Nonclassical CD4\(^+\)CD49b\(^+\) Regulatory T Cells as a Better Alternative to Conventional CD4\(^+\)CD25\(^+\) T Cells To Dampen Arthritis Severity

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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,*†‡† and Pascale Louis-Plence*†‡†

Promising immunotherapeutic strategies are emerging to restore tolerance in autoimmune diseases by triggering an increase in the number and/or the function of endogenous regulatory T (Treg) cells, which actively control pathological immune responses. Evidence suggests a remarkable heterogeneity in peripheral Treg cells that warrants their better characterization in terms of phenotype and suppressive function, to determine which subset may be optimally suitable for a given clinical situation. We found that repetitive injections of immature dendritic cells expanded Foxp3-negative CD49b⁺ Treg cells that displayed an effector memory phenotype. These expanded Treg cells were isolated ex vivo for transcriptome analysis and found to contain multiple transcripts of the canonical Treg signature shared mainly by CD25⁺ but also by other subphenotypes. We characterized the CD49b⁺ Treg cell phenotype, underscoring its similarities with the CD25⁺ Treg cell phenotype and highlighting some differential expression patterns for several markers, including lymphocyte activation gene 3, KLRG1, CD103, ICOS, CTLA-4, and granzyme B. Comparison of the canonical Treg signature shared mainly by CD25⁺ but also by other subphenotypes. We characterized the CD49b⁺ Treg cell markers and suppressive function could be Foxp3 independent, and underscore the therapeutic potential of IL-10–secreting CD49b⁺ Treg cells in arthritis. The Journal of Immunology, 2016, 196: 298–309.

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(32, 33). Similarly, Benoit and colleagues (34) demonstrated that CD4+CD49b+ Treg cells, present in naive mice, were more efficient in suppressing the onset of diabetes than CD4+CD25+ Treg cells. As with the cell population we described, these cells’ effect was IL-4 and IL-10 dependent. Recently, Gagliani et al. (35) showed that CD49b+ T cells isolated from naive mice were more efficiently suppressive against IL-10- or IL-4-producing Treg cells.

Altogether, these data reveal a remarkable heterogeneity in Treg cell populations and define the CD49b molecule as a relevant marker for specific Treg cell subsets. Interestingly, recent studies challenged the notion that Foxp3 expression is uniquely responsible for all aspects of the transcriptional signature of CD4+CD25+ Treg cells and showed that Foxp3-independent epigenetic changes are required for Treg cell function (36, 37). These results underscore the need to better characterize the nonclassical CD49b+-induced Treg cells, which are mainly Foxp3 negative. We therefore investigated their suppressive mechanism in vivo and compared it with that of CD25+ Treg cells to determine their respective therapeutic capacities.

Materials and Methods

Mice

DBA/1 mice were obtained from Harlan Laboratories and were bred in our own animal facility. Transgenic mice carrying the rearranged Vβ11.1 and Vβ8.2 TCR gene family were isolated from a collagen type II (Col II)–specific T cell hybridoma and were provided by R. Toes (Leiden University Medical Center, Leiden, the Netherlands) with the approval of W. Ladiges. C57BL/6 T cell hybridoma were provided by R. Toes (Leiden University Medical Center, Leiden, the Netherlands) with the approval of W. Ladiges. C57BL/6 KO and wild-type littermates with the same genetic background. Experiments were performed in accordance with national guidelines and approved by the Ethics Committee for Animal Research of Languedoc-Roussillon (CEEA-LR-1067) and French Health Authorities (C34-172-36).

DC generation and injections

DCs were generated as previously described (30). Briefly, bone marrow cells were harvested from the femur and tibiae of mice and washed in RPMI 1640 following RBC lysis. T and B cells were depleted using mouse pan T and pan B Dynabeads (Dynal), and monocytes were removed by 4-h plate adhesion. The remaining cells were cultured in complete medium (RPMI 1640 supplemented with 5% FCS, 2 mmL-glutamine, 5 x 10^-5 M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, essential aminos acids, and 1 μm sodium pyruvate) with 1000 IU/ml murine rIL-4 (R&D Systems) and 1000 IU/ml murine rIL-12 (R&D Systems) at 5 x 10^5 cells/ml in 24-well plates. Culture medium was renewed at days 2 and 4. For in vivo experiments, DCs were harvested at day 7. Syngeneic DBA/1, IL-10 KO, or wild-type littermates were injected i.p. with 0.5 x 10^6 DCs in 100 μl PBS, 7, 5, and 3 d before euthanasia for splenic T cell purification.

Abs and FACS analysis

Spleens were harvested, and single-cell suspensions were obtained by gentle passage through 70-μm nylon mesh filters (BD Biosciences). Following RBC lysis using ACK buffer, suspensions were preblocked using purified anti-CD16/32 Ab (2.4G2) for 10 min. For intracellular cytokine staining, cells were stimulated during 48 h at 37°C with anti-CD3/anti-CD28 Ab-coated Dynabeads (Dynal Biotech ASA, Oslo, Norway). During the last 4 h of stimulation, 50 ng/ml PMA, 1 μg/ml ionomycin, and 10 μg/ml brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added. Subsequently, cells were stained with surface Abs (20 min, on ice). Cells were fixed using the eBioscience permeabilization kit according to the manufacturer’s procedure and subsequently stained for intracellular markers. Data acquisition was performed on a Canto II or LSRII Flow cytometer (BD Biosciences, Mountain View, CA), and analyses were performed using FlowJo software.

Treg cell isolation and adoptive cell transfer experiments

Spleenocytes from DC-vaccinated mice were recovered by filtration on cell nylon mesh filters (BD Biosciences). Following RBC lysis using ACK buffer, suspensions were preblocked using purified anti-CD16/32 Ab (2.4G2) for 10 min. For intracellular cytokine staining, cells were stimulated during 48 h at 37°C with anti-CD3/anti-CD28 Ab-coated Dynabeads (Dynal Biotech ASA, Oslo, Norway). During the last 4 h of stimulation, 50 ng/ml PMA, 1 μg/ml ionomycin, and 10 μg/ml brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added. Subsequently, cells were stained with surface Abs (20 min, on ice). Cells were fixed using the eBioscience permeabilization kit according to the manufacturer’s procedure and subsequently stained for intracellular markers. Data acquisition was performed on a Canto II or LSRII Flow cytometer (BD Biosciences, Mountain View, CA), and analyses were performed using FlowJo software.

In vitro suppressive experiments

CFSE-labeled CD4+ T cells (10^5) were cultured with titrated numbers of either FACS-sorted Treg cells or unlabeled T effector cells in the presence of irradiated allogeneic splenocytes (5 x 10^5) and 2-5 μg/ml anti-CD3e mAb (145-2C11). After 4 d of culture, proliferation of effector cells was assessed by FACS. Data were analyzed using FlowJo software.

CIA induction and evaluation

Male 9- to 12-wk-old mice were immunized at the base of the tail with 100 μg bovine or chicken Col II (BD Biosciences) emulsified in CFA (Pierce; complemented to 4 mg/ml with Mycobacterium tuberculosis H37RA) for DBA/1 or C57BL/6, respectively. To boost immunization, C57BL/6 mice received an i.v. injection of one million chicken Col II (2 μg/ml)-loaded mature DCs on day 0. On day 21, DBA/1 or C57BL/6 mice received a booster immunization at the base of the tail with 100 μg bovine Col II emulsified in IFA or chicken Col II emulsified in CFA, respectively. Mice were i.v. injected with the FACS-sorted Treg cells on day 28. From day 21, the thickness of each hind paw was measured three times per week with a caliper, and the severity of arthritis was graded according to the clinical scale previously described (40) with some modifications. Ankylosis was graded (score 5), and number of inflamed digits was also added to obtain a maximal score of 10 per paw and 40 per mouse. Clinical scores are represented as means ± SEM on a given day.

Cytokine secretion profile

Supernatants of FACS-sorted T cells (100,000 cells/well) were harvested 48 h following in vitro stimulation and stored at -20°C until tested for the presence of IL-12, IL-4, IL-5, and IL-13. All these cytokines were quantified by ELISA kits according to the manufacturer recommendations (R&D Systems).

Th1 or Th2 polarization of T cells

For in vitro differentiation, naïve OVA-specific CD4+ T cells from DO11.10 transgenic mice were cultured during 3 d in Th1 (rIL-12, 10 ng/ml + anti-IL-4 Ab, 5 μg/ml) or Th2 (rIL-4, 10 ng/ml + anti–IFN-γ Ab, 1.25 μg/ml) polarizing conditions with irradiated splenocytes in the presence of OVA peptide (1 μg/ml) provided by A. Chavanieu (Institut des Biomolecules Max Mousseron, Montpellier, France).

Statistics

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

**Results**

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

As we previously published (30, 32) and as clearly shown in Supplemental Fig. 1, repetitive injections of immature DCs significantly induced CD4+CD49b+ cells (from 5 ± 0.2% to 9 ± 0.4%, p < 0.0001) without modifying CD4+CD25+ cell frequencies (12 ± 0.2% to 12 ± 0.1%, NS; Supplemental Fig. 1B). In naive mice, the CD4+CD49b+ cells are a heterogeneous population containing Foxp3+ cells (57 ± 2%) and activated CD25+Foxp3neg cells (7 ± 0.4%). After repeated DC injection, the expanded CD4+CD49b+ population showed a significant decrease in the percentage of Foxp3-expressing cells (24 ± 1%, p < 0.0001; Supplemental Fig. 1B) and a significant increase in the percentage of CD25negFoxp3neg cells (69 ± 0.9%, p < 0.0001). These results demonstrate that the induced CD49b+ cells were mostly CD25neg and Foxp3neg. Although the frequency of CD4+CD25+ cells did not significantly change after repeated DC injection, we observed a slight increase in the frequency of these cells expressing Foxp3 (67 ± 1% to 76 ± 2%, p < 0.0001). As shown in Supplemental Fig. 1A, the analyzed populations were gated as CD4+CD49b+CD25neg, CD4+CD25+, and CD4+CD49b+Foxp3neg cells and hereafter referred to as gated CD49b, CD49b, and CD49b cells, respectively. The same gating strategy was used to sort the three populations.

To better characterize the DC-induced CD49b+ cells, we compared their cell surface phenotype with those of CD25+ and CD4+ cells. We first compared the frequency of naïve T cells (defined as CD44lowCD62Lhigh and effector memory T cells (CD44high CD62Llow) within the gated CD4, CD25, and CD49b cell populations in noninjected and DC-injected mice (Fig. 1A, lower right and upper left quadrant, respectively). The percentage of naïve T cells (Fig. 1B, top panels) was considerably lower both in the CD25+ (44 ± 1%) and CD49b+ (26 ± 1%) cell populations, than in the CD4+ cell population (65 ± 1%) of noninjected mice. Concomitantly, the percentages of effector memory T cells (Fig. 1B, bottom panels) were found to be higher in the CD49b+ cell population (48 ± 0.5%) than in the CD25+ and CD4+ cell populations (25 ± 1% and 16 ± 1%, respectively) of noninjected mice. Following DC vaccination, we observed a slight, but significant decrease in the percentage of cells with a naïve phenotype within the CD25+ cell population (44 ± 1% and 36 ± 1%, p < 0.05) and more importantly within the CD49b+ cell population (26 ± 1% to 10 ± 1%, p < 0.0001). These significant decreases in cells with naïve phenotype were associated with significant increases in cells with effector memory phenotype in the CD49b+ (48 ± 0.5% to 66 ± 2%, p < 0.0001) and to a lesser extent the CD25+ (25 ± 1% to 33 ± 1%, p < 0.05) cell populations. These data demonstrate that the CD49b+ T cell population induced by DC vaccination clearly displayed an effector memory phenotype, whereas the CD25+ T cell phenotype was less impacted.

**The transcriptional profiles of CD49b+ T cells contain multiple transcripts of the canonical Treg cell signature shared either by CD25+ or other Treg subphenotypes**

To identify the genes differentially expressed by CD25+ and CD49b+, defined as prototypical Treg transcripts, we compared the gene expression patterns of highly purified T cells. The gating strategy and purity of FACS-sorted CD49b+, CD25+, and CD4+ populations are given in Supplemental Fig. 1. We determined the differential transcriptional profiles associated with the DC vaccination protocol by comparative analysis of the FACS-sorted CD4+ cells isolated from noninjected and DC-injected mice. The transcriptional profiles of DC-induced CD25+ and CD49b+ included both the transcriptional profile associated with the CD4+ cell subset and the DC vaccination-induced transcripts. To focus our analysis only on CD49b+ and CD25+ specific transcripts, we removed the transcripts associated with DC vaccination found in CD4+. We were therefore able to compare these CD25+ and CD49b+ differential gene expression profiles with the canonical Treg cell expression signature consisting of 603 probe sets (16, 37). These 603 probe sets defined by Hill et al. (37) correspond to 431 transcripts (138 downregulated and 293 upregulated) that revealed a mean probe set redundancy of 1.4 in their study. In our study, the precise and robust analysis of the differentially expressed transcripts is underscored by the mean global score for probe redundancy of 2.0. We found 79 differentially expressed transcripts in the CD49b+ cells (18 downregulated and 61 upregulated) and 128 differentially expressed transcripts in the CD25+ cells (28 downregulated and 100 upregulated) all in common with the canonical Treg signature, with similar modulation described by Hill et al. (37) (Fig. 2A). Interestingly, the CD25+ and CD49b+ cell populations shared 59 differentially expressed transcripts (11 downregulated and 48 upregulated) (Fig. 2B), corresponding to 74.6% of the differentially expressed transcripts found in CD49b+, therefore underscoring the similarities between CD49b+ and CD25+ Treg cells. Similar transcriptional expression variations were observed between CD49b+ and CD25+ Treg cells with similar modulations to those described by Hill et al. (37). The common transcriptional pattern between CD49b+ and CD25+ contained several prototypical Treg transcripts, including Igae, Krig1, Nr1p1, Gemb, Ebi3, Enup1, Dusp4, Socs2, Ahr, and Swap70.

We also found that each cell population uniquely expressed several canonical Treg cell signature transcripts, as follows: 69 for CD25+ (17 downregulated and 52 upregulated) and 20 for CD49b+ (7 downregulated and 13 upregulated) (Fig. 2A, Supplemental Fig. 2). Interestingly, among the transcripts specific for CD49b+, we found AcoT7, Lxn, 5830474E16Rik, Gpr34, Pros1, and Ndr1. These transcripts have previously been described as differentially expressed in conventional Treg cells isolated from spleen, and highly expressed in CD103+ and KLRG1+ Treg cells (26). Altogether, our results demonstrate that the CD49b+ transcriptional signature contains prototypical Treg cell transcripts shared by either CD25+ or other Treg cell subphenotypes.

**CD49b+ T cells express several canonical markers of CD25+Foxp3+ Treg cells**

To further characterize and compare the phenotypes of the DC-induced CD49b+ and CD25+ cells isolated from the same DC-vaccinated mice, we performed 6–10 color cytometric analyses. We showed that, despite weakly expressing CD25 and the master regulator transcription factor Foxp3 (Supplemental Fig. 1), CD49b+ cells express markers commonly used to characterize CD25+Foxp3+ Treg cells, including CD103, KLRG1, CTLA-4, latency-associated peptide (LAP), and glucocorticoid-induced TNFR family–related gene (GITR) (Fig. 3). Interestingly, CD49b+ cells also expressed programmed cell death-1, shown to play an important role in pTreg cell induction and function (41), although at a lower level than that in CD25+ cells (Fig. 3A). Moreover, expression of LAG-3, KLRG1, and CD103 molecules was in contrast significantly higher in CD49b+ than in CD25+ cells (Fig. 3A). Within the CD49b+ cell population, we noted that LAG-3 expression was mostly restricted to the Foxp3neg cells, whereas KLRG1 and CD103 expressions were found in both Foxp3+ and Foxp3neg cells.

Several molecules sustaining the Treg cell–suppressive function are known to be highly expressed following activation. As CD25
and CD49b expressions are also modulated following activation, we first purified the T cells from DC-vaccinated mice by FACS sorting them (Supplemental Fig. 1), and the three resulting populations were analyzed 48 h following in vitro stimulation. Phenotypic analysis of activated T cells clearly showed that all T cells acquired CD25 expression, and that half of the CD25+ Treg cells were Foxp3+ compared with < 4% of the CD49b+ Treg cells (Fig. 3B). Interestingly, compared with the CD25+ T cell population, that of the CD49b+ T cells displayed higher percentages or mean fluorescence intensity of several markers commonly expressed by Treg cells. These markers included granzyme B (GrB), GITR, ICOS, LAP, and IL-10 in terms of percentages, and CTLA-4 for mean fluorescence intensity. We narrowed our focus down to effector memory cells. Percentage of naive (CD44lowCD62Lhigh) and effector memory (CD44highCD62Llow) T cells within the CD4-, CD25-, and CD49b-gated populations (for gating strategy, see Supplemental Fig. 1) was analyzed by flow cytometry for DC-injected (DC-Inj, n = 10) and noninjected mice (Non-Inj, n = 3). (A) Representative dot plots within gated CD4 (left), CD25 (middle), and CD49b (right)-positive cells in noninjected (top panels) and DC-injected mice (bottom panels). (B) Percentages of naive and effector memory cells within the gated CD4 (left), CD25 (middle), and CD49b (right) cell population. Each symbol represents an individual mouse, and bars show the mean ± SEM. Data are representative of two independent experiments. *p < 0.05, ****p < 0.0001 by repeated-measures two-way ANOVA (Bonferroni’s multiple comparisons test).

FIGURE 1. DC-induced CD49b+ cells display an effector memory phenotype. Percentage of naive (CD44lowCD62Lhigh) and effector memory (CD44highCD62Llow) T cells within the CD4-, CD25-, and CD49b-gated populations (for gating strategy, see Supplemental Fig. 1A) was analyzed by flow cytometry for DC-injected (DC-Inj, n = 10) and noninjected mice (Non-Inj, n = 3). (A) Representative dot plots within gated CD4 (left), CD25 (middle), and CD49b (right)-positive cells in noninjected (top panels) and DC-injected mice (bottom panels). (B) Percentages of naive and effector memory cells within the gated CD4 (left), CD25 (middle), and CD49b (right) cell population. Each symbol represents an individual mouse, and bars show the mean ± SEM. Data are representative of two independent experiments. *p < 0.05, ****p < 0.0001 by repeated-measures two-way ANOVA (Bonferroni’s multiple comparisons test).

Peripheral induced CD49b cells express Neuropilin-1 without coexpressing Helios

Neuropilin-1 (Nrp-1) was proposed as a Treg cell surface marker in 2004 (42), and its coordinated expression along with Helios, an Ikaros family transcription factor, was more recently suggested for use in distinguishing thymic derived from inducible Foxp3+CD25+ Treg cells (43–46). Indeed, pTreg cell populations generated in vivo displayed reduced Nrp-1 expression compared with Treg cells, indicating Nrp-1 as a tTreg-specific marker (26). As previously published for NOD and C57BL/6 mice (43), we showed in DBA/1 mice that the majority of CD25+ cells expressed concurrently Nrp-1+ and Helios (56 ± 3%) (Fig. 4). Interestingly, Nrp-1 expression was higher (58 ± 1%) than Helios expression was significantly lower (15 ± 1%) in CD49b+ cells. Moreover, we observed that CD49b+Helios+ cells coexpressed Nrp-1+ and Foxp3+, suggesting that, among the CD49b+ cell population, almost 20% of cells could be considered as natural Treg cells based on the concomitant expression of Helios, Nrp-1, and Foxp3 (Fig. 4). Altogether, our results show that induced Foxp3+CD49b+ Treg cells are positive for Nrp-1, but do not coexpress Helios, as expected for induced pTreg cells.

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

Recent evidence suggests that the capacity of Treg cells to control polarized settings can be associated with the expression of specific transcription factors, such as T-bet, IFN regulatory factor 4, and STAT3 to control Th1, Th2, and Th17 responses, respectively (47–49). Treg cells expressing these transcription factors can partially mimic the phenotype of the effector T cells, providing them with particular homing, survival, or functional properties (50). It has been demonstrated that 25% of Foxp3+ compared with only 5% of Foxp3+ Treg cells isolated from spleen express the canonical Th2 transcription factor Gata3 (51). These authors showed that the expression of Gata3 controlled unbalanced polarization and inflammatory cytokine production in Treg cells, and that it was required for the maintenance of Foxp3 high-level expression and promoted the accumulation of Treg cells at inflamed sites (51). In our study in DBA/1 mice, we observed Gata3 expression in 8 ± 1% of the CD25+ cells and in 47 ± 2% of the CD49b+ cell population (Fig. 5A). Furthermore, we observed that only the DC-induced CD49b+ cells displayed a considerable proportion of double-positive staining for T-bet and Gata3 (25 ± 1.5%), in contrast with the CD4+ and CD25+ cells (2 ± 0.2% for both populations) (Fig. 5A, right panel). This DC-induced increase in the number of CD49b+ cells expressing both T-bet and Gata3 was statistically significant (25 ± 1.5% versus 2 ± 0.2%, p < 0.0001).

C-Maf was the first Th2-specific transcription factor identified and has been shown to play a critical role in trans-activating IL-4 and IL-10 expression during Th17 polarization. The ligand-activated transcription factor aryl hydrocarbon receptor (AhR), like the proto-oncogene Maf, was shown to be strongly induced during Th1 cell differentiation with similarly high levels of expression found in both Th1 and Th17 cells (52). We thus evaluated the expression of c-Maf and AhR in CD49b+ cells and found them in 53 ± 2% and 82 ± 1%, respectively, compared with in only 38 ± 1% and 39 ± 2%, respectively, of the CD25+ population (Fig. 5B).
To further characterize the cytokine secretion profile, we quantified the level of cytokine secretion in the supernatant of highly purified FACS-sorted cells following their in vitro activation. Besides the high level of IL-10 secretion (19 ± 7 ng/ml), we measured significantly elevated secretion levels of other type 2 cytokines, including IL-4 (10 ± 2 ng/ml), IL-5 (18 ± 2 ng/ml), and IL-13 (31 ± 0.5 ng/ml), as well as a relatively high amount of IFN-γ (3 ± 1 ng/ml) in the supernatant of the CD49b+ T cell population. These results revealed an obvious type 2 dominant cytokine profile for the CD49b+ Treg cells and underscored their dissimilarity with Tr1 cells, which secrete high levels of IL-10 without concomitant secretion of IL-4 (40, 53).

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

We compared the in vitro potential of CD49b+ and CD25+ Treg cells to functionally suppress the proliferation of CD4+ T cells by cocultivating Treg and responder cell populations stimulated by a polyclonal TCR stimulator (anti-CD3 mAb) and in the presence of APCs. Addition of CD49b+ or CD25+ Treg cells reduced the proliferation, as
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 of cells are shown within each quadrant. Each symbol represents a pool of two mice, and bars show the mean ± SEM. ****p < 0.0001, **p = 0.0005, *p = 0.01, and *p = 0.013 by repeated-measures two-way ANOVA (Tukey’s multiple comparisons test). (B) The FACS-sorted CD4, CD25, and CD49b T cell populations from DC-vaccinated mice (n = 18) were analyzed by FACS 48 h following in vitro stimulation. Gates and quadrants were set as indicated, and frequencies of cells are shown. Each symbol represents a pool of six mice, and bars show the mean ± SEM. ****p < 0.0001, **p = 0.0001, *p = 0.002, and *p = 0.03 by repeated-measures two-way ANOVA (Tukey’s multiple comparisons test).
measured by the CFSE dilution, in a dose-dependent manner, thus confirming their potent in vitro suppressive capacities (Fig. 6A).

We previously demonstrated the in vivo therapeutic potential of CD49b+ Treg cells to protect against (30) as well as to improve the condition of established arthritis (32). To further investigate the therapeutic potential of CD49b+ Treg cells, we compared their protective effect with that of CD25+ Treg cells isolated from the same DBA/1 mice and with CD49b+ Treg cells isolated from Col II-specific T cell transgenic mice (TBC). We repeatedly injected syngeneic mice i.p. with 0.5 × 10^6 DCs the week before their euthanasia. CD4+ T cells were purified, and the Treg cells were FACs sorted to obtain ≥98% pure population. The FACs-sorted populations were adaptively transferred i.v. into CIA mice on day 28, at the onset of the clinical signs. In this experimental setting that mimics the clinical situation, we observed a significant decrease of arthritis severity in mice injected with either of the polyclonal Treg cells, CD49b+ or CD25+, isolated from the same DBA/1 mouse, or with the Ag-specific CD49b+ Treg cells (CD49b TBC; Fig. 6B). Similar results were obtained in several independent experiments, and we performed robust statistical analyses using relative arthritic scores calculated using the mean of the PBS-treated mice as 100% disease severity for each experiment. We included in these experiments a control group of mice, which were injected with the CD4+ cell population. As shown in Fig. 6C, injection of polyclonal CD49b+ Treg cells markedly and significantly decreased the disease severity compared with PBS-treated or CD4+ mice. We observed a trend toward decreased disease severity after injection of the CD25+ Treg cells or of the Ag-specific CD49b+ Treg cells isolated from TBC mice; however, these decreases were not significant. These results in the CIA experimental model suggest that the impaired Treg cell differentiation may be corrected by adoptive transfer of in vitro generated autologous Treg cells or by immunotherapeutic strategies triggering an increase in the number and/or an improved functioning of endogenous Treg cells.

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

However, some evidence suggests that Treg cells generated in vitro are phenotypically and functionally unstable, whereas those induced in vivo are epigenetically more stable and would lead to a longer-lasting therapeutic effect (4, 58, 59). The in vivo induced Treg cells are usually Ag specific, which implies a likely more efficient effect in treating autoimmune diseases. In RA patients, TNF-α blocking Abs have been described as an effective way to stimulate the induction of peripheral Foxp3+ Treg cells, overcoming the impaired peripheral Treg cell differentiation (60). For all these reasons, the development of strategies to promote in vivo

**FIGURE 4.** Peripherally induced CD49b+ cells express Nrp-1 without coexpressing Helios. Representative flow cytometry analyses of splenocytes from DC-injected mice (n = 18) within the gated CD4 (left), CD25 (middle), and CD49b (right) cell population. Quadrants were set as indicated, and percentages of Helios, Nrp-1, or double-positive cells were analyzed. Each symbol represents a pool of two mice, and bars show the mean ± SEM. ****p < 0.0001 by repeated-measures two-way ANOVA (Tukey’s multiple comparisons test).
FIGURE 5. Peripherally induced CD49b+ cells express Th2-specific transcriptional factors and display a dominant Th2 cytokine profile. Percentages of cells expressing T-bet, Gata3, and transcriptional factors associated with IL-10 production, including c-Maf and AhR, were analyzed within the gated CD4, CD25, and CD49b cell populations from DC-injected splenocytes (n = 18). (A) Representative histogram plots of T-bet and Gata3 staining in gated CD4, CD25, and CD49b cell populations were compared with in vitro polarized Th1 and Th2 cells. Percentages of Gata3+ and double-positive Gata3+T-bet+ cells are represented with mean ± SEM, each symbol representing a pool of two mice. Data are representative of two independent experiments. ****p < 0.0001, ***p = 0.0003 by repeated-measures two-way ANOVA (Tukey’s multiple comparisons test). (B) Representative dot plots and percentages of AhR+ and c-Maf+ cells in gated CD4, CD25, and CD49b cell populations. Each symbol represents a pool of two mice, and bars show the mean ± SEM. Data are representative of two independent experiments. ****p < 0.0001 by repeated-measures one-way ANOVA (Tukey’s multiple comparisons test). (C) Level of cytokine secretion by highly purified cells following in vitro activation.
generation of Ag-specific Treg cells appears crucial for the treatment of autoimmune diseases.

The aim of our study was to better characterize a particular subpopulation of in vivo induced CD49b+ Treg cells. We demonstrated that this particular Treg cell subset expresses several canonical markers of Treg cells while being mostly negative for CD25 and Foxp3, which are routinely used to identify Treg cells. We first demonstrated that 30% of the Treg cell signature was found in the CD25+ Treg cell–specific expression profile. Indeed, the Treg cell canonical signature is a composite signature derived from Treg cells isolated from several lymphoid organs (37). This bulk of Treg cell subphenotypes could explain the lack of complete overlapping with the specific transcriptional profile of CD25+ cells in our study. A similar lack of complete overlapping has previously been observed when comparing the transcriptional profile of converted Foxp3+ Treg cells with the canonical Treg cell signature (26). Interestingly, the induced CD49b+ Treg cells shared a transcriptional profile common to CD25+ Treg cells and the canonical Treg cell signature. We showed that 75% of the differentially expressed transcripts found in CD49b+ T cells were common with those found in CD25+ T cells, underscoring the similarities between CD49b+ and CD25+ Treg cells. Indeed, we demonstrated that these cells share a common signature of 59 prototypical Treg cell transcripts, including effector molecules and transcription factors. Several transcripts from this common signature have been proposed as promising candidates to specifically discriminate between Ag-induced and homeostatically converted Treg cells, including Itgae, Cita4, Entpd1 (CD39), Ebi3 (a component of IL35), Ifi4, αEB7 (CD103), and Klrg1 (a member of the killer cell lectin-like receptor family). The CD49b+ Treg cell transcriptional profile also contained several specific transcripts in common with the canonical Treg cell signature. These results suggest an overlap of the transcriptional profile of CD49b+ Treg cells with several other Treg subphenotypes.
We validated by FACS analyses the common expression of several markers between CD25+ and CD49b+ cells that were differentially expressed compared with CD4+. Among these markers, CD49b and KLRG1, both considered as NK cell markers and minimally expressed on conventional CD4+ T cells, were previously observed in an extrathymically derived subset of CD4+CD25Foxp3+ Treg cells (61). Within the subpopulation of CD25+Foxp3− Treg cells in the spleen, KLRG1+ Treg cells were previously shown to display a more activated phenotype (CD69+CD62L+CD103+CD44high) than KLRG1− Foxp3+ Treg cells. Furthermore, cell surface staining of homeostatically converted Foxp3+ cells revealed them as uniformly CD103+, an excellent marker for identifying in vivo activated Foxp3+CD4+ Treg cells, and that 50% of the cells expressed KLRG1 (26). We showed that the two markers, KLRG1 and CD103, were expressed on CD49b+ and CD25+ cells and, as previously observed for the CD4+ CD25+Foxp3+ Treg cells, were associated with an activated phenotype for the CD49b cells. Similarly, Nrp-1 was previously described on a population of activated/memory Foxp3−Nrp-1+ in secondary lymphoid organs and inflamed tissues, which could imply that the expression of Nrp-1 is associated with the CD49b+ activated/memory phenotype. Finally, the lack of concomitant expression of Nrp-1 and Helios as well as their effector/memory phenotype confirm the peripheral origin of these cells.

Initially characterized as a Th2-specific cytokine, IL-10 has since been found expressed by almost all CD4+ T cells, including CD25+Foxp3+ Treg cells and Th1 cells, but also Th1, Th2, and Th17 cells, to promote immune homeostasis. Previous mouse studies have described the collaborative actions of c-Maf with AhR and the ICOS receptor ligation that drive IL-10 expression and promote Tr1 differentiation (52, 62). We demonstrated in this study that the CD49b+ Treg cells highly express these three molecules, suggesting that, similarly to Tr1 cells, several transcriptional pathways, associated with high secretion of IL-10, are activated. CD49b+ cells are also positive for the Th2-specific transcription factor Gata3, and 30% of the cells are double positive for T-bet and Gata3 with concomitant secretion of IFN-γ and Th2 cytokines. The coexpression of T-bet and Gata3 has been previously observed in vivo following viral infection, and this hybrid phenotype appeared to be stable (63). Altogether, our results suggest that the CD49b+ cells display a balanced Th2/Th1 phenotype that could endow them with specific properties to better control effector T cell responses.

Other similarities and differences between IL-10–secreting CD49b+ Treg cells and Tr1 cells can be discussed. Coexpression of CD49b and LAG-3 has been recently proposed as specific for Tr1 cells (35). In our experimental setting, only 5–10% of CD49b+ Treg cells were positive for LAG-3 before in vitro activation, and, interestingly, CD49b+LAG-3+ cells are mostly Foxp3-negative cells like Tr1 cells. Furthermore, Tr1 cells are reported to be induced at mucosal sites in response to Ag stimulation in the presence of IL-10. We observed that IL-10–deficient DCs promoted IL-10–secreting CD49b+ Treg cell expansion in several lymphoid organs of wild-type animals, suggesting that, in contrast to Tr1 cells (64), the IL-10 secretion by DCs is dispensable for the expansion of CD49b+ (P. Louis-Plence, unpublished observations). Altogether, our results suggest that the CD49b+ Treg cells constitute a Treg subphenotype that shares similarities with the CD25+ Treg cells as well as with the Tr1 cells, and should be considered alongside other subphenotypes as homeostatically converted or Ag induced.

In this study, we have investigated the suppressive function of CD25+ and CD49b+ Treg cell populations in vitro and in vivo, in the experimental model of CIA. In vitro, both Treg cell populations similarly suppressed the T cell proliferation. To compare their therapeutic potential in CIA, we injected CD25+, polyclonal CD49b+, or Col II-specific CD49b+ Treg cells at the onset of clinical signs of arthritis. As previously described (32), we demonstrated a significant reduction of these clinical signs following injection of polyclonal CD49b+ Treg cells. Although not significant, we also observed decreased clinical signs following injection of CD25+ or Col II-specific CD49b+ Treg cells. Our results suggest that, following their activation by self-Ag(s), the CD49b+ Treg cells display a potent bystander suppressive function, and, as polyclonal in vivo expanded Treg cells, they could be a better alternative to classical Treg cells for arthritis treatment. The suppressive function of CD49b+ Treg cells was found to be partially dependent on IL-10 secretion. Moreover, expression of several canonical Treg markers, implicated in the Treg-suppressive function, suggests that other molecules might also play a role in the CD49b+ suppressive activity. Indeed, GrB and CTLA-4 have been shown to play a crucial role in the suppressive function of conventional CD25+Foxp3+ Treg cells and thus might also play an important role in the suppressive function of CD49b+ Treg cells. Furthermore, CD103 expression could also be implicated in their suppressive function as its expression was shown to be responsible for the retention of Treg cells in inflamed tissue by interaction with its ligand E-cadherin (65, 66). Finally, expression of the α2 integrin CD49b itself could also be important for their function because it was demonstrated that this integrin is required for the migration of memory CD4 T cell precursors into their survival niches of the bone marrow (67). Because VLA-2 also binds Col II, expression of CD49b could provide Treg cells with particular homing, survival, or more potent suppressive function in the context of arthritis because Col II is expressed by the damaged cartilage.

In this study, we have provided an in-depth characterization of the CD49b+ Treg cells, underscoring their similarities with other Treg subphenotypes and highlighting specific expression patterns for several markers, including ICOS, CTLA-4, and GrB. The expression of these canonical Treg markers strongly supports the notion that several suppressive mechanisms could be Foxp3 independent. Their potent suppressive activity in vivo, higher than that of the classical CD25+ Treg cells, underscores the need to select appropriate Treg subsets for a given clinical application and supports their therapeutic application in RA.

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Disclosures
The authors have no financial conflicts of interest.

References
The text contains a list of scientific references with varying formatting and styles. Here is a structured representation of the text:

collagen-II specific IL-10 producing Tr1 cell clones from rheumatoid arthritis blood. *Int. Immunopharmacol.* 11: 1074–1078.


Supplemental Figure 1. Repetitive injections of immature DCs induce FoxP3 CD49b+ Treg cells. Mice were repetitively injected or not with 0.5 x 10^6 immature DCs at days 7, 5 and 3 before euthanasia. Flow cytometry analyses of CD25, CD49b and FoxP3 expression were performed at day 0 on splenocytes. (A) Representative dot plots showing CD4 staining (left panels), CD49b and CD25 staining in gated CD4 cells (middle panels) and CD25 and FoxP3 staining in gated CD49b cell populations (right panels) in non-injected mice (Non-Inj, n=9) and DC-injected mice (DC-Inj, n=9). Gated CD4^+CD49b^+CD25^+, CD4^+CD25^+ and CD4^+CD25^+CD49b^- are called CD49b, CD25 and CD4 respectively in all subsequent experiments. (B) Frequencies of CD25, CD49b cells and FoxP3 positive cells within the gated populations (labeled above each plot). Each symbol represents an individual mouse and bars show the mean ± SEM. Data are representative of multiple independent experiments. **** p<0.0001 by repeated measures two-way ANOVA (Bonferroni’s multiple comparisons test). (C) Gating strategy and purity of FACS-sorted CD49b^+, CD25^+ and CD4^+ populations. Sort gates for the FACS purification and re-analytics of FACS-sorted CD49b^+ and CD25^+ are displayed. One representative out of 9 independent experiments is shown.
Supplemental Figure 2: Transcriptional profiles of FACS-sorted CD25\(^+\) and CD49b\(^+\) T cells contain specific transcripts common with the canonical Treg cell signature. A, Bar graphs show the transcriptional expression variation of the genes differentially expressed within the CD25\(^+\) signature common with the canonical Treg cell signature. B, Bar graphs show the transcriptional expression variation of the genes differentially expressed within the CD49b\(^+\) signature common with the canonical Treg cell signature.
Supplemental Figure 3. IL-10-secreting CD49b+ Treg cells highly expressed CTLA-4 and GrB. CD25+ and CD49b+ cell populations from DC-vaccinated mice (n=18) were analyzed by FACS 48 hours following in vitro stimulation. Gates were set as indicated on IL-10 secreting or FoxP3-positive cells for the CD49b and CD25 sorted population respectively. Frequencies of cells are given within each quadrant. Each symbol represents a pool of 6 mice and bars show the mean ± SEM. ** p<0.01 * p<0.05 by Repeated measures two-way ANOVA (Tukey’s multiple comparisons test).