

# Nonclassical CD4 + CD49b + Regulatory T Cells as a Better Alternative to Conventional CD4 + CD25 + T Cells To Dampen Arthritis Severity

Rita Vicente, Julie Quentin, Anne-Laure Mausset-Bonnefont, Paul Chuchana, Delphine Martire, Maïlys Cren, Christian Jorgensen, Pascale Louis-Pence

► **To cite this version:**

Rita Vicente, Julie Quentin, Anne-Laure Mausset-Bonnefont, Paul Chuchana, Delphine Martire, et al.. Nonclassical CD4 + CD49b + Regulatory T Cells as a Better Alternative to Conventional CD4 + CD25 + T Cells To Dampen Arthritis Severity. *Journal of Immunology*, Publisher : Baltimore : Williams & Wilkins, c1950-. Latest Publisher : Bethesda, MD : American Association of Immunologists, 2015, 196 (1), pp.298 - 309. 10.4049/jimmunol.1501069 . hal-01834236

**HAL Id: hal-01834236**

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

Submitted on 18 Dec 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3  
4 Rita Vicente<sup>1\*†‡</sup>, Julie Quentin<sup>1\*†‡</sup>, Anne-Laure Mausset-Bonnefont<sup>\*†‡</sup>, Paul  
5 Chuchana<sup>\*†‡</sup>, Delphine Martire<sup>\*†‡</sup>, Maïlys Cren<sup>†‡</sup>, Christian Jorgensen<sup>\*†‡</sup>, and  
6 Pascale Louis-Plence<sup>\*†‡</sup>

7  
8 \* Inserm, U1183, Institute of Regenerative Medicine and Biotherapies, Montpellier,  
9 France

10 † University of Montpellier, Montpellier, France.

11 ‡ CHU Saint Eloi, Institute of Regenerative Medicine and Biotherapies, Montpellier,  
12 France

13  
14 <sup>1</sup> equally contributed to the study

15  
16 Address correspondence to: Dr. Pascale Louis-Plence, Inserm U1183, IRMB, 80 rue  
17 Augustin Fliche, 34295 Montpellier cedex 05, France.

18 Phone: (+33) 467 33 57 21/ Fax: (+33) 467 33 01 13

19 Email: [pascale.plence@inserm.fr](mailto:pascale.plence@inserm.fr)

20  
21  
22 Running title: CD49b<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> but also by other sub-phenotypes. We characterized the CD49b<sup>+</sup> Treg cell  
43 phenotype, underscoring its similarities with the CD25<sup>+</sup> 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<sup>+</sup> and  
46 CD49b<sup>+</sup> 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<sup>+</sup> 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  $\alpha$  chain) and thus denoted as  
60 CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> 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- $\beta$  (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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>CD49b<sup>+</sup> Treg cells, present in naïve mice,  
87 were more efficient in suppressing the onset of diabetes than CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 $\alpha$ 11.1 and V $\beta$ 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<sup>-/-</sup> knockout mice (KO) were obtained from Janvier  
109 (B6.129P2-IL10<sup>tm1Cgn</sup>/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<sup>-5</sup> M  $\beta$ -mercaptoethanol, 100U/ml penicillin, 100  $\mu$ 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 10<sup>5</sup> 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  $0.5 \times 10^6$  DCs in 100  $\mu$ 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  $\mu$ 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  $\mu$ g/ml of ionomycin and 10  $\mu$ 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<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup>CD49b<sup>+</sup> T cells (purity  $>95 \pm 2\%$ ), CD4<sup>+</sup>CD25<sup>+</sup>CD49b<sup>-</sup>  
151 (purity  $>96 \pm 1\%$ ) or CD4<sup>+</sup>CD25<sup>-</sup>CD49b<sup>-</sup> T cells (purity  $>96 \pm 1\%$ ), herein called



152 CD49b<sup>+</sup>, CD25<sup>+</sup> and CD4<sup>+</sup> cells, respectively, were washed and 1.5 x 10<sup>5</sup> 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<sup>+</sup>, CD25<sup>+</sup> and CD49b<sup>+</sup> T cells isolated from DC-injected mice and  
157 non-injected mice (CD4<sup>+</sup> 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<sup>+</sup> and CD49b<sup>+</sup> cell groups as  
160 well as two replicates for CD4<sup>+</sup> 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<sup>+</sup> cells isolated from non-injected mice, and to calculate the differential  
171 expression variation using the transcriptional profiles of CD4<sup>+</sup>, CD25<sup>+</sup> and CD49b<sup>+</sup> 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  $\geq 1.15$  and occurrence of 4/4 for  
176 CD4<sup>+</sup> and 6/6 for CD25<sup>+</sup> and CD49b<sup>+</sup>) (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 ( $5 \times 10^5$ ) and 2-5  $\mu\text{g}/\text{ml}$  of anti-CD3 $\epsilon$  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  $\mu\text{g}$  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 $\mu\text{g}/\text{ml}$ )-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  $\mu\text{g}$  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- $\gamma$ , 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<sup>+</sup> 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  $\mu$ g/  
209 ml) or Th2 (rIL-4, 10 ng/ml + anti-IFN- $\gamma$  antibody, 1.25  $\mu$ g/ml) polarizing conditions  
210 with irradiated splenocytes in the presence of OVA peptide (1 $\mu$ 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<sup>+</sup>CD49b<sup>+</sup> cells (from 5  
224 ± 0.2% to 9 ± 0.4%, p<0.0001) without modifying CD4<sup>+</sup>CD25<sup>+</sup> cell frequencies (12 ±  
225 0.2% to 12 ± 0.1%, NS; Fig. S1B). In naïve mice the CD4<sup>+</sup>CD49b<sup>+</sup> cells are a  
226 heterogeneous population containing FoxP3<sup>+</sup> cells (57 ± 2%) and activated  
227 CD25<sup>+</sup>FoxP3<sup>neg</sup> cells (7 ± 0.4%). After repeated DC injection, the expanded  
228 CD4<sup>+</sup>CD49b<sup>+</sup> 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<sup>neg</sup>FoxP3<sup>neg</sup> cells (69 ± 0.9%, p<0.0001). These results  
231 demonstrate that the induced CD49b<sup>+</sup> cells were mostly CD25<sup>neg</sup> and FoxP3<sup>neg</sup>.  
232 Although the frequency of CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD49b<sup>+</sup>CD25<sup>+/-</sup>, CD4<sup>+</sup>CD25<sup>+</sup> and  
236 CD4<sup>+</sup>CD25<sup>neg</sup>CD49b<sup>neg</sup> 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<sup>+</sup> cells, we compared their cell  
240 surface phenotype with those of CD25<sup>+</sup> and CD4<sup>+</sup> cells. We first compared the  
241 frequency of naïve T cells (defined as CD44<sup>low</sup>CD62L<sup>high</sup>) and effector memory T cells  
242 (CD44<sup>high</sup>CD62L<sup>low</sup>) 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<sup>+</sup> (44 ± 1%) and CD49b<sup>+</sup> (26 ± 1%) cell populations, than in the  
246 CD4<sup>+</sup> 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<sup>+</sup> cell population (48 ± 0.5%) than in the CD25<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> cell population (44 ± 1% and 36 ± 1%, p<0.05) and more  
252 importantly within the CD49b<sup>+</sup> 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<sup>+</sup> (48 ± 0.5% to 66 ±  
255 2%, p<0.0001) and to a lesser extent the CD25<sup>+</sup> (25 ± 1% to 33 ± 1%, p<0.05) cell  
256 populations. These data demonstrate that the CD49b<sup>+</sup> T cell population induced by  
257 DC vaccination clearly displayed an effector memory phenotype whereas the CD25<sup>+</sup>  
258 T cells phenotype was less impacted.

259

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

263 To identify the genes differentially expressed by CD25<sup>+</sup> and CD49b<sup>+</sup>, 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<sup>+</sup>, CD25<sup>+</sup> and  
266 CD4<sup>+</sup> 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<sup>+</sup> cells isolated from non-injected and DC-injected  
269 mice. The transcriptional profiles of DC-induced CD25<sup>+</sup> and CD49b<sup>+</sup> included both

270 the transcriptional profile associated with the CD4<sup>+</sup> cell subset and the DC-  
271 vaccination induced transcripts. To focus our analysis only on CD49b<sup>+</sup> and CD25<sup>+</sup>  
272 specific transcripts, we removed the transcripts associated with DC vaccination found  
273 in CD4<sup>+</sup>. We were therefore able to compare these CD25<sup>+</sup> and CD49b<sup>+</sup> 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<sup>+</sup> cells (18 down-regulated and 61 up-regulated) and 128  
281 differentially expressed transcripts in the CD25<sup>+</sup> 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<sup>+</sup> and CD49b<sup>+</sup> 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<sup>+</sup>, therefore underscoring the similarities between CD49b<sup>+</sup>  
287 and CD25<sup>+</sup> Treg cells. Similar transcriptional expression variations were observed  
288 between CD49b<sup>+</sup> and CD25<sup>+</sup> Treg cells with similar modulations to those described  
289 by Hill *et al.* The common transcriptional pattern between CD49b<sup>+</sup> and CD25<sup>+</sup>  
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<sup>+</sup> (17 down-regulated and 52 up-  
294 regulated) and 20 for CD49b<sup>+</sup> (7 down-regulated and 13 up-regulated) (Fig. 2A and

295 Fig. S2). Interestingly among the transcripts specific for CD49b<sup>+</sup>, we found *Aco77*,  
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<sup>+</sup> and KLRG1<sup>+</sup> Treg cells (26). Altogether, our  
299 results demonstrate that the CD49b<sup>+</sup> transcriptional signature contains prototypical  
300 Treg cell transcripts shared by either CD25<sup>+</sup> or other Treg cell sub-phenotypes.

301

302 **CD49b<sup>+</sup> Treg cells express several canonical markers of CD25<sup>+</sup>FoxP3<sup>+</sup> Treg**  
303 **cells.**

304 To further characterize and compare the phenotypes of the DC-induced  
305 CD49b<sup>+</sup> and CD25<sup>+</sup> 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<sup>+</sup> cells express  
308 markers commonly used to characterize CD25<sup>+</sup>FoxP3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells (Fig.  
313 3A). Moreover, expression of LAG-3, KLRG1 and CD103 molecules was in contrast  
314 significantly higher in CD49b<sup>+</sup> than in CD25<sup>+</sup> cells (Fig. 3A). Within the CD49b<sup>+</sup> cell  
315 population, we noted that LAG-3 expression was mostly restricted to the FoxP3<sup>neg</sup>  
316 cells, whereas KLRG1 and CD103 expressions were found in both FoxP3<sup>+</sup> and  
317 FoxP3<sup>neg</sup> 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<sup>+</sup> Treg  
324 cells were FoxP3<sup>+</sup> compared to less than 4% of the CD49b<sup>+</sup> Treg cells (Fig. 3B).  
325 Interestingly, compared to the CD25<sup>+</sup> T cell population, that of the CD49b<sup>+</sup> 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<sup>+</sup> cell population) with that of FoxP3<sup>+</sup> cells (gated within the CD25<sup>+</sup> cell  
332 population) (Fig. S3). Interestingly, the FoxP3<sup>+</sup> 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<sup>+</sup> Treg cells than in the FoxP3<sup>+</sup>CD25<sup>+</sup> cell sub-population. These  
338 results suggest that, besides IL-10, these three molecules could play an important  
339 role in the CD49b<sup>+</sup> suppressive function (Fig. S3).

340

### 341 **Peripherally induced CD49b<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells. Moreover,  
352 we observed that CD49b<sup>+</sup>Helios<sup>+</sup> cells co-expressed Nrp-1<sup>+</sup> and FoxP3<sup>+</sup> suggesting  
353 that among the CD49b<sup>+</sup> 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<sup>neg</sup>CD49b<sup>+</sup> 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<sup>+</sup> compared to only 5% of FoxP3<sup>neg</sup> 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<sup>+</sup> cells and in  $47 \pm 2\%$  of the CD49b<sup>+</sup> cell population (Fig. 5A). Furthermore,  
373 we observed that only the DC-induced CD49b<sup>+</sup> 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<sup>+</sup> and CD25<sup>+</sup> cells ( $2 \pm 0.2\%$  for both populations) (Fig. 5A, right panel).  
376 This DC-induced increase in the number of CD49b<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 ( $19 \pm 7$  ng/ml), we  
389 measured significantly elevated secretion levels of other type 2 cytokines, including  
390 IL-4 ( $10 \pm 2$  ng/ml), IL-5 ( $18 \pm 2$  ng/ml) and IL-13 ( $31 \pm 0.5$  ng/ml), as well as a  
391 relatively high amount of IFN- $\gamma$  ( $3 \pm 1$  ng/ml) in the supernatant of the CD49b<sup>+</sup> T cell  
392 population. These results revealed an obvious type 2 dominant cytokine profile for  
393 the CD49b<sup>+</sup> 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<sup>+</sup> Treg cells have potent *in vitro* and *in vivo***  
397 **suppressive capacities**

398 We compared the *in vitro* potential of CD49b<sup>+</sup> and CD25<sup>+</sup> Treg cells to  
399 functionally suppress the proliferation of CD4<sup>+</sup> 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<sup>+</sup> or  
402 CD25<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> Treg cells, we  
408 compared their protective effect with that of CD25<sup>+</sup> Treg cells isolated from the same  
409 DBA/1 mice and with CD49b<sup>+</sup> Treg cells isolated from Col II-specific T cell transgenic  
410 mice (TBC). We repeatedly injected syngeneic mice intraperitoneally with  $0.5 \times 10^6$   
411 DCs the week before their euthanasia. CD4<sup>+</sup> 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<sup>+</sup> or CD25<sup>+</sup>, isolated  
417 from the same DBA/1 mouse, or with the antigen-specific CD49b<sup>+</sup> 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<sup>+</sup> cell population. As shown in figure 6C, injection of polyclonal  
423 CD49b<sup>+</sup> Treg cells markedly and significantly decreased the disease severity  
424 compared with PBS-treated or CD4<sup>+</sup>-treated mice. We observed a tendency towards  
425 decreased disease severity after injection of the CD25<sup>+</sup> Treg cells or of the antigen-  
426 specific CD49b<sup>+</sup> 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<sup>+</sup> Treg cells may represent the best therapeutic strategy, over the use of  
429 natural CD25<sup>+</sup> Treg cells.

430 Finally, to further investigate the *in vivo* suppressive mechanism, we  
431 performed similar adoptive transfer experiments with CD49b<sup>+</sup> Treg cells isolated from  
432 IL-10 KO mice. In this experimental setting, CD49b<sup>+</sup> 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<sup>+</sup> isolated from wild-type  
435 littermates significantly protected mice from arthritis whereas IL-10 deficient CD49b<sup>+</sup>  
436 Treg cells were less protective. These results underscore the partial involvement of  
437 IL-10 secretion in the CD49b<sup>+</sup> 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- $\alpha$  blocking  
463 antibodies have been described as an effective way to stimulate the induction of  
464 peripheral FoxP3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells in our study. A similar lack of  
477 complete overlapping has previously been observed when comparing the  
478 transcriptional profile of converted FoxP3<sup>+</sup> Treg cells with the canonical Treg cell  
479 signature (26). Interestingly, the induced CD49b<sup>+</sup> Treg cells shared a transcriptional  
480 profile common to CD25<sup>+</sup> Treg cells and the canonical Treg cell signature. We  
481 showed that 75% of the differentially expressed transcripts found in CD49b<sup>+</sup> T cells  
482 were common with those found in CD25<sup>+</sup> T cells, underscoring the similarities  
483 between CD49b<sup>+</sup> and CD25<sup>+</sup> 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*,  $\alpha$ E $\beta$ 7 (*CD103*) and *Klrg1* (a member of the  
489 killer cell lectin-like receptor family). The CD49b<sup>+</sup> 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<sup>+</sup> Treg cells  
492 with several other Treg sub-phenotypes.

493 We validated by FACS analyses the common expression of several markers  
494 between CD25<sup>+</sup> and CD49b<sup>+</sup> cells that were differentially expressed compared with  
495 CD4<sup>+</sup>. Among these markers, CD49b and KLRG1, both considered as NK cell  
496 markers and minimally expressed on conventional CD4<sup>+</sup> T cells, were previously  
497 observed in an extrathymically derived subset of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells (61).  
498 Within the sub-population of CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells in the spleen, KLRG1<sup>+</sup> Treg  
499 cells were previously shown to display a more activated phenotype  
500 (CD69<sup>+</sup>CD62L<sup>low</sup>CD103<sup>+</sup>CD44<sup>high</sup>) than KLRG1<sup>neg</sup> Treg cells. Furthermore, cell-  
501 surface staining of homeostatically converted FoxP3<sup>+</sup> cells revealed them as  
502 uniformly CD103<sup>+</sup>, an excellent marker for identifying *in vivo*-activated FoxP3<sup>+</sup>CD4<sup>+</sup>  
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<sup>+</sup> and CD25<sup>+</sup> cells and, as  
505 previously observed for the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> 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<sup>neg</sup>Nrp-1<sup>+</sup> in secondary lymphoid organs  
508 and inflamed tissues, which could imply that the expression of Nrp-1 is associated  
509 with the CD49b<sup>+</sup> 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<sup>+</sup> T cells, including CD25<sup>+</sup>FoxP3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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- $\gamma$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> Treg cells were positive for LAG-3 before *in vitro* activation and  
531 interestingly CD49b<sup>+</sup>LAG-3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> (P. Louis-Pence, unpublished data).  
537 Altogether our results suggest that the CD49b<sup>+</sup> Treg cells constitute a Treg sub-  
538 phenotype that shares similarities with the CD25<sup>+</sup> 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<sup>+</sup> and CD49b<sup>+</sup>  
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<sup>+</sup>, polyclonal CD49b<sup>+</sup> or Col II-  
545 specific CD49b<sup>+</sup> 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<sup>+</sup> Treg cells. Although not significant, we also  
548 observed decreased clinical signs following injection of CD25<sup>+</sup> or Col II-specific  
549 CD49b<sup>+</sup> Treg cells. Our results suggest that following their activation by self-Ag(s),  
550 the CD49b<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>FoxP3<sup>+</sup> Treg cells and thus might also play an important role in  
558 the suppressive function of CD49b<sup>+</sup> 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<sup>+</sup>  
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<sup>+</sup> 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

## 585 **References**

- 586 1. Klein, L., and K. Jovanovic. Regulatory T cell lineage commitment in the thymus.  
587 *Semin Immunol* 23:401-409.
- 588 2. Wing, K., and S. Sakaguchi. Regulatory T cells exert checks and balances on self  
589 tolerance and autoimmunity. *Nat Immunol* 11:7-13.
- 590 3. Ziegler, S. F. 2006. FOXP3: of mice and men. *Annu Rev Immunol* 24:209-226.

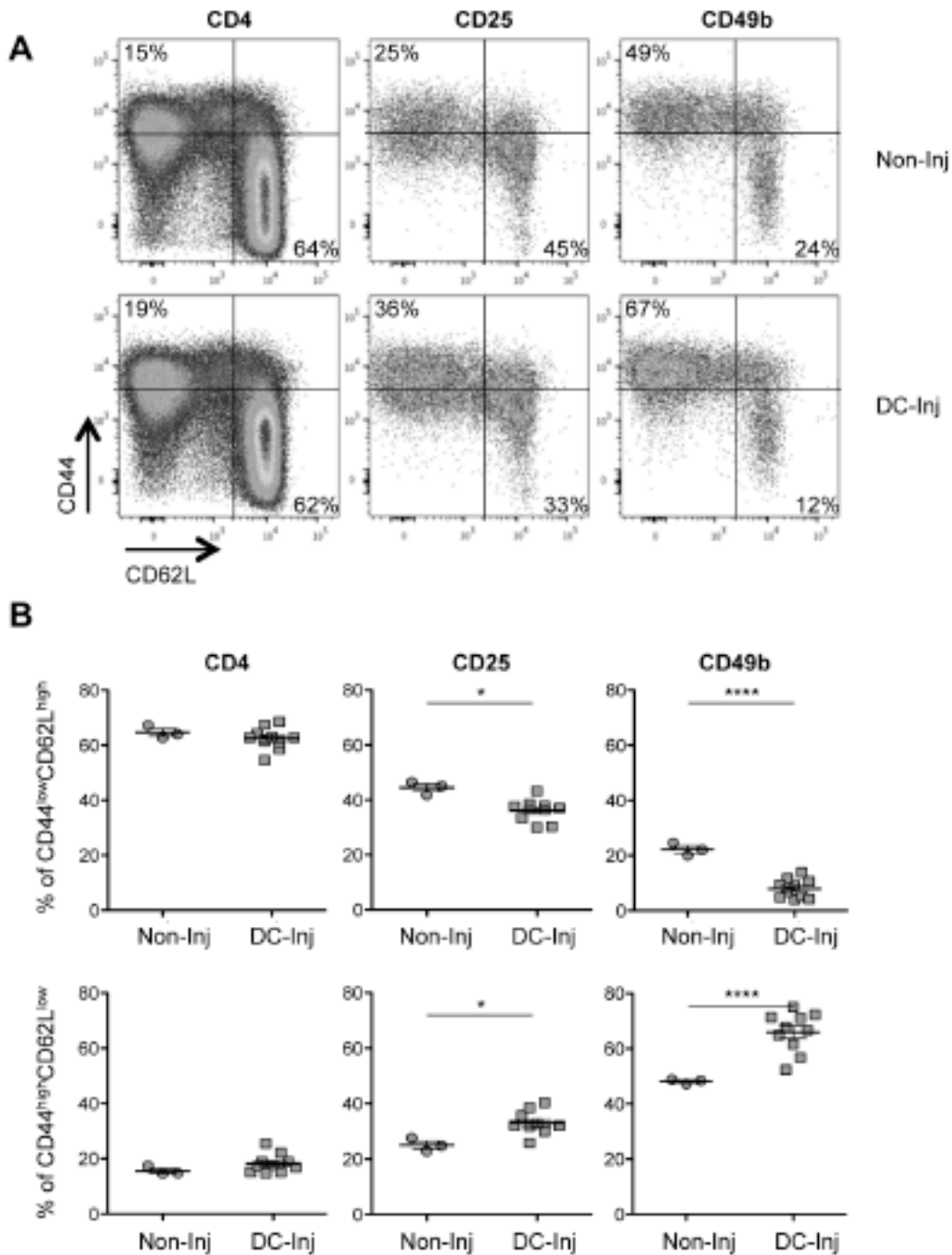
- 591 4. Chen, Q., Y. C. Kim, A. Laurence, G. A. Punkosdy, and E. M. Shevach. 2003. IL-2  
592 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. *J*  
593 *Immunol* 186:6329-6337.
- 594 5. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+  
595 regulatory T cells: more of the same or a division of labor? *Immunity* 30:626-635.
- 596 6. Zheng, S. G., J. Wang, P. Wang, J. D. Gray, and D. A. Horwitz. 2007. IL-2 is essential for  
597 TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for  
598 expansion of these cells. *J Immunol* 178:2018-2027.
- 599 7. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune  
600 system control. *Nat Med* 10:801-805.
- 601 8. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting  
602 regulatory T cells in allergy and asthma. *Nat Rev Immunol* 5:271-283.
- 603 9. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K.  
604 Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and  
605 humans. *Immunol Rev* 212:28-50.
- 606 10. Verginis, P., K. A. McLaughlin, K. W. Wucherpfennig, H. von Boehmer, and I.  
607 Apostolou. 2008. Induction of antigen-specific regulatory T cells in wild-type mice:  
608 visualization and targets of suppression. *Proc Natl Acad Sci U S A* 105:3479-3484.
- 609 11. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von  
610 Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign  
611 antigen. *Nat Immunol* 6:1219-1227.
- 612 12. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor  
613 commitment in naive T cells. *J Exp Med* 199:1401-1408.
- 614 13. Mucida, D., N. Kutchukhidze, A. Erazo, M. Russo, J. J. Lafaille, and M. A. Curotto de  
615 Lafaille. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest*  
616 115:1923-1933.
- 617 14. Curotto de Lafaille, M. A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J. J. Lafaille.  
618 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of  
619 allergic inflammation. *Immunity* 29:114-126.
- 620 15. Knoechel, B., J. Lohr, E. Kahn, J. A. Bluestone, and A. K. Abbas. 2005. Sequential  
621 development of interleukin 2-dependent effector and regulatory T cells in response  
622 to endogenous systemic antigen. *J Exp Med* 202:1375-1386.
- 623 16. Haribhai, D., W. Lin, B. Edwards, J. Ziegelbauer, N. H. Salzman, M. R. Carlson, S. H. Li,  
624 P. M. Simpson, T. A. Chatila, and C. B. Williams. 2009. A central role for induced  
625 regulatory T cells in tolerance induction in experimental colitis. *J Immunol* 182:3461-  
626 3468.
- 627 17. Curotto de Lafaille, M. A., A. C. Lino, N. Kutchukhidze, and J. J. Lafaille. 2004. CD25- T  
628 cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J Immunol*  
629 173:7259-7268.
- 630 18. Benson, M. J., K. Pino-Lagos, M. Roseblatt, and R. J. Noelle. 2007. All-trans retinoic  
631 acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face  
632 of high levels of co-stimulation. *J Exp Med* 204:1765-1774.
- 633 19. Edwards, A. J., and S. L. Pender. Histone deacetylase inhibitors and their potential  
634 role in inflammatory bowel diseases. *Biochem Soc Trans* 39:1092-1095.
- 635 20. Amodio, G., A. Mugione, A. M. Sanchez, P. Vigano, M. Candiani, E. Somigliana, M. G.  
636 Roncarolo, P. Panina-Bordignon, and S. Gregori. HLA-G expressing DC-10 and CD4(+) T  
637 cells accumulate in human decidua during pregnancy. *Hum Immunol* 74:406-411.

- 638 21. Gross, C. C., and H. Wiendl. Dendritic cell vaccination in autoimmune disease. *Curr Opin Rheumatol* 25:268-274.
- 639
- 640 22. Hilkens, C. M., J. D. Isaacs, and A. W. Thomson. 2010. Development of dendritic cell-based immunotherapy for autoimmunity. *Int Rev Immunol* 29:156-183.
- 641
- 642 23. Mahnke, K., Y. Qian, J. Knop, and A. H. Enk. 2003. Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 101:4862-4869.
- 643
- 644
- 645 24. Kretschmer, K., T. S. Heng, and H. von Boehmer. 2006. De novo production of antigen-specific suppressor cells in vivo. *Nat Protoc* 1:653-661.
- 646
- 647 25. Idoyaga, J., C. Fiorese, L. Zbytnuik, A. Lubkin, J. Miller, B. Malissen, D. Mucida, M. Merad, and R. M. Steinman. Specialized role of migratory dendritic cells in peripheral tolerance induction. *J Clin Invest* 123:844-854.
- 648
- 649
- 650 26. Feuerer, M., J. A. Hill, K. Kretschmer, H. von Boehmer, D. Mathis, and C. Benoist. 2010. Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A* 107:5919-5924.
- 651
- 652
- 653 27. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A. H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192:1213-1222.
- 654
- 655
- 656
- 657 28. Menges, M., S. Rossner, C. Voigtlander, H. Schindler, N. A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M. B. Lutz. 2002. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J Exp Med* 195:15-21.
- 658
- 659
- 660
- 661 29. Petzold, C., J. Riewaldt, T. Koenig, S. Schallenberg, and K. Kretschmer. Dendritic cell-targeted pancreatic beta-cell antigen leads to conversion of self-reactive CD4(+) T cells into regulatory T cells and promotes immunotolerance in NOD mice. *Rev Diabet Stud* 7:47-61.
- 662
- 663
- 664
- 665 30. Charbonnier, L. M., L. M. van Duivenvoorde, F. Apparailly, C. Cantos, W. G. Han, D. Noel, C. Duperray, T. W. Huizinga, R. E. Toes, C. Jorgensen, and P. Louis-Plence. 2006. Immature dendritic cells suppress collagen-induced arthritis by in vivo expansion of CD49b+ regulatory T cells. *J Immunol* 177:3806-3813.
- 666
- 667
- 668
- 669 31. van Duivenvoorde, L. M., P. Louis-Plence, F. Apparailly, E. I. van der Voort, T. W. Huizinga, C. Jorgensen, and R. E. Toes. 2004. Antigen-specific immunomodulation of collagen-induced arthritis with tumor necrosis factor-stimulated dendritic cells. *Arthritis Rheum* 50:3354-3364.
- 670
- 671
- 672
- 673 32. Charbonnier, L. M., W. G. Han, J. Quentin, T. W. Huizinga, J. Zwerina, R. E. Toes, C. Jorgensen, and P. Louis-Plence. 2010. Adoptive transfer of IL-10-secreting CD4+CD49b+ regulatory T cells suppresses ongoing arthritis. *J Autoimmun* 34:390-399.
- 674
- 675
- 676
- 677 33. Han, W. G., E. I. van der Voort, H. el Bannoudi, P. Louis-Plence, T. W. Huizinga, and R. E. Toes. 2010. DX5(+)CD4(+) T cells modulate cytokine production by CD4(+) T cells towards IL-10 via the production of IL-4. *Eur J Immunol* 40:2731-2740.
- 678
- 679
- 680 34. Gonzalez, A., I. Andre-Schmutz, C. Carnaud, D. Mathis, and C. Benoist. 2001. Damage control, rather than unresponsiveness, effected by protective DX5+ T cells in autoimmune diabetes. *Nat Immunol* 2:1117-1125.
- 681
- 682
- 683 35. Gagliani, N., C. F. Magnani, S. Huber, M. E. Gianolini, M. Pala, P. Licona-Limon, B. Guo, D. R. Herbert, A. Bulfone, F. Trentini, C. Di Serio, R. Bacchetta, M. Andreani, L.
- 684

- 685 Brockmann, S. Gregori, R. A. Flavell, and M. G. Roncarolo. 2013. Coexpression of  
686 CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med*  
687 19:739-746.
- 688 36. Ohkura, N., M. Hamaguchi, H. Morikawa, K. Sugimura, A. Tanaka, Y. Ito, M. Osaki, Y.  
689 Tanaka, R. Yamashita, N. Nakano, J. Huehn, H. J. Fehling, T. Sparwasser, K. Nakai, and  
690 S. Sakaguchi. T cell receptor stimulation-induced epigenetic changes and Foxp3  
691 expression are independent and complementary events required for Treg cell  
692 development. *Immunity* 37:785-799.
- 693 37. Hill, J. A., M. Feuerer, K. Tash, S. Haxhinasto, J. Perez, R. Melamed, D. Mathis, and C.  
694 Benoist. 2007. Foxp3 transcription-factor-dependent and -independent regulation of  
695 the regulatory T cell transcriptional signature. *Immunity* 27:786-800.
- 696 38. Chuchana, P., D. Marchand, M. Nugoli, C. Rodriguez, N. Molinari, and J. A. Garcia-  
697 Sanz. 2007. An adaptation of the LMS method to determine expression variations in  
698 profiling data. *Nucleic Acids Res* 35:e71.
- 699 39. Chuchana, P., P. Holzmuller, F. Vezilier, D. Berthier, I. Chantal, D. Severac, J. L.  
700 Lemesre, G. Cuny, P. Nirde, and B. Bucheton. 2010. Intertwining threshold settings,  
701 biological data and database knowledge to optimize the selection of differentially  
702 expressed genes from microarray. *PLoS One* 5:e13518.
- 703 40. Asnagli, H., D. Martire, N. Belmonte, J. Quentin, H. Bastian, M. Boucard-Jourdin, P. B.  
704 Fall, A. L. Mausset-Bonnefont, A. Mantello-Moreau, S. Rouquier, I. Marchetti, C.  
705 Jorgensen, A. Foussat, and P. Louis-Plence. 2014. Type 1 regulatory T cells specific for  
706 collagen-type II as an efficient cell-based therapy in arthritis. *Arthritis Res Ther*  
707 16:R115.
- 708 41. Francisco, L. M., V. H. Salinas, K. E. Brown, V. K. Vanguri, G. J. Freeman, V. K. Kuchroo,  
709 and A. H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function  
710 of induced regulatory T cells. *J Exp Med* 206:3015-3029.
- 711 42. Bruder, D., M. Probst-Kepper, A. M. Westendorf, R. Geffers, S. Beissert, K. Loser, H.  
712 von Boehmer, J. Buer, and W. Hansen. 2004. Neuropilin-1: a surface marker of  
713 regulatory T cells. *Eur J Immunol* 34:623-630.
- 714 43. Yadav, M., C. Louvet, D. Davini, J. M. Gardner, M. Martinez-Llordella, S. Bailey-  
715 Bucktrout, B. A. Anthony, F. M. Sverdrup, R. Head, D. J. Kuster, P. Ruminiski, D. Weiss,  
716 D. Von Schack, and J. A. Bluestone. 2012. Neuropilin-1 distinguishes natural and  
717 inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med*  
718 209:1713-1722, S1711-1719.
- 719 44. Weiss, J. M., A. M. Bilate, M. Gobert, Y. Ding, M. A. Curotto de Lafaille, C. N.  
720 Parkhurst, H. Xiong, J. Dolpady, A. B. Frey, M. G. Ruocco, Y. Yang, S. Floess, J. Huehn,  
721 S. Oh, M. O. Li, R. E. Niec, A. Y. Rudensky, M. L. Dustin, D. R. Littman, and J. J. Lafaille.  
722 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not  
723 mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 209:1723-1742, S1721.
- 724 45. Thornton, A. M., P. E. Korty, D. Q. Tran, E. A. Wohlfert, P. E. Murray, Y. Belkaid, and E.  
725 M. Shevach. Expression of Helios, an Ikaros transcription factor family member,  
726 differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J*  
727 *Immunol* 184:3433-3441.
- 728 46. Singh, K., M. Hjort, L. Thorvaldson, and S. Sandler. Concomitant analysis of Helios and  
729 Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naive mice. *Sci*  
730 *Rep* 5:7767.

- 731 47. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T. T. Chu, L. Corcoran, P.  
732 Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program  
733 co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458:351-356.
- 734 48. Koch, M. A., G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, and D. J.  
735 Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis  
736 and function during type 1 inflammation. *Nat Immunol* 10:595-602.
- 737 49. Chaudhry, A., D. Rudra, P. Treuting, R. M. Samstein, Y. Liang, A. Kas, and A. Y.  
738 Rudensky. 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent  
739 manner. *Science* 326:986-991.
- 740 50. Campbell, D. J., and M. A. Koch. Phenotypical and functional specialization of FOXP3+  
741 regulatory T cells. *Nat Rev Immunol* 11:119-130.
- 742 51. Wohlfert, E. A., J. R. Grainger, N. Bouladoux, J. E. Konkel, G. Oldenhove, C. H. Ribeiro,  
743 J. A. Hall, R. Yagi, S. Naik, R. Bhairavabhotla, W. E. Paul, R. Bosselut, G. Wei, K. Zhao,  
744 M. Oukka, J. Zhu, and Y. Belkaid. GATA3 controls Foxp3(+) regulatory T cell fate  
745 during inflammation in mice. *J Clin Invest* 121:4503-4515.
- 746 52. Pot, C., H. Jin, A. Awasthi, S. M. Liu, C. Y. Lai, R. Madan, A. H. Sharpe, C. L. Karp, S. C.  
747 Miaw, I. C. Ho, and V. K. Kuchroo. 2009. Cutting edge: IL-27 induces the transcription  
748 factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately  
749 act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol*  
750 183:797-801.
- 751 53. Magnani, C. F., G. Alberigo, R. Bacchetta, G. Serafini, M. Andreani, M. G. Roncarolo,  
752 and S. Gregori. 2011. Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a  
753 novel mechanism of suppression by human Tr1 cells. *Eur J Immunol* 41:1652-1662.
- 754 54. Selvaraj, R. K., and T. L. Geiger. 2008. Mitigation of experimental allergic  
755 encephalomyelitis by TGF-beta induced Foxp3+ regulatory T lymphocytes through the  
756 induction of energy and infectious tolerance. *J Immunol* 180:2830-2838.
- 757 55. Weber, S. E., J. Harbertson, E. Godebu, G. A. Mros, R. C. Padrick, B. D. Carson, S. F.  
758 Ziegler, and L. M. Bradley. 2006. Adaptive islet-specific regulatory CD4 T cells control  
759 autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo.  
760 *J Immunol* 176:4730-4739.
- 761 56. Zhang, Q., F. Cui, L. Fang, J. Hong, B. Zheng, and J. Z. Zhang. TNF-alpha impairs  
762 differentiation and function of TGF-beta-induced Treg cells in autoimmune diseases  
763 through Akt and Smad3 signaling pathway. *J Mol Cell Biol* 5:85-98.
- 764 57. Brun, V., V. Neveu, Y. M. Pers, S. Fabre, B. Quatannens, H. Bastian, N. Clerget-  
765 Chossat, C. Jorgensen, and A. Foussat. 2011. Isolation of functional autologous  
766 collagen-II specific IL-10 producing Tr1 cell clones from rheumatoid arthritis blood. *Int*  
767 *Immunopharmacol* 11:1074-1078.
- 768 58. Floess, S., J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polansky, K. Schlawe, H. D. Chang,  
769 T. Bopp, E. Schmitt, S. Klein-Hessling, E. Serfling, A. Hamann, and J. Huehn. 2007.  
770 Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol* 5:e38.
- 771 59. Polansky, J. K., K. Kretschmer, J. Freyer, S. Floess, A. Garbe, U. Baron, S. Olek, A.  
772 Hamann, H. von Boehmer, and J. Huehn. 2008. DNA methylation controls Foxp3 gene  
773 expression. *Eur J Immunol* 38:1654-1663.
- 774 60. Nadkarni, S., C. Mauri, and M. R. Ehrenstein. 2007. Anti-TNF-alpha therapy induces a  
775 distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-  
776 beta. *J Exp Med* 204:33-39.

- 777 61. Stephens, G. L., J. Andersson, and E. M. Shevach. 2007. Distinct subsets of FoxP3+  
778 regulatory T cells participate in the control of immune responses. *J Immunol*  
779 178:6901-6911.
- 780 62. Apetoh, L., F. J. Quintana, C. Pot, N. Joller, S. Xiao, D. Kumar, E. J. Burns, D. H. Sherr,  
781 H. L. Weiner, and V. K. Kuchroo. The aryl hydrocarbon receptor interacts with c-Maf  
782 to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat*  
783 *Immunol* 11:854-861.
- 784 63. Hegazy, A. N., M. Peine, C. Helmstetter, I. Panse, A. Frohlich, A. Bergthaler, L. Flatz, D.  
785 D. Pinschewer, A. Radbruch, and M. Lohning. Interferons direct Th2 cell  
786 reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined  
787 Th2 and Th1 cell functions. *Immunity* 32:116-128.
- 788 64. Amodio, G., M. Comi, D. Tomasoni, M. E. Gianolini, R. Rizzo, J. LeMaout, M. G.  
789 Roncarolo, and S. Gregori. 2015. HLA-G expression levels influence the tolerogenic  
790 activity of human DC-10. *Haematologica*.
- 791 65. Suffia, I., S. K. Reckling, G. Salay, and Y. Belkaid. 2005. A role for CD103 in the  
792 retention of CD4+CD25+ Treg and control of Leishmania major infection. *J Immunol*  
793 174:5444-5455.
- 794 66. Cepek, K. L., S. K. Shaw, C. M. Parker, G. J. Russell, J. S. Morrow, D. L. Rimm, and M. B.  
795 Brenner. 1994. Adhesion between epithelial cells and T lymphocytes mediated by E-  
796 cadherin and the alpha E beta 7 integrin. *Nature* 372:190-193.
- 797 67. Hanazawa, A., K. Hayashizaki, K. Shinoda, H. Yagita, K. Okumura, M. Lohning, T. Hara,  
798 S. Tani-ichi, K. Ikuta, B. Eckes, A. Radbruch, K. Tokoyoda, and T. Nakayama. CD49b-  
799 dependent establishment of T helper cell memory. *Immunol Cell Biol* 91:524-531.  
800  
801



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

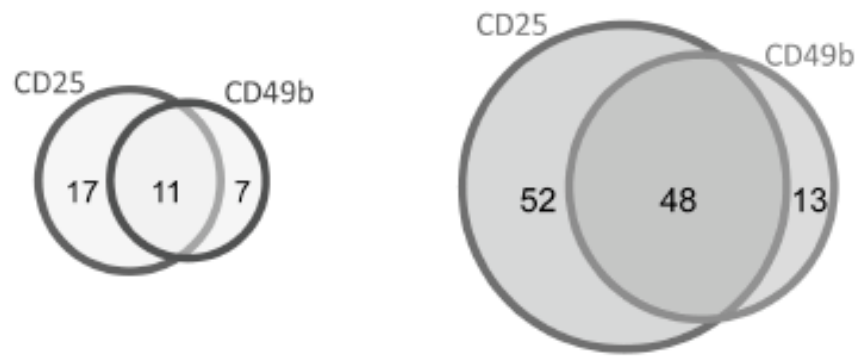
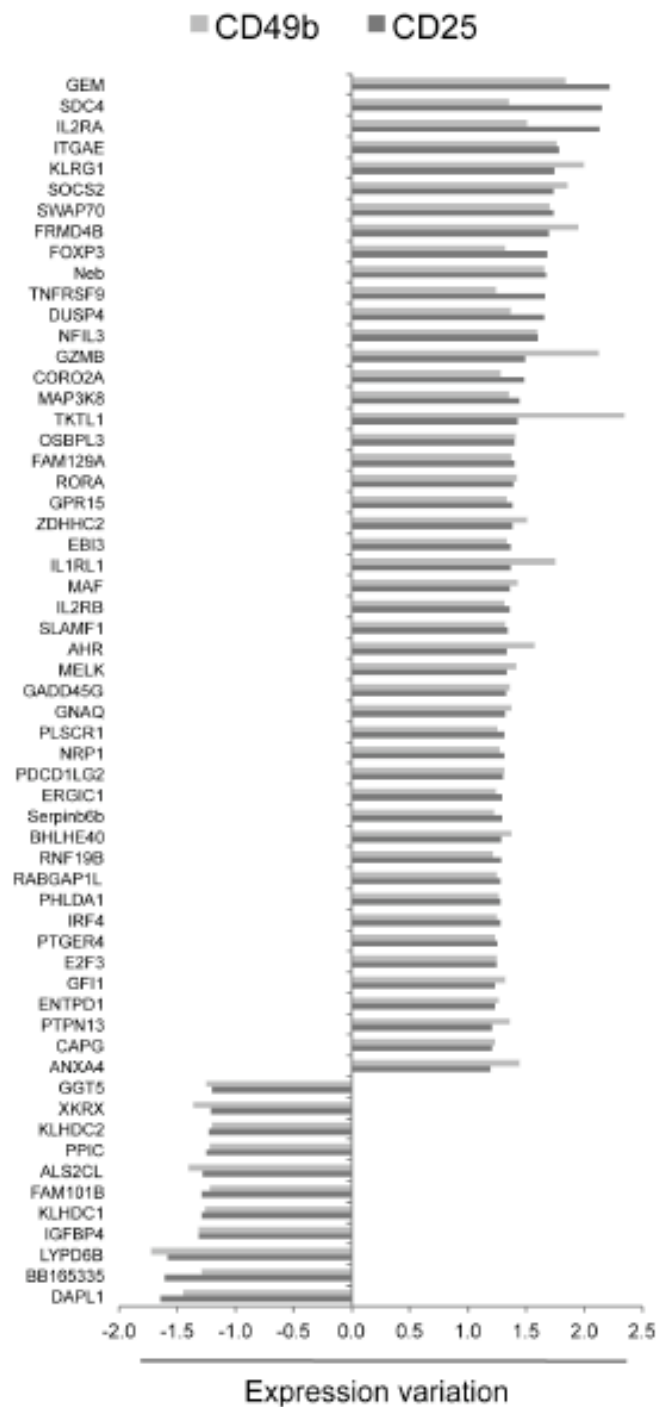
806 Percentage of naïve (CD44<sup>low</sup>CD62L<sup>high</sup>) and effector memory (CD44<sup>high</sup>CD62L<sup>low</sup>) T

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



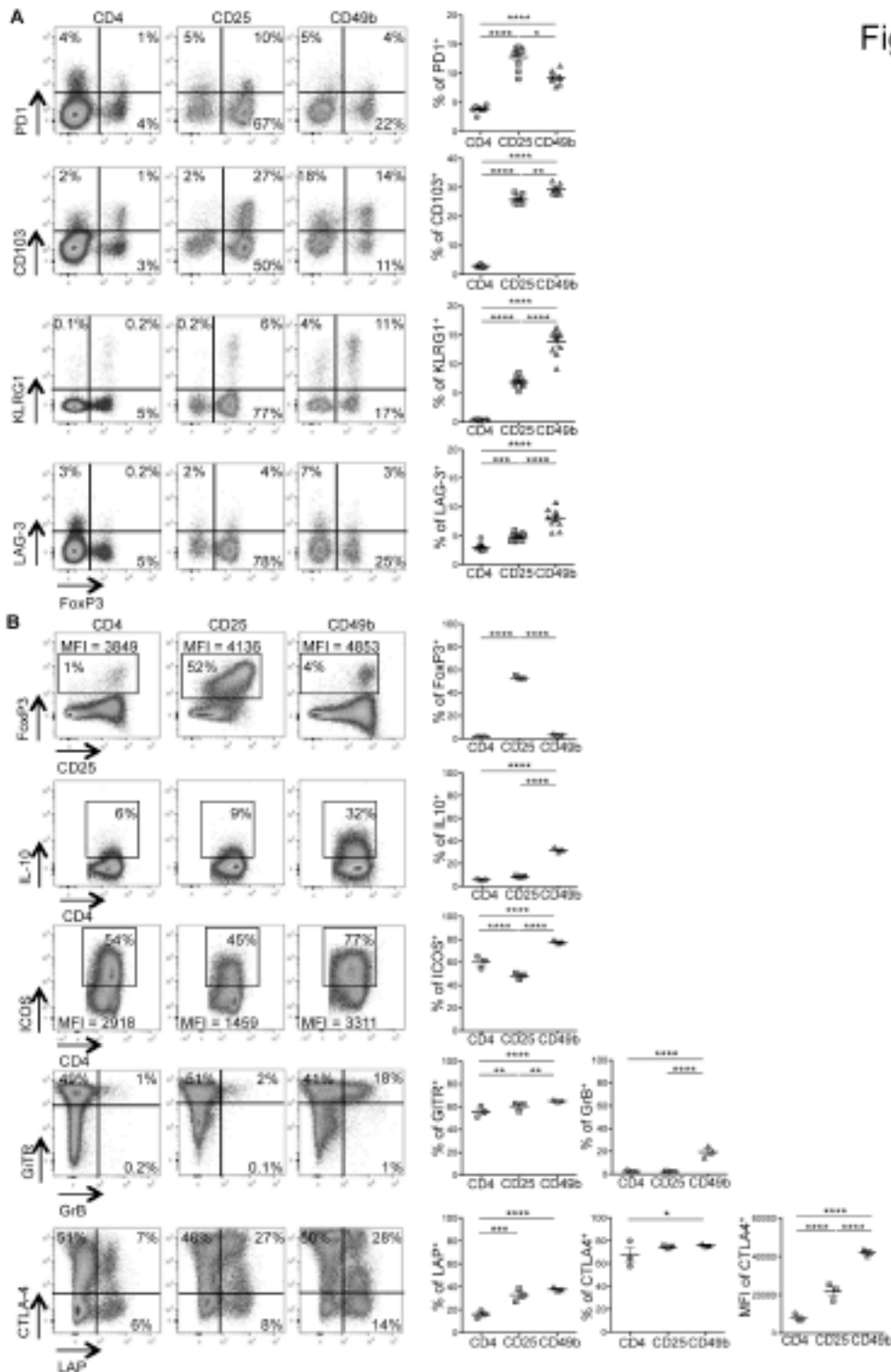
808 Fig S1A) were analyzed by flow cytometry for DC-injected (DC-Inj, n=10) and non-  
809 injected mice (Non-Inj, n=3). A, Representative dot plots within gated CD4 (left),  
810 CD25 (middle) and CD49b (right) positive cells in non-injected (top panels) and DC-  
811 injected mice (bottom panels). 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<sup>+</sup> 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<sup>+</sup> and CD25<sup>+</sup> 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<sup>+</sup>, CD25<sup>+</sup> and the canonical Treg  
824 signature.  
825

Figure 3

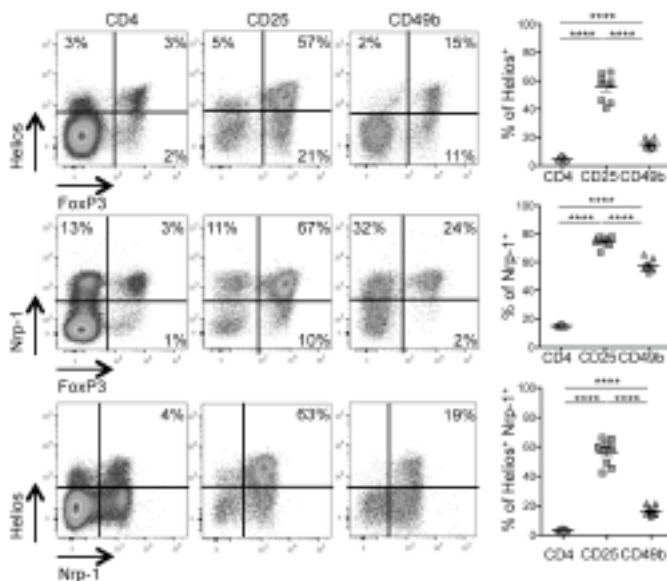


826  
827

**FIGURE 3.** Several canonical markers of CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells are expressed by CD49b<sup>+</sup> 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

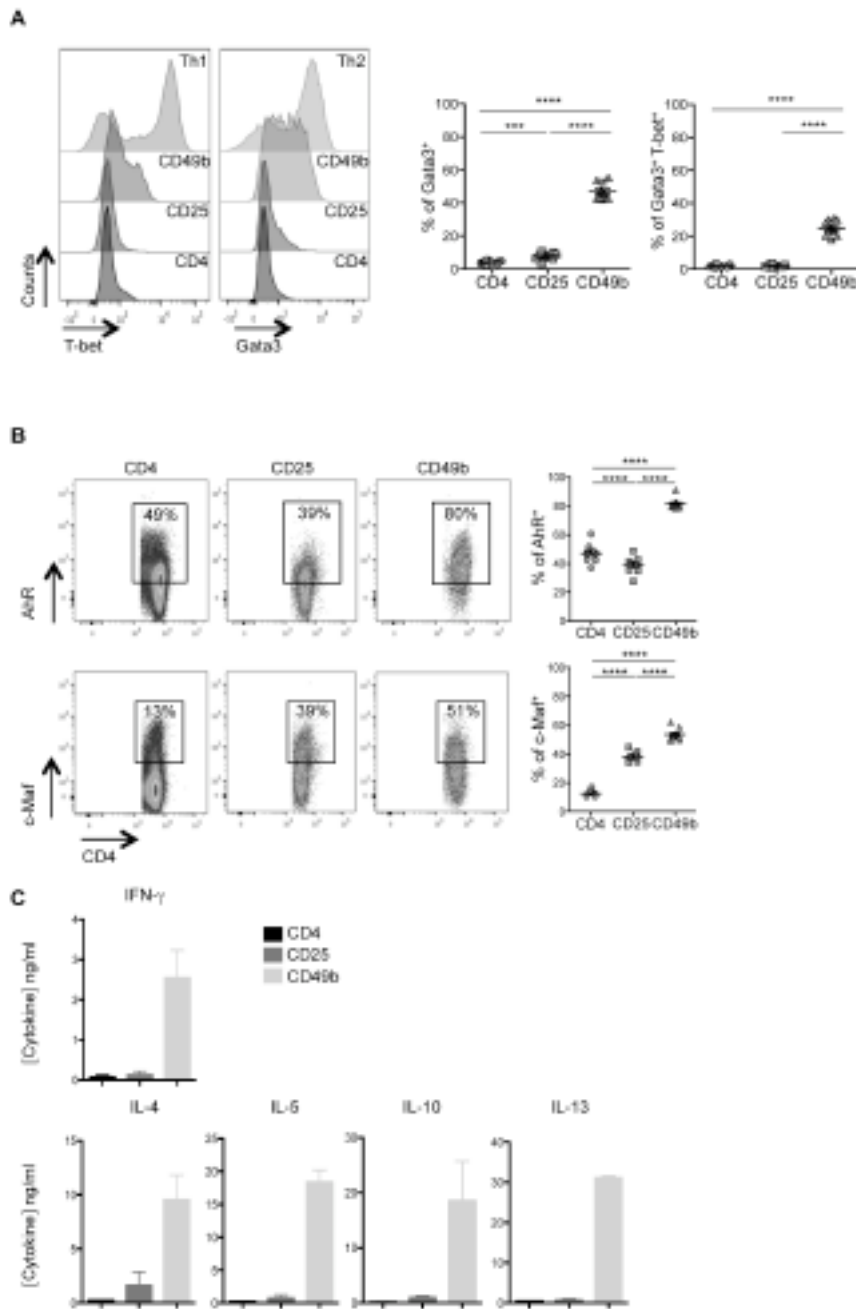
831 of cells are shown within each quadrant. Each symbol represents a pool of 2 mice  
 832 and bars show the mean  $\pm$  SEM. \*\*\*\*  $p < 0.0001$ , \*\*\*  $p = 0.0005$ , \*\*  $p = 0.01$  and \*  
 833  $p = 0.013$  by repeated measures two-way ANOVA (Tukey's multiple comparisons test).  
 834 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).

840



841

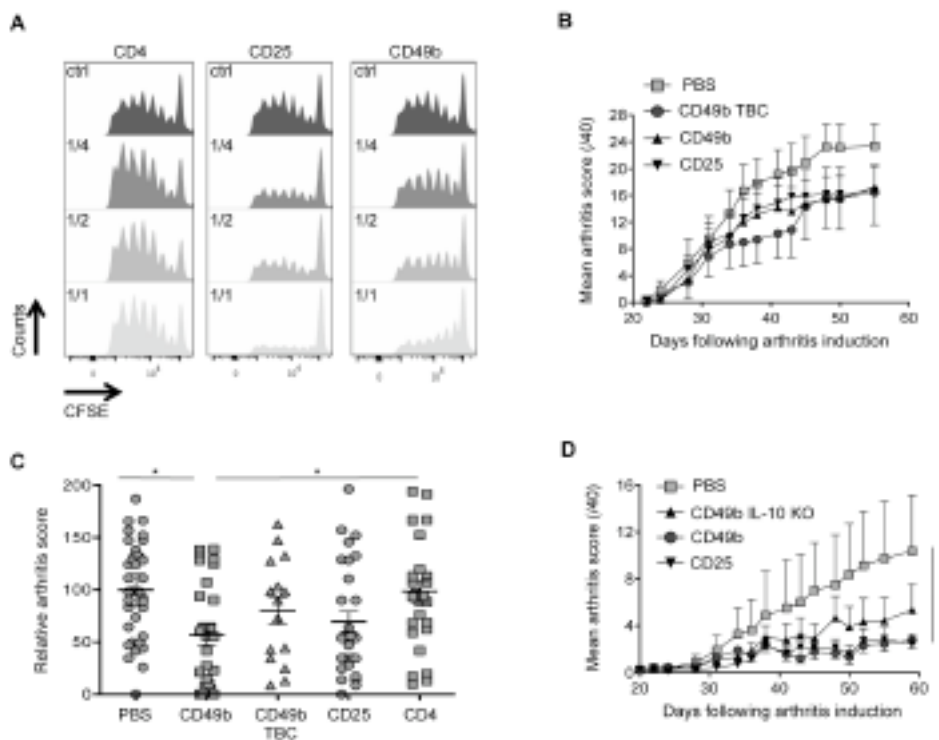
842 **FIGURE 4.** Peripherally induced CD49b<sup>+</sup> cells express Neuropilin-1 without co-  
 843 expressing Helios. Representative flow cytometry analyses of splenocytes from DC-  
 844 injected mice ( $n = 18$ ) within the gated CD4 (left), CD25 (middle) and CD49b (right)  
 845 cell population. 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<sup>+</sup> 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<sup>+</sup> and double  
 857 positive Gata3<sup>+</sup>T-bet<sup>+</sup> 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<sup>+</sup> and c-Maf<sup>+</sup> 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<sup>+</sup> T reg cells display potent *in vitro* and  
 869 *in vivo* suppressive capacities. A, Comparable suppressive capacities of FACS-  
 870 sorted CD25<sup>+</sup> and CD49b<sup>+</sup> Treg cells *in vitro*. CFSE-labeled effector T (Teff) cells

871 were cultured in activating conditions with titrated numbers of CD4<sup>+</sup>, CD25<sup>+</sup> or  
872 CD49b<sup>+</sup> 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<sup>+</sup> (CD49b) and CD25<sup>+</sup> (CD25) Treg cells isolated from  
880 syngeneic DBA/1 mice as well as Ag-specific CD49b<sup>+</sup> 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<sup>+</sup> (CD49b, 4 independent experiments), Ag-specific  
886 CD49b<sup>+</sup> (CD49bTBC, 2 independent experiments), and CD25<sup>+</sup> (CD25, 4 independent  
887 experiments) Treg cells as well as CD4<sup>+</sup> 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<sup>+</sup> Treg cells is partially  
890 dependent on IL-10. Therapeutic potential of CD49b<sup>+</sup> Treg cells FACS-sorted from  
891 IL-10 KO mice (CD49b IL-10 KO) or from wild-type littermates (CD49b), and CD25<sup>+</sup>  
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