expanded autologous
457 Ag-specific Treg cells in RA.

458 However, some evidence suggests that Treg cells generated *in vitro* are
459 phenotypically and functionally unstable, whereas those induced *in vivo* are
460 epigenetically more stable and would lead to a longer-lasting therapeutic effect (4,
461 58, 59). The *in vivo*-induced Treg cells are usually Ag specific, which implies a likely
462 more efficient effect in treating autoimmune diseases. In RA patients, TNF- α blocking
463 antibodies have been described as an effective way to stimulate the induction of
464 peripheral FoxP3⁺ Treg cells, overcoming the impaired peripheral Treg cell

465 differentiation (60). For all these reasons, the development of strategies to promote *in*
466 *vivo* generation of Ag-specific Treg cells appears crucial for the treatment of
467 autoimmune diseases.

468 The aim of our study was to better characterize a particular sub-population of
469 *in vivo*-induced CD49b⁺ Treg cells. We demonstrated that this particular Treg cell
470 subset expresses several canonical markers of Treg cells while being mostly
471 negative for CD25 and FoxP3, which are routinely used to identify Treg cells. We first
472 demonstrated that 30% of the Treg cell signature was found in the CD25⁺ Treg cell
473 specific expression profile. Indeed, the Treg cell canonical signature is a composite
474 signature derived from Treg cells isolated from several lymphoid organs (37). This
475 bulk of Treg cell sub-phenotypes could explain the lack of complete overlapping with
476 the specific transcriptional profile of CD25⁺ cells in our study. A similar lack of
477 complete overlapping has previously been observed when comparing the
478 transcriptional profile of converted FoxP3⁺ Treg cells with the canonical Treg cell
479 signature (26). Interestingly, the induced CD49b⁺ Treg cells shared a transcriptional
480 profile common to CD25⁺ Treg cells and the canonical Treg cell signature. We
481 showed that 75% of the differentially expressed transcripts found in CD49b⁺ T cells
482 were common with those found in CD25⁺ T cells, underscoring the similarities
483 between CD49b⁺ and CD25⁺ Treg cells. Indeed, we demonstrated that these cells
484 share a common signature of 59 prototypical Treg cell transcripts including effector
485 molecules and transcription factors. Several transcripts from this common signature
486 have been proposed as promising candidates to specifically discriminate between
487 Ag-induced and homeostatically converted Treg cells, including *Itgae*, *Ctla4*, *Entpd1*
488 (*CD39*), *Ebi3* (a component of *IL35*), *Irf4*, α E β 7 (*CD103*) and *Klrg1* (a member of the
489 killer cell lectin-like receptor family). The CD49b⁺ Treg cell transcriptional profile also

490 contained several specific transcripts in common with the canonical Treg signature.
491 These results suggest an overlap of the transcriptional profile of CD49b⁺ Treg cells
492 with several other Treg sub-phenotypes.

493 We validated by FACS analyses the common expression of several markers
494 between CD25⁺ and CD49b⁺ cells that were differentially expressed compared with
495 CD4⁺. Among these markers, CD49b and KLRG1, both considered as NK cell
496 markers and minimally expressed on conventional CD4⁺ T cells, were previously
497 observed in an extrathymically derived subset of CD4⁺CD25⁺FoxP3⁺ Treg cells (61).
498 Within the sub-population of CD25⁺FoxP3⁺ Treg cells in the spleen, KLRG1⁺ Treg
499 cells were previously shown to display a more activated phenotype
500 (CD69⁺CD62L^{low}CD103⁺CD44^{high}) than KLRG1^{neg} Treg cells. Furthermore, cell-
501 surface staining of homeostatically converted FoxP3⁺ cells revealed them as
502 uniformly CD103⁺, an excellent marker for identifying *in vivo*-activated FoxP3⁺CD4⁺
503 Treg cells, and that 50% of the cells expressed KLRG1 (26). We showed that the two
504 markers, KLRG1 and CD103, were expressed on CD49b⁺ and CD25⁺ cells and, as
505 previously observed for the CD4⁺CD25⁺FoxP3⁺ Treg cells, were associated with an
506 activated phenotype for the CD49b cells. Similarly, Nrp-1 was previously described
507 on a population of activated/memory FoxP3^{neg}Nrp-1⁺ in secondary lymphoid organs
508 and inflamed tissues, which could imply that the expression of Nrp-1 is associated
509 with the CD49b⁺ activated/memory phenotype. Finally, the lack of concomitant
510 expression of Nrp-1 and Helios as well as their effector/memory phenotype confirm
511 the peripheral origin of these cells.

512 Initially characterized as a Th2 specific cytokine, IL-10 has since been found
513 expressed by almost all CD4⁺ T cells, including CD25⁺FoxP3⁺ Treg cells and Tr1
514 cells, but also Th1, Th2 and Th17 cells, in order to promote immune homeostasis.

515 Previous mouse studies have described the collaborative actions of c-Maf with AhR
516 and the ICOS receptor ligation that drive IL-10 expression and promote Tr1
517 differentiation (52, 62). We demonstrated in this study that the CD49b⁺ Treg cells
518 highly express these three molecules suggesting that, similarly to Tr1 cells, several
519 transcriptional pathways, associated with high secretion of IL-10, are activated.
520 CD49b⁺ cells are also positive for the Th2 specific transcription factor Gata3 and 30%
521 of the cells are double positive for T-bet and Gata3 with concomitant secretion of
522 IFN- γ and Th2 cytokines. The co-expression of T-bet and Gata3 has been previously
523 observed *in vivo* following viral infection and this hybrid phenotype appeared to be
524 stable (63). Altogether our results suggest that the CD49b⁺ cells display a balanced
525 Th2/Th1 phenotype that could endow them with specific properties to better control
526 effector T cell responses.

527 Other similarities and differences between IL-10-secreting CD49b⁺ Treg cells
528 and Tr1 cells can be discussed. Co-expression of CD49b and LAG-3 has been
529 recently proposed as specific for Tr1 cells (35). In our experimental setting, only 5 to
530 10% of CD49b⁺ Treg cells were positive for LAG-3 before *in vitro* activation and
531 interestingly CD49b⁺LAG-3⁺ cells are mostly FoxP3 negative cells like Tr1 cells.
532 Furthermore Tr1 cells are reported to be induced at mucosal sites in response to
533 antigen stimulation in the presence of IL-10. We observed that IL-10 deficient DCs
534 promoted IL-10 secreting CD49b⁺ Treg cell expansion in several lymphoid organs of
535 wild type animals suggesting that, in contrast to Tr1 cells (64), the IL-10 secretion by
536 DCs is dispensable for the expansion of CD49b⁺ (P. Louis-Pence, unpublished data).
537 Altogether our results suggest that the CD49b⁺ Treg cells constitute a Treg sub-
538 phenotype that shares similarities with the CD25⁺ Treg cells as well as with the Tr1

539 cells, and should be considered alongside other sub-phenotypes as homeostatically
540 converted or antigen-induced.

541 Here we have investigated the suppressive function of CD25⁺ and CD49b⁺
542 Treg cell populations *in vitro* and *in vivo*, in the experimental model of CIA. *In vitro*,
543 both Treg cell populations similarly suppressed the T cell proliferation. To compare
544 their therapeutic potential in CIA, we injected CD25⁺, polyclonal CD49b⁺ or Col II-
545 specific CD49b⁺ Treg cells at the onset of clinical signs of arthritis. As previously
546 described (32), we demonstrated a significant reduction of these clinical signs
547 following injection of polyclonal CD49b⁺ Treg cells. Although not significant, we also
548 observed decreased clinical signs following injection of CD25⁺ or Col II-specific
549 CD49b⁺ Treg cells. Our results suggest that following their activation by self-Ag(s),
550 the CD49b⁺ regulatory T cells display a potent bystander suppressive function and as
551 polyclonal *in vivo*-expanded Treg cells, they could be a better alternative to classical
552 Treg cells for arthritis treatment. The suppressive function of CD49b⁺ Treg cells was
553 found to be partially dependent on IL-10 secretion. Moreover expression of several
554 canonical Treg markers, implicated in the Treg suppressive function, suggests that
555 other molecules might also play a role in the CD49b suppressive activity. Indeed, GrB
556 and CTLA-4 have been shown to play a crucial role in the suppressive function of
557 conventional CD25⁺FoxP3⁺ Treg cells and thus might also play an important role in
558 the suppressive function of CD49b⁺ Treg cells. Furthermore, CD103 expression could
559 also be implicated in their suppressive function as its expression was shown to be
560 responsible for the retention of Treg cells in inflamed tissue by interaction with its
561 ligand E-cadherin (65, 66). Finally, expression of the alpha2 integrin CD49b itself,
562 could also be important for their function since it was demonstrated that this integrin
563 is required for the migration of memory CD4 T-cell precursors into their survival

564 niches of the bone marrow (67). Since VLA-2 also binds collagen II, expression of
565 CD49b could provide Treg cells with particular homing, survival, or more potent
566 suppressive function in the context of arthritis since collagen II is expressed by the
567 damaged cartilage.

568 In this study, we have provided an in-depth characterization of the CD49b⁺
569 Treg cells, underscoring their similarities with other Treg sub-phenotypes and
570 highlighting specific expression patterns for several markers including ICOS, CTLA-4
571 and GrB. The expression of these canonical Treg markers strongly supports the
572 notion that several suppressive mechanisms could be FoxP3-independent. Their
573 potent suppressive activity *in vivo*, higher than that of the classical CD25⁺ Treg cells,
574 underscores the need to select appropriate Treg subsets for a given clinical
575 application and supports their therapeutic application in RA.

576

577 **Acknowledgments**

578 We thank Myriam Boyer and Christophe Duperray (Montpellier RIO Imaging
579 platform) for performing cell-sorting experiments with the FACS Aria and the
580 ECELLFRANCE national infrastructure for providing the LSR Fortessa cytometer. We
581 also thank Véronique Pantesco for gene-chip hybridization and the animal facility
582 staff located at the INM institute in Montpellier (RAM network) for their expert care of
583 the mice colonies.

584

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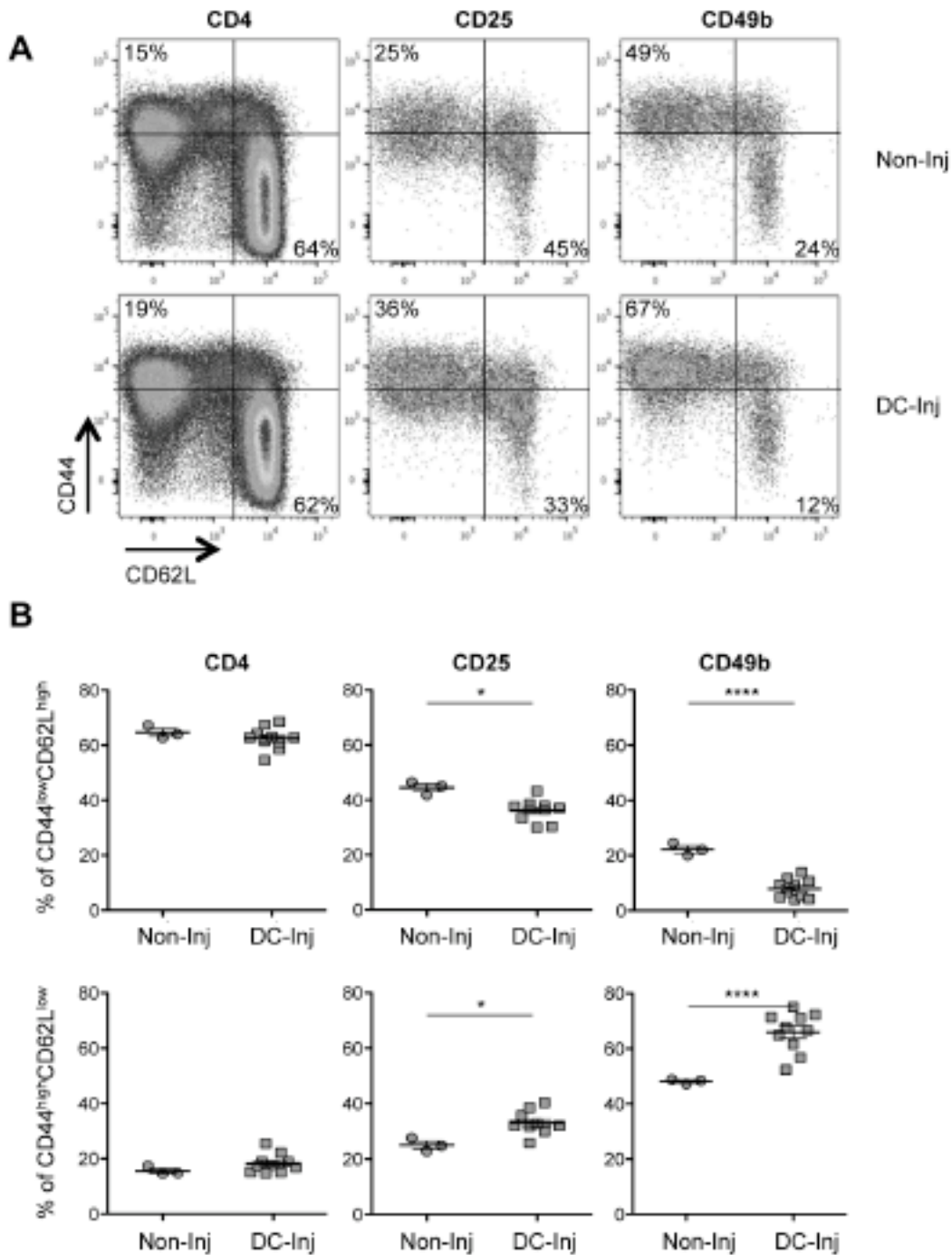
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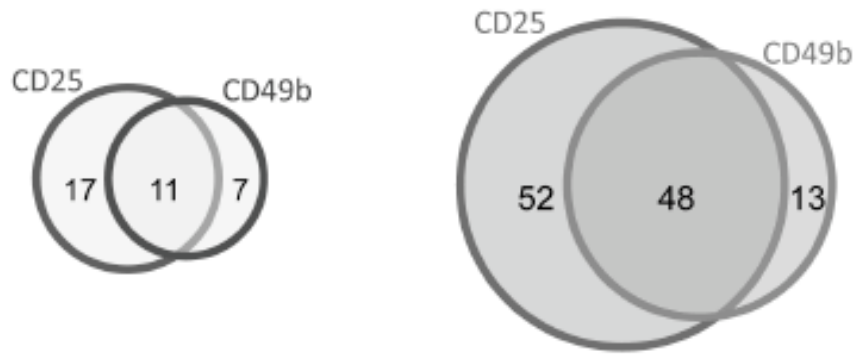
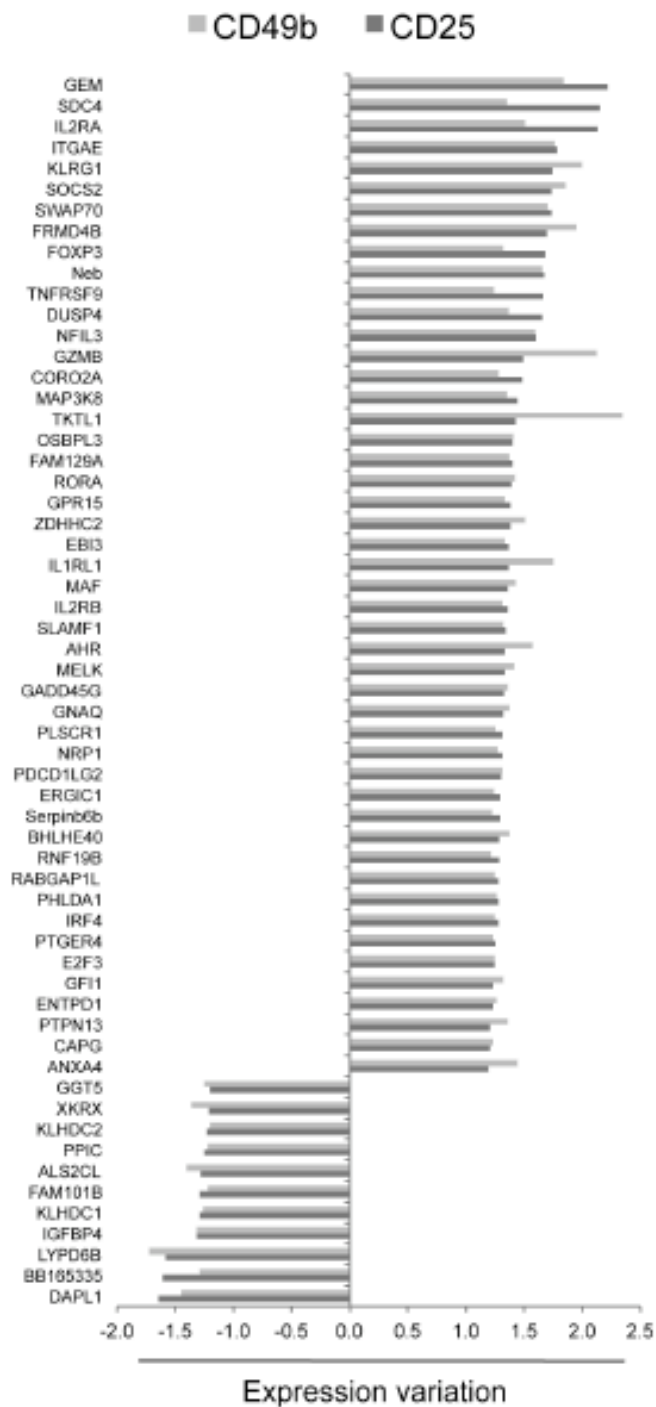
805 **FIGURE 1.** DC-induced CD49b⁺ cells display an effector memory phenotype.

806 Percentage of naïve (CD44^{low}CD62L^{high}) and effector memory (CD44^{high}CD62L^{low}) T

807 cells within the CD4, CD25 and CD49b gated populations (For gating strategy see

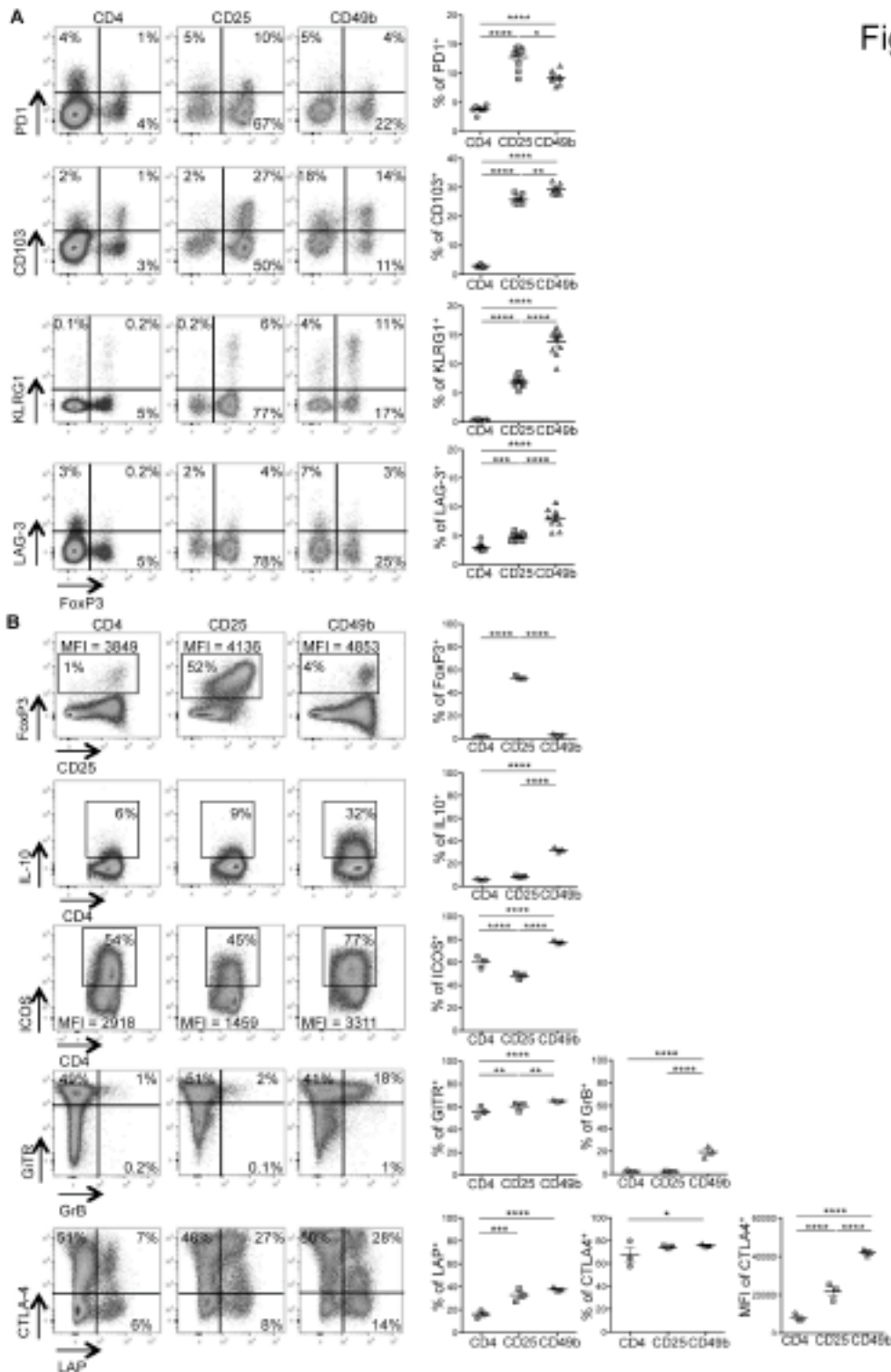
808 Fig S1A) were analyzed by flow cytometry for DC-injected (DC-Inj, n=10) and non-
809 injected mice (Non-Inj, n=3). A, Representative dot plots within gated CD4 (left),
810 CD25 (middle) and CD49b (right) positive cells in non-injected (top panels) and DC-
811 injected mice (bottom panels). B, Percentages of naïve and effector memory cells
812 within the gated CD4 (left), CD25 (middle) and CD49b (right) cell population. Each
813 symbol represents an individual mouse and bars show the mean \pm SEM. Data are
814 representative of two independent experiments. * $p < 0.05$, **** $p < 0.0001$ by repeated
815 measures two-way ANOVA (Bonferroni's multiple comparisons test).

816

A**B**

818 **FIGURE 2.** Transcriptional profile of CD49b⁺ Treg cells contains multiple transcripts
819 of the canonical Treg signature. A, Venn diagram depicting the number of commonly
820 and uniquely down-regulated (left) or up-regulated (right) transcripts, in the FACS-
821 sorted CD49b⁺ and CD25⁺ Treg cell populations, with the canonical Treg signature.
822 B, Bar graphs show the transcriptional expression variation of the differentially
823 expressed genes common among the CD49b⁺, CD25⁺ and the canonical Treg
824 signature.
825

Figure 3

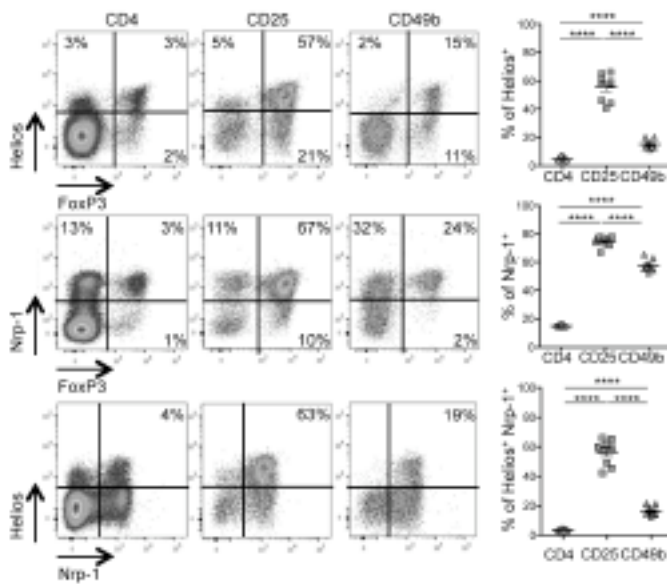


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FIGURE 3. Several canonical markers of CD25⁺FoxP3⁺ Treg cells are expressed by CD49b⁺ effector memory cells. A, Representative flow cytometry analyses of splenocytes from DC-injected mice (n=18) within the gated CD4 (left), CD25 (middle) and CD49b (right) cell populations. Quadrants were set as indicated and frequencies

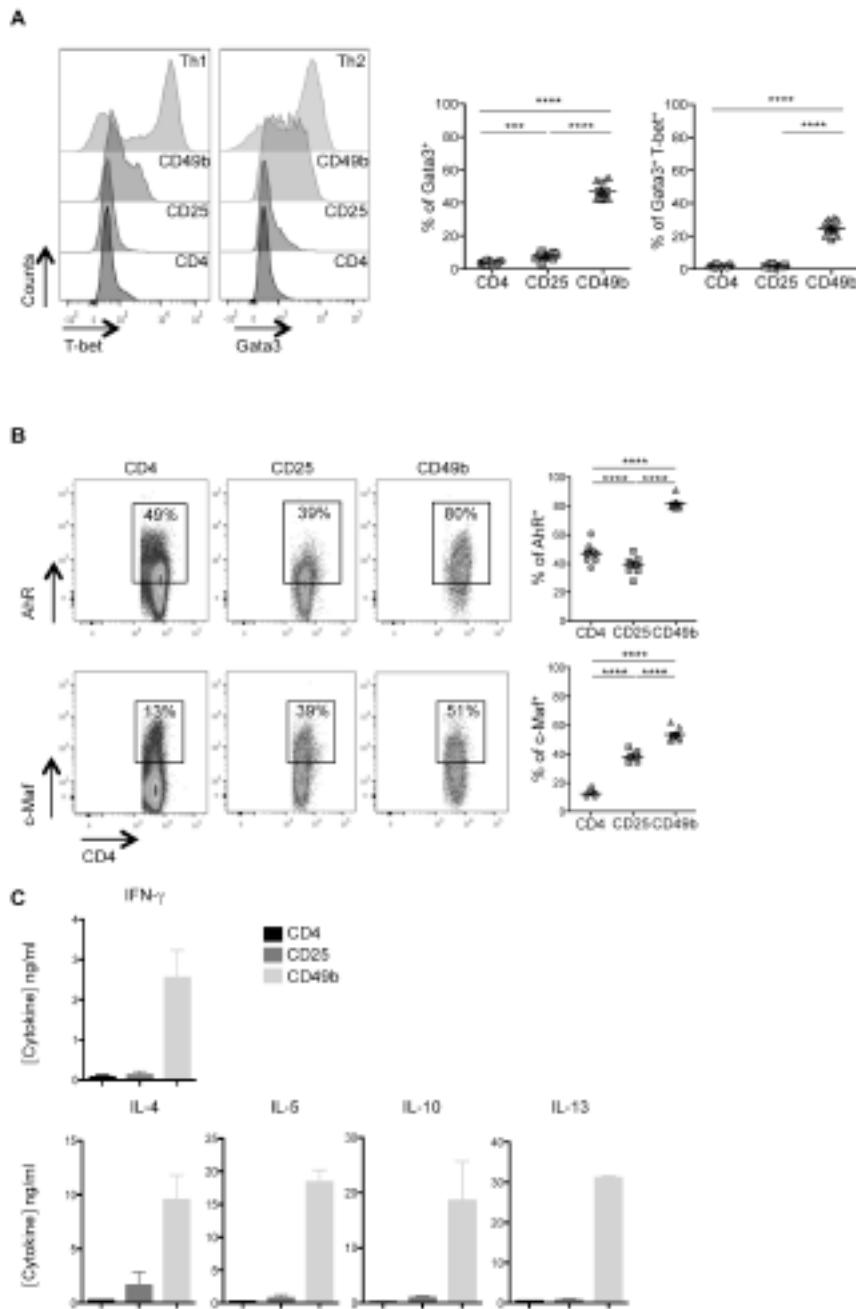
831 of cells are shown within each quadrant. Each symbol represents a pool of 2 mice
 832 and bars show the mean \pm SEM. **** $p < 0.0001$, *** $p = 0.0005$, ** $p = 0.01$ and *
 833 $p = 0.013$ by repeated measures two-way ANOVA (Tukey's multiple comparisons test).
 834 B, The FACS-sorted CD4, CD25 and CD49b T cell populations from DC-vaccinated
 835 mice ($n = 18$) were analyzed by FACS 48 hours following *in vitro* stimulation. Gates
 836 and quadrants were set as indicated and frequencies of cells are shown. Each
 837 symbol represents a pool of 6 mice and bars show the mean \pm SEM. **** $p < 0.0001$,
 838 *** $p = 0.0001$, ** $p = 0.002$ and * $p = 0.03$ by repeated measures two-way ANOVA
 839 (Tukey's multiple comparisons test).

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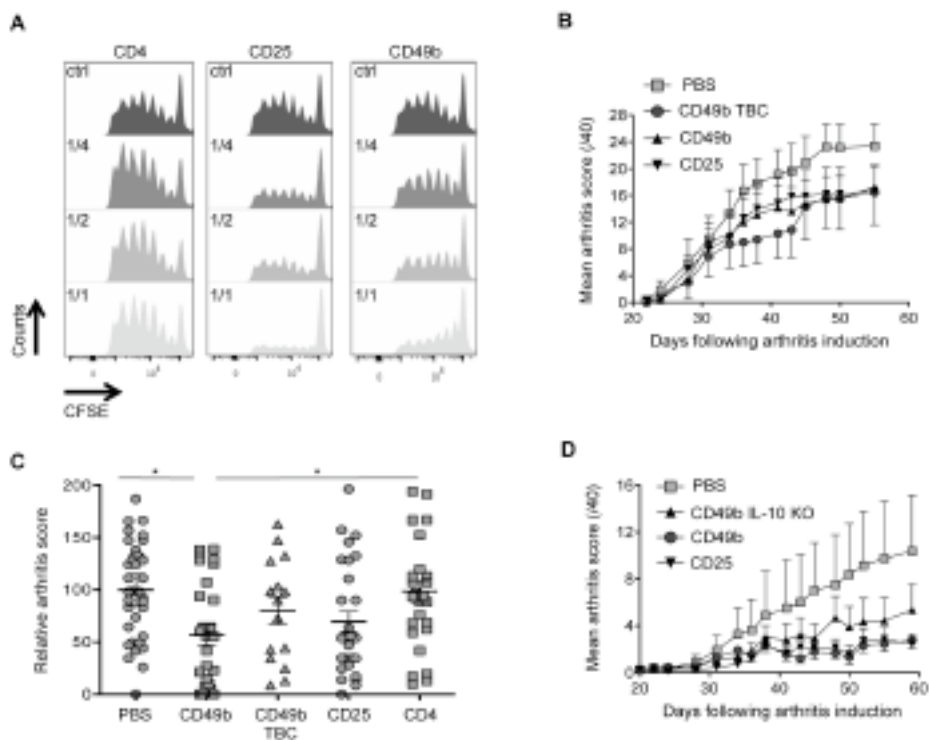
842 **FIGURE 4.** Peripherally induced CD49b⁺ cells express Neuropilin-1 without co-
 843 expressing Helios. Representative flow cytometry analyses of splenocytes from DC-
 844 injected mice ($n = 18$) within the gated CD4 (left), CD25 (middle) and CD49b (right)
 845 cell population. Quadrants were set as indicated and percentages of Helios, Nrp-1 or
 846 double positive cells were analyzed. Each symbol represents a pool of 2 mice and
 847 bars show the mean \pm SEM. **** $p < 0.0001$ by repeated measures two-way ANOVA
 848 (Tukey's multiple comparisons test).



849

850 **FIGURE 5.** Peripherally induced CD49b⁺ cells express Th2-specific transcriptional
 851 factors and display a dominant Th2 cytokine profile. Percentages of cells expressing
 852 T-bet, Gata3 and transcriptional factors associated with IL-10 production, including c-
 853 Maf and AhR, were analyzed within the gated CD4, CD25 and CD49b cell
 854 populations from DC-injected splenocytes (n=18). A, Representative histogram plots
 855 of T-bet and Gata3 staining in gated CD4, CD25 and CD49b cell populations were

856 compared to *in vitro* polarized Th1 and Th2 cells. Percentages of Gata3⁺ and double
 857 positive Gata3⁺T-bet⁺ cells are represented with mean ± SEM, each symbol
 858 representing a pool of 2 mice. Data are representative of two independent
 859 experiments. **** p<0.0001, *** p=0.0003 by repeated measures two-way ANOVA
 860 (Tukey's multiple comparisons test). B, Representative dot plots and percentages of
 861 AhR⁺ and c-Maf⁺ cells in gated CD4, CD25 and CD49b cell populations. Each symbol
 862 represents a pool of 2 mice and bars show the mean ± SEM. Data are representative
 863 of two independent experiments. **** p<0.0001 by repeated measures one-way
 864 ANOVA (Tukey's multiple comparisons test). C, Level of cytokine secretion by highly
 865 purified cells following *in vitro* activation.
 866



867
 868 **FIGURE 6.** Polyclonal and Ag-specific CD49b⁺ T reg cells display potent *in vitro* and
 869 *in vivo* suppressive capacities. A, Comparable suppressive capacities of FACS-
 870 sorted CD25⁺ and CD49b⁺ Treg cells *in vitro*. CFSE-labeled effector T (Teff) cells

871 were cultured in activating conditions with titrated numbers of CD4⁺, CD25⁺ or
872 CD49b⁺ T cell population at different Treg/Teff ratios. As positive control for T cell
873 proliferation, Teff cells were cultured alone in activating conditions (ctrl). Results are
874 representative of three independent experiments. B, C, D, Comparison of the
875 suppressive function of Treg cells *in vivo* in the experimental model of CIA. Mice
876 were intravenously injected with 150,000 FACS-sorted T cells or with PBS on day 28
877 after immunization. Means of the severity scores of arthritis are represented for each
878 group with a maximal score of 40 per mouse. B, Comparison of the therapeutic
879 potential of polyclonal CD49b⁺ (CD49b) and CD25⁺ (CD25) Treg cells isolated from
880 syngeneic DBA/1 mice as well as Ag-specific CD49b⁺ cells (CD49b TBC) isolated
881 from TBC mice. Data are represented as mean \pm SEM of each group. C, Relative
882 arthritic scores were calculated for each independent experiment using the mean of
883 the PBS-treated mice (PBS, 5 independent experiments) as 100% disease severity.
884 Each symbol represents a mouse and bars represent the mean \pm SEM. Results
885 obtained with polyclonal CD49b⁺ (CD49b, 4 independent experiments), Ag-specific
886 CD49b⁺ (CD49bTBC, 2 independent experiments), and CD25⁺ (CD25, 4 independent
887 experiments) Treg cells as well as CD4⁺ T cells treated mice (CD4, 4 independent
888 experiments) are represented. * $p < 0.05$ by one-way ANOVA (Tukey's multiple
889 comparisons test). D, The suppressive mechanism of CD49b⁺ Treg cells is partially
890 dependent on IL-10. Therapeutic potential of CD49b⁺ Treg cells FACS-sorted from
891 IL-10 KO mice (CD49b IL-10 KO) or from wild-type littermates (CD49b), and CD25⁺
892 Treg cells FACS-sorted from wild-type littermates (CD25) were compared in CIA (n=7
893 to 9 mice per group). Data are represented as mean \pm SEM of each group, and are
894 representative of 2 independent experiments. * $p < 0.05$ by repeated measures two-
895 way ANOVA (Tukey's multiple comparisons test). Significant differences were

896 observed from days 50 to 59 for CD49b treated mice compared with PBS-injected
897 mice; and from day 59 for CD25 treated mice compared with PBS-injected mice.

898