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Abstract

We have previously demonstrated, in the collagen-induced arthritis model (CIA), that repetitive injections of immature bone-marrow-derived dendritic cells (iDCs) induce the expansion of a population of CD4CD49b-expressing cells, and that their adoptive transfer results in protection against CIA in a prophylactic setting. However, the in vivo mechanism responsible for their expansion, as well as their therapeutic potential in established disease remains to be defined. In the present study, we show that expression of the MHC class II molecules on iDCs is required for their expansion thus identifying these cells as MHC class II-restricted T cells. Using adoptive transfer of Thy1.1 positive cells, it is shown that iDC-induced $CD4^+CD49b^+$ T cells home to the lymph nodes draining the inflamed tissue. The high immuno-modulatory potential of these cells was underscored following their adoptive transfer in a model of contact hypersensitivity. Finally, we assessed and compared the therapeutic potential of iDC-inducible $CD4^+CD49b^+$ T cells with that of iDCs in established CIA. Repetitive injections of iDCs in arthritic mice failed to decrease the severity of established disease. In contrast however, a single injection of iDC-induced $CD4^+CD49b^+$ T cells reversed clinical symptoms of arthritis and provided long-lasting protection. Together, our data indicate that iDC-induced $CD4^+CD49b^+$ T cells are bona fide T regulatory cells with strong immunomodulatory properties that are not only able to prevent disease onset, but also to interfere with an ongoing inflammatory immune response.

Keywords: Arthritis; Regulatory T cells; IL-10; Dendritic Cells; Immunomodulation; Autoimmunity

1. Introduction

It has become increasingly clear that the balance between pathogenic and immune regulatory responses underlies disease progression in many autoimmune disorders. Compelling evidence is now accumulating that naturally-occurring, as well as inducible T regulatory (Treg) cells, represent an important component of the immune system to maintain self tolerance [1]. Among the various subpopulations of Treg cells described at present, the most studied cells are the so-called $CD4^+CD25^+$ natural suppressor cells that, in mice, are characterized by the expression of the Foxp3 transcription factor, the constitutive expression of several cell surface molecules including GITR, CTLA-4, OX40, CD62L, as well as by a low expression of CD127, the IL-7Rα chain [2-5]. Although various mechanisms have been proposed to explain the immunosuppressive effects of these cells, their effector mechanism has not yet been clearly determined [6-15]. In contrast to natural $CD4^+CD25^+$ Treg cells, that are selected in the thymus due to high affinity interactions of their TCR with MHC molecules and an intermediate affinity for antigen presented by thymic dendritic cells, populations of IL-10-producing Treg cells are induced *in vivo* in the periphery, in particular under various tolerogenic conditions [16-21]. *In vitro*, the inducible Treg cells are generated following Ag-specific or non-specific stimulation in the presence of immunosuppressive drugs such as 1,25(OH)2-vitaminD3 and Dexamethasone [22,23], IL-10 in the absence or presence of IL-4 [24-26] or IFN-α [27], respectively, and co-engagement of CD3 and CD46 [28]. It is still unclear to what extent these populations of inducible IL-10-secreting T cells are identical, as T cells induced in the presence of either immunosuppressive drugs, soluble protein or peptide antigen, do not produce IL-2, IL-4, IL-5, IFN-γ or other effector cytokines, while others, like T regulatory 1 (Tr1) cells, produce IFN-γ and IL-5 in addition to IL-10.

There is increasing evidence that dendritic cells (DCs) can contribute to the expansion and differentiation of Treg cells that regulate or suppress the activity of other

immunocompetent cells. Such tolerogenic DCs are able to expand and/or activate naturally occurring Treg cells [29-31] or inducible IL10-secreting T cells [17,18,32]. In the collagen-induced arthritis model (CIA), the experimental model of rheumatoid arthritis (RA), we recently demonstrated that repetitive injections of immature bone marrow-derived DCs (iDCs) induced the expansion of IL-10 secreting $CD4^+CD49b^+$ cells in the liver and spleen of DC-injected mice. We also showed that the adoptive transfer of $CD4^+CD49b^+$ cells before immunization of the mice resulted in a complete protection against arthritis [33]. This protective effect was associated with an attenuation of the B and T cell responses and a secretion of IL-10 in the lymph nodes draining the inflamed paws. We showed that these $CD4^+CD49b^+$ cells did not recognize the glycolipid α-galactosylceramide in the context of the CD1d molecule, identifying them as a population of non-NKT type I cells. Because $CD49b^+$ NKT/T cells have been shown to mediate protection in models for EAE and diabetes [34-37], we investigated in the present study the involvement of the CD1d or MHC class II molecules in the expansion process of these induced $CD4^+CD49b^+$ cells in order to distinguish between type II NKT or T cells respectively. Moreover, we evaluated the homing capacity to inflamed tissue, and challenged their suppressive activity on antigen sensitization in a model of epicutaneous contact hypersensitivity (CHS). Finally, in order to be used as therapeutic agent in autoimmune settings, Treg cells must be able to inhibit ongoing T-cell responses and to suppress established pathology. Therefore, we assessed their therapeutic potential in a curative strategy in arthritic mice.

2. Materials and Methods

2.1 Mice.

C57BL/6, DBA/1 and BALB/c mice were obtained from Harlan (France), and congenic Thy1.1 were kindly provided by Javier Hernandez [38]. The B6.129-H2-Ab1tm1Gru N12 mice (Taconic; Denmark) contain a disruption of the H2-Ab1 gene and do not express I-A and I-E molecules. They are referred in this manuscript as MHC class II knock-out mice (MHC II -/-). Experiments with animals were conducted in accordance with the national guidelines for animal care and approved by the Ethic committee on Animal Research of the Languedoc-Roussillon (CE-LR-0505).

2.2 Generation and injections of DCs.

DCs were generated as previously described [33]. Briefly, bone marrow cells were harvested from the femur and tibiae of DBA/1 mice and washed in RPMI following lysis of red blood cells. T and B cells were depleted using mouse pan T and pan B Dynabeads® (Invitrogen) and monocytes were removed by adhesion in RPMI 5% FCS. Remaining cells were culture in complete medium (RPMI 1640 supplemented with 5% FCS, 2mM L-glutamin, 5 x 10^{-5} M β-mercaptoethanol, 100U/ml penicillin, 100 µg/ml streptomycin, non essential amino acids and 1 mM sodium pyruvate) with 1,000 IU/ml of rmGM-CSF (R&D Systems) and 1,000 IU/ml of rmIL-4 (R&D Systems) at 5 x 10^5 cells/ml in 24-well plate. Culture medium was renewed at day 2 and 4. For in vivo experiments, immature DCs (iDCs) were harvested at day 7, washed twice. Mice were injected intraperitonealy with 0.5 x 10^6 iDCs in 100 µl PBS, 7, 5 and 3 days before purification of $CD4^+CD49b^+$ T cells.
Bone marrow from CD1d knock-out mice was kindly provided by Dr. Kuiper (division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden, The Netherlands).

2.3 Antibodies and FACS analyses.

The following Abs were purchased from BD Pharmingen: FITC-labeled anti-Vα2 (B20.1), anti-Vα3.2 (RR3-16), anti-Vα8.3 (B21.14), anti-Vα11 (RR8-1), anti-Vβ2 (B20.6), anti-Vβ6 (RR4-7), anti V-β8 (F23.1), anti-CD90.1 (HIS51); PE-labelled anti-CD49b (DX5); PE-Cy5 labelled anti-CD4; PerCP labelled anti-CD4, APC-conjugated anti-IFN-γ (XMG1.2), anti-IL-5 (TRFK5), anti-IL-10 (JES5-16E3) antibodies or APC-conjugated isotypes controls (R3-34 and A95-1). The anti-MHC class II antibodies (M5/114.15.2) were obtained from Miltenyi Biotec (Miltenyi, France).

Cell suspensions from blood, spleen and lymph nodes were prepared according to standard procedures. Lymphocytes from liver were obtained as previously reported [39]. Briefly, the liver was pressed through a 100 µm cell strainer and suspended in PBS. After treatment with red cell lysis buffer, cells were washed 3 times with PBS. Mononuclear cells were isolated after centrifugation in isotonic 33.8% Percoll (Amersham Biosciences, Orsay, France) for 12 min at 693g. Recovered leucocytes were washed before labeling.
For intracellular cytokine staining (ICS), liver mononuclear cells (0.5 x 10^6 cells/well in 96-wells plate) were stimulated overnight with syngenic 0.1 x 10^6 iDC/well. During the last 3 hours of stimulation, 10 µg/ml brefeldin A (Sigma-Aldrich) was added. Subsequently, cells were stained for cell-surface markers (20 minutes, on ice). After washing step with PBA (PBS containing 0.5% BSA, 0.02% azide), cells were fixed in 4% paraformaldehyde for 5 minutes on ice. Cells were subsequently permeabilized in PBA containing 0.1% saponin (PBA-sap) supplemented with 10% FCS for 20 minutes on ice. Intracellular cytokine staining was performed with APC-conjugated Abs diluted in PBA-sap for 20 minutes on ice. Cells were washed with PBA and fixed in 1% paraformaldehyde. Data acquisition and analysis was performed on a FACSCalibur using CellQuestPro software (BD Biosciences).

2.4 $CD4^+CD49b^+$ cell isolation and adoptive cell transfer experiments.

Mononuclear cells from the liver were isolated after centrifugation in isotonic Percoll as described above. Cell suspensions from spleen were prepared according to standard procedures and after treatment with red cell lysis buffer; cells were washed 3 times with PBS. $CD4^+$ cells from liver and spleen were purified by negative selection using Dynal® CD4 negative isolation kit (Invitrogen). Purified CD4 cells were stained with anti CD4 and CD49b-conjugated antibodies before cell sorting on FacsAria (Becton Dickinson, CA San Jose). The usual purity of the sorted cells was more than 99% for the $CD4^+CD49b^-$ and 96 to 98% for the $CD4^+CD49b^+$ cells. After cell sorting, cells were washed and injected i.v. in the tail vein of recipient mice.

2.5 Contact sensitivity to oxazolone.

The adoptive transfer of the purified $CD4^+CD49b^+$ cells was performed the day of the sensitization of the mice with 2% oxazolone/acetone on the shaved abdomen on day -7. Mice were painted with 0.04% oxazolone/acetone on the inner side of the right ears on days 0 and a second challenge was performed at day 5 when indicated. Left ears were challenged by vehicles only and served as controls. Contact sensitivity was assessed by measuring ear swelling, which was determined before and after each challenge using a micrometer.

2.6 Induction and clinical evaluation of arthritis.

Male 9-weeks-old DBA/1 were immunized with bovine type II collagen (bCII) (MD Biosciences, Zurich, Suitzerland) as previously described [40]. From day 21, the thickness of each paw was measured with a caliper 3 times a week and the severity of arthritis was graded according to the following scale: 0 = normal with no increase in joint thickness; 1 = slight

swelling and erythema with 0.1- to 0.15-mm increase in joint diameter; 2 = significant swelling and redness with 0.15- to 0.25-mm increase in joint thickness; 3 = severe swelling and redness from joint to digit with 0.25- to 0.45-mm increase in joint thickness ; and 4 = maximal swelling, deformity and ankylosis with an increase in paw swelling above 0.45 mm. Each limb was graded, resulting in a maximal clinical score of 16 per animal and expressed as the mean score ± SEM on a given day. Hind paws were collected for histologic examination, fixed in 4% paraformaldehyde, EDTA-decalcified, embedded in paraffin, and 5-µm sections were stained with hematoxylin/eosin (HE) and tartrate-resistant acid phosphatase (TRAP). HE-stained sections were examined by 2 independent observers and scored for histopathologic features such as inflammatory infiltrates, synovial hyperplasia, cartilage erosion, and bone destruction. Quantitative analysis of TRAP staining (leucocyte acid phosphatase kit, Sigma) was performed using a Nikon microscope equipped with a video image system (Osteomeasure, Osteometrics, Georgia).

2.7 Measurement of serum anti-bCII levels.

Serum level of antibodies against bCII was measured by a standard ELISA as previously described [40,41] during disease course. Antibody units for bCII were determined using a reference serum created from pooled sera of arthritic mice and assigned an arbitrary level of antigen-specific antibodies.

2.8 Statistical analyses.

Statistical analyses were performed using the non-parametric Mann-Whitney U test or Student’s paired t test as appropriate according to data distribution.

3. Results

3.1 Generation of CD4$^+$CD49b$^+$ requires the expression of MHC Class II molecules.

In a first series of experiments, we analyzed the T cell receptor (TCR) repertoire of in vivo-induced CD4$^+$CD49b$^+$ cells following repetitive injections of iDCs. To this purpose, liver mononuclear cells from iDC-vaccinated and control mice were compared for the expression of various TCR Vα and Vβ chain gene products by flow cytometry. As previously reported, repetitive injections of iDC resulted in a significant increase in the frequency of CD49b$^+$ cells within the CD4 population (20.8% ± 2.4 in iDC-treated group versus 5.7 ± 0.3% in controls, P=0.0002) (Fig.1A). The analysis of expression of several TCR Vα and Vβ chains within the population of CD4$^+$CD49b$^+$ cells showed no major variation of their frequencies between control and iDC-treated mice (Fig. 1B). A slight decrease in the percentage of Vβ2 and Vβ8 positive cells were observed within the population of iDC-induced CD4$^+$CD49b$^+$ cells (4.3% ± 0.2 versus 6.1% ± 0.5 in control mice for Vβ2 and 25.7% ± 2.5 versus 30.5% ± 1.2 in control mice for Vβ8, P<0.05). As these two TCR Vβ chains are preferentially associated with the Vα14 chain on NKT type 1 cells, the observed decrease of the Vβ2 and Vβ8 frequencies are likely to reflect the relative decrease in the NKT type 1 cell population in the iDC-vaccinated mice due to the expansion of other CD4$^+$ cells. Likewise, no important variation in the frequencies of the TCR α- and β-chain expression was observed in the spleen of iDC-injected mice (data not shown). The absence of obvious alteration of the TCR chain frequencies assessed does not support a skew of the TCR repertoire suggesting that "conventional" CD4$^+$ T cells but not type I NKT cells, are activated upon transfer of iDC. To substantiate these findings and to obtain a better appreciation of the restriction molecules used by CD4$^+$CD49b$^+$ T-cells, we generated iDCs from CD1d (CD1d$^{-/-}$) and MHC class II (MHCII$^{-/-}$) knock-out mice. These DC were subsequently injected into syngeneic wild type mice and were evaluated for their ability to drive the expansion of IL-10-secreting CD4$^+$CD49b$^+$ cells.

As shown in figure 2, the in vivo generation/expansion of the $CD4^+CD49b^+$ T cells was found to be dependent on the expression of MHC class II molecules, as their expansion was lost after repetitive injections of iDCs isolated from $MHCII^{-/-}$ mice (top panel), while conserved after injections of iDCs from $CD1d^{-/-}$ mice (data not shown). We next assessed the cytokine secretion profile of the expanded cells and demonstrated that whereas wild type iDCs drive the expansion of IFNγ- and IL10-secreting $CD49b^+$ T cells, the iDCs derived from $MHCII^{-/-}$ mice failed to induce the $CD4^+CD49b^+$ T cells producing both cytokines (Fig. 2A and B). Taken together, these results show that iDC-expanded $CD4^+CD49b^+$ cells correspond to a MHC class II restricted, $CD4^+$ T cell and not to type I or type II NKT cells.

3.2 iDC-induced $CD49b^+$ Treg cells are present in lymph nodes that drain the site of inflammation.

We next evaluated whether iDC-induced $CD49b^+$ Treg cells were able to home to lymph nodes draining the site of the immunization. $CD4^+CD49b^-$ and $CD4^+CD49b^+$ T cells were isolated from iDCs-injected Thy1.1 mice, purified by cell sorting, and adoptively transferred into Thy1.2 mice. The same day, the mice were immunized with complete Freund adjuvant (CFA) into their right foot pad. Two days later, we examined the distribution of the adoptively transferred Thy1.1 positive cells by immunofluorescent cell analysis. The $Thy1.1^+$ cells were detected in the blood, liver, spleen, as well as in the inguinal and popliteal lymph nodes draining (DLN) or not (NDLN) the inflamed paws (Fig. 3 and not shown). Next, we compared the homing capacity of the $CD4^+CD49b^-$ and $CD4^+CD49b^+$ cells in CFA-immunized Thy1.2 mice using the Thy1.1 marker as a read-out. The percentage of the $Thy1.1^+$ cells in the blood and spleen was comparable following adoptive transfer of either $CD4^+CD49b^+$ or $CD4^+CD49b^-$ T cells. However, the percentage of $Thy1.1^+$ cells was lower in the blood

(0.13% versus 0.18%) and higher in the draining lymph nodes (0.22% versus 0.13%) following adoptive transfer of $CD4^+CD49b^+$ T cells compared with $CD4^+CD49b^-$ cells. Moreover, the percentage of $Thy1.1^+$ cells in the contra-lateral lymph nodes was lower in mice adoptively transferred with $CD4^+CD49b^+$ cells, as compared with those that had received $CD4^+CD49b^-$ T cells, as well as in non-immunized animals injected with $CD4^+CD49b^+$ T cells (0.03% versus 0.12% and 0.10% respectively). Percentages of $Thy1.1^+$ cells in the blood and spleen were similar in immunized and non-immunized mice after adoptive transfer of $CD4^+CD49b^+$ T cells. Taken together, these results suggest a systemic distribution of the injected $CD4^+CD49b^+$ cells as they are present in the blood and secondary lymphoid organs of the injected-mice and a homing capacity to the lymph nodes draining the immunization site.

3.3 Injection of $CD4^+CD49b^+$ T cells decreases contact sensitivity to oxazolone.

In order to investigate the anti-inflammatory potential of iDCs-induced $CD4^+CD49b^+$ Treg cells on antigen sensitization and the subsequent consequence on the immune response, we used a model of epicutaneous contact sensitivity in DBA/1 mice. Just before sensitization with oxazolone, the mice were injected with increasing numbers of iDCs-induced $CD4^+CD49b^+$ T cells ranging from $0.7x10^5$ to $2.1x10^5$ cells. Five to 7 days later, antigen challenge was performed consisting in the local application of oxazolone on one ear and vehicle on the other. The ear swelling was monitored with a caliper and a second challenge was performed after recovery. Although not significant, a dose-dependent trend in reducing ear swelling appeared to be present in groups of mice that were injected with $CD4^+CD49b^+$ T cells during both primary and secondary antigen challenge (Fig. 4A). To further confirm the immunosuppressive function of the $CD4^+CD49b^+$ T cells, we compared the effect of their adoptive transfer with the transfer of $CD4^+CD49b^-$ T cells as control. A significant decrease

of the ear swelling was observed at day 2 following the challenge, in the group of mice transferred with $CD4^+CD49b^+$ cells compared with $CD4^+CD49b^-$ cells (Fig. 4B). Altogether these results underscored the immunosuppressive potential of the $CD4^+CD49b^+$ inducible T cell population.

3.4 $CD49b^+$ Treg cells are able to decrease severity of ongoing arthritis.

We have previously demonstrated, in a prophylactic setting, that adoptive transfer of as few as 6 x 10^4 $TCR\beta^+CD49b^+$ cells isolated from iDCs-vaccinated mice conferred a complete protection against arthritis induction [33]. In order to mimic the treatment of human disease, we tested the therapeutic potential of these Treg cells in a curative therapeutic strategy i.e. on ongoing arthritis. $CD4^+CD49b^+$ and $CD4^+CD49b^-$ T cells were purified by cell sorting from DBA/1 mice that had been repetitively injected with iDCs and both T cell populations were adoptively transferred into DBA/1 mice on day 28 following arthritis induction. In CIA, on day 28, bCII-specific T cells are already primed and anti-CII antibodies are present in the sera of the mice with more than 50% of the mice showing clinical signs of arthritis with either mild or severe symptoms of the disease. As shown in figure 5A, a single injection of 10^5 $CD4^+CD49b^+$ T cells was able to control the progression of the disease with a stabilization of the disease severity, whereas the same number of $CD4^+CD49b^-$ T cells was ineffective. To further confirm our clinical data, histological examination of arthritic joints were performed and revealed a decreased inflammatory synovitis, pannus formation, and inflammatory cell infiltration in the mice adoptively transferred with $CD4^+CD49b^+$ T cells compared with $CD4^+CD49b^-$-injected or control mice. Bone resorption area was measured on hind paws section and tartrate-resistant acid phosphatase activity was stained in order to assess osteoclastogenesis. A decrease in the resorption area as well as a lower osteoclast number was

quantified on EDTA-decalcified hind paws of the $CD4^+CD49b^+$ Treg treated mice compared with control mice (Fig. 5B).

In order to evaluate the best strategy, it was important to compare the therapeutic potentials of $CD49b^+$ Treg cells and iDCs on ongoing arthritis. In these experiments, repetitive injections of iDCs were performed in arthritic mice on days 21, 23 and 25, in order to evaluate their impact on the ongoing immune response, after antigen immunization and recall, and to compare their efficacy with the one induced by the injection on day 28 of $CD4^+CD49b^+$ Treg cells (Fig. 5C). We show that the injections of iDCs just after the recall of the immunization decreased only transiently the severity of arthritis, but the disease flared again from day 36, and reached a higher severity score than the control group. However, a single injection of 10^5 $CD4^+CD49b^+$ Treg cells decreased significantly the severity of the disease (from day 32 to 45, $P<0.05$) and protected the mice from severe arthritis until the end of the experiment. Because severity of CIA correlates with anti-CII IgG2a antibody level, we quantified the anti-bCII antibodies titers into mice sera. We showed that the protective effect induced by the $CD4^+CD49b^+$ Treg cells was associated with decreased IgG2a anti-bCII antibody levels compared with controls and iDC-treated mice whereas the anti-bCII IgG1 levels were similar between controls and Treg-treated mice (Fig. 5D).

4. Discussion

Results from several preclinical animal studies have established that the adoptive transfer of Treg cells can prevent induction of various autoimmune diseases including autoimmune diabetes [42-49], EAE [22,50-52], CIA arthritis [33,53,54], inflammatory bowel disease [24,53,55,56] and systemic lupus erythematosus [57]. In contrast, only few studies have shown that transfer of Treg-cells is also efficient in reverting active disease [46,47,49,54-56]. In the majority of these studies, the suppression of the disease was mediated by transgenic Treg cells expressing a T-cell receptor (TCR) specific for the pathogenic antigen. Moreover, also the transfer of natural $CD4^+CD25^+$ polyclonal Treg cells in these models was found to be efficient in inhibiting established disease [54,56]. However, it should be noted that suppression of autoimmunity after adoptive transfer of the latter cells was obtained in lymphopenic hosts in which activation or expansion of Treg-cell subsets can be influenced by homeostatic proliferation. In this study we showed that inducible regulatory T cells adoptively transferred into non-lymphopenic syngenic mice were able to not only dampen immune response in a model of contact hypersensitivity but also was able to reverse clinical symptoms of established arthritis.

We have previously demonstrated that repetitive injections of iDCs drive the expansion of IL-10-secreting $CD4^+CD49b^+$ Treg cells. It has also been shown that NKT cells and in particular type II NKT cells can exert potent immunoregulatory effects in experimental autoimmune encephalitis (EAE) [58]. In order to better define the $CD4^+CD49b^+$ T cells population, and particularly to distinguish these cells from type II NKT cells that are restricted by the MHC class-I like molecule CD1d but do not recognize the synthetic glycolipid α-galactosylceramide [59], it was crucial to analyze the MHC molecule responsible for their expansion. We demonstrate here that in vivo generation/expansion of the IL10-secreting $CD49b^+$ T cells is dependent on the expression of the MHC class II molecules, as their

expansion was lost after repetitive injections of iDCs isolated from MHCII$^{-/-}$ mice. These results demonstrate that IL-10-secreting CD49b^{+} Treg cells are not type I or type II NKT but act as an inducible regulatory T cell population without preferential usage of their TCR Vα and Vβ gene product. The expansion of such CD4^{+}CD49b^{+} T cells seems to be independent on the IL-10 secretion by iDCs since no IL-10 was measured in the supernatant of the iDCs in our experimental conditions. In accordance with this result, repetitive injections of iDCs generated from IL-10 knock-out mice were able to expand the IL10-secreting CD4^{+}CD49b^{+} T cells in wild type mice (data not shown).

These regulatory T cells are CD62L^{low} and CD44^{high} indicating that they are antigen-experienced. CD4^{+}CD49b^{+} T cells are induced by repetitive injections of both immature and mature DCs, however the IL-10 secretion was only observed following immature DC vaccination [60]. CD4^{+}CD49b^{+} T cells do not seem to represent a particular lineage of IL10-secreting Treg cells but are rather a result of an altered activation of CD4^{+} T cells that endowed them with the capacity to dampen inflammation. Interestingly the expansion of such IL-10-secreting CD4^{+}CD49b^{+} regulatory T cells was observed in the spleen of the DO11.10 mice following repetitive injections of unloaded iDCs suggesting that the expansion of such regulatory T cells is not driven by the antigen-specificity of the TCR. The molecular mechanism underlying the expansion of the IL-10-secreting CD4^{+}CD49b^{+} T cells is not clearly defined and the MHC-class II restricted antigen(s) driving their expansion is still unknown as it is for the conventional FoxP3^{+}CD4CD25^{+} Treg cells.

The homing properties of CD4^{+}CD49b^{+} and CD4^{+}CD49b^{-} cells purified from congenic mice were compared following their adoptive transfer using Thy1.1 marker as a read out. The percentage of both populations was found to be similar in the various organs tested, except a higher percentage of CD4^{+}CD49b^{+} T cells in the lymph nodes draining the immunized paws. These results suggest that the CD4^{+}CD49b^{+} cells may act in periphery by homing to inflamed

tissues and secondary lymphoid organs. Since the $CD4^+CD49b^+$ T cells are negative for CCR7 and positive for CXCR3 (data not shown), the receptor for inflammatory chemokines, their homing to the inflamed lymph nodes could be dependent on CXCR3 expression as it was previously demonstrated for NK cells [61].

To further validate the immunoregulatory potential of $CD4^+CD49b^+$ T cells, their impact of on the immune response during antigen sensitization was assessed using a model for epicutaneous CHS following the adoptive transfer of $0.7x10^5$ to $2.1x10^5$ cells into syngenic mice. The augmented ear swelling was inhibited at the lower dose, and in a dose-dependent manner, by the selective adoptive transfer of $CD4^+CD49b^+$ T cells compared with $CD4^+CD49b^-$ cells. Similar results have been obtained following adoptive transfer of natural suppressor $CD4^+CD25^+$ T cells, although the latter cells required a five-fold higher cell number in order to be efficacious, thereby underscoring the potent immunomodulatory potential of the $CD4^+CD49b^+$ T cell population [62].

Suppression of an already primed immune response is a major challenge in the treatment of chronic autoimmune diseases such as RA. Our results indicate that vaccination with iDCs after disease onset decreases only transiently the severity of the disease and suggest that such DC are not likely to be effective in a clinical situation of chronic established disease. The lack of efficacy could be explained by a possible in-vivo maturation of the injected iDCs or by the absence of Treg cell induction in an inflammatory environment. The poor therapeutic effect obtained following repetitive injections of iDCs under these experimental conditions underscores the notion that in vivo-maturation and stabilization of the tolerogenic potential of the injected-iDCs has to be carefully controlled. In this respect, using various immunomodulatory DCs in the context of established arthritis, recent results revealed that IL-10 modulated DC represents a promising strategy to reduce disease severity in the context of an ongoing immune response [63].

Finally, in the present study we validated the therapeutic potential of the IL-10-secreting $CD4^+CD49b^+$ T cells in established arthritis. Indeed, adoptive transfer of 10^5 iDCs-induced $CD49b^+$ Treg cells were able to decrease severity and to reverse symptoms of arthritis in mice whereas $CD4^+CD49b^-$ isolated from iDC-vaccinated mice did not. These results underscored the therapeutic potential of the $CD4^+CD49b^+$ regulatory T cells in non lymphopenic host and validate the use of such IL-10 secreting Treg cells in autoimmune diseases such arthritis. The molecular and cellular mechanisms underlying the clinical effect of the induced-$CD4^+CD49b^+$ Treg cells are still unclear. IL-10 could play a key role in the protection as this cytokine acts directly on various immune cells, inhibiting the production of IL-2 and TNFα by T cells, or, alternatively, via the inhibition of the production of pro-inflammatory cytokines such as IL-12 by antigen-presenting cells. However, the small number of adoptively transferred $CD4^+CD49b^+$ T cells did not allow the detection of IL10-secreting T cells in the draining lymph nodes of the protected animal. To unravel the molecular mechanisms responsible for the anti-arthritic effect provided by these regulatory T cells, the IL-10 blockade after the adoptive transfer of the $CD49b^+$ Tregs, would be informative. However all attempts to down-regulate IL-10 in CIA, already revealed a disease worsening [64-66] and thus such experimental procedure will not solve this issue. The clinical effect following the adoptive transfer of $CD4^+CD49b^+$ Treg cells was associated with a less severe inflammatory synovitis and a decrease in osteoclastogenesis and bone erosion suggesting that $CD4^+CD49b^+$ Treg cells inhibit osteoclastogenesis *in vivo* as previously reported for natural Treg cells [67,68]. The homing ability of the $CD4^+CD49b^+$ T cells suggests their migration into the lymph nodes draining the inflamed paws during arthritis, whereas the lower anti-bCII Ab titers in the sera of the protected mice suggest an impact of the $CD4^+CD49b^+$ Treg cells on the B cell immune response. Following adoptive transfer of the $CD4^+CD49b^+$ Treg cells, no decrease of the IL-6 level in the sera of the mice was

correlated with the improvement of the clinical symptoms (data not shown) as it was recently reported for the natural $CD4^+CD25^+$ suppressor cells [67].

Given the efficacy of the $CD4^+CD49b^+$ T-cells in various murine disease models [33,34] it would be interesting to identify their human counterpart. Indeed, recently, it has been shown that two integrin chains, CD18 and CD49b are co-expressed on human IL-10-secreting Treg cells with a frequency of this $CD18^{hi}CD49b^+$ T cell population varying between 1 and 4%, in peripheral blood of healthy individuals [69]. The secretion of IL-10 associated with the expression of the CD49b and an elevated expression of the CD18 integrins therefore might serve as useful biological marker for the identification of such IL-10-producing Treg cells. In a recent review [26], Roncarolo *et al*. proposed to use the term Tr1 cells for all IL-10-producing T cell populations that are induced by IL-10 and that have regulatory activity. However, the full biological characterization of Tr1 has been hampered by the lack of specific markers. The inducible IL-10-secreting $CD4^+CD49b^+$ Treg cells described in this study fulfil these criteria.

Although little information is available at present about a possible protective role of Treg cells in RA, it is of note that infliximab treatment of RA patients have been implicated in the induction of differentiation of a Treg cell population through the conversion of $CD4^+CD25^-$ T cells [70]. These cells express FoxP3 and low levels of CD62L and are therefore different from natural $CD4^+CD25^+$ suppressor Treg cells [71]. These induced Treg cells were found to mediate immune suppression via the production of both IL-10 and TGF-β. It is currently not known whether RA patients would benefit from interventions targeting inducible- or natural $CD4^+CD25^+$ Treg cells. However, the results from Ehrenstein et al. have emphasized an interest for inducible Treg cell populations for the treatment of RA. The various inducible Treg cells described so far support the need to precisely define which Treg cells are able to dampen efficiently the immune response in the disease settings of RA and the

present work brings a first proof of concept for the potential use of $CD4^+CD49b^+$ inducible Treg cells in arthritis.

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Figure Captions

Fig 1. Analyses of the T cell repertoire of the $CD4^+CD49b^+$ T cells. DBA/1 mice (*n*=10/group) were injected intraperitonaly 3 times (days -7, -5, and -3) with PBS or 0.5 x 10^6 iDCs. At day 0, mononuclear cells from the liver were analyzed by FACS for the staining of CD4 (CD4-Cy5), CD49b (DX5-PE) and various α and β chains of the TCR (FITC). A) Characteristic dot plots showing the expansion of the $CD4^+CD49b^+$ cells after injection of iDCs . B) The percentages of positive cells for the various Vα and Vβ, gated on the $CD4^+CD49b^+$ cells, were compared between PBS and iDCs-injected mice. Similar results were obtained in two independent experiments.

Fig. 2. Expansion of IL10-secreting $CD4^+CD49b^+$ T cells is dependent on the expression of the MHC class II molecules. A) C57BL/6 mice (*n*=3/group) were injected intraperitonaly 3 times (days -7, -5, and -3) with PBS or 0.5 x 10^6 iDCs derived from MHC class II knock-out ($iDC^{MHC\ II-/-}$) or wild type (iDC^{WT}) mice. At day 0, mononuclear cells from the liver were analyzed by FACS for the staining of CD4 and CD49b. The percentages of double positive cells are labelled in top pannels. The cytokine production by liver mononuclear cells was assessed by intracellular cytokine staining. The plots are gated on $CD4^+CD49b^+$ cells, and the percentage of IFN-γ and IL-10 secreting cells are labelled in the upper quadrants. B) Percentage of $CD4^+CD49b^+$T cells (left), IFNγ (middle) and IL-10 (right) producing cells of $CD4^+CD49b^+$cells, in the liver of mice following injections of PBS, iDC^{WT} or $iDC^{MHC\ II-/}$. Graph shows combined data from two independent experiments. Differences were analyzed with the nonparametric Mann-Whitney test (* = $p < 0.05$ and ** = $p < 0.005$ with a 95% confidence interval).

Fig. 3. $CD4^+CD49b^+$ T cells preferentially home to draining lymph nodes. $CD4^+CD49b^-$ and $CD4^+CD49b^+$ T cells were isolated from iDCs-vaccinated Thy1.1 mice, purified by cell sorting and 1 million of cells were adoptively transfered into Thy1.2 mice (n=3 per group) the same day of immunization into the right foot pad. Two days after, the distribution of the adoptively transferred $Thy1.1^+$ cells was evaluated by FACS analysis in blood, spleen, lymph nodes draining the immunized paw (DLN) or contralateral lymph nodes (NDLN). Non-immunized mice transferred with $CD4^+CD49b^+$ cells were used as controls (n=3). Dot plots are gated on $CD4^+$ cells and percentages of $Thy1.1^+$ cells are labelled in the upper quadrants. Results are representative of two independent experiments.

Fig. 4. $CD4^+CD49b^+$ T cells inhibit contact hypersensitivity to oxazolone**.** iDCs were generated and repetitively injected into syngenic mice the week before the isolation and purification of $CD4^+CD49b^+$ T cells from liver mononuclear cells. $CD4^+CD49b^+$ T cells were adoptively transferred the day of the sensitization of the mice with oxazolone (day -7). After repeated oxazolone challenges (day 0 and 5), the ear thickness in sensitized mice were compared with control mice, data are expressed as mean differences between the hapten-and vehicle-challenged ears. A) The augmented ear swelling in control mice (n=2) was reversed by the transfer of $0.7x10^5$ (n=3) or $2x10^5$ (n=3) in a dose dependent manner. Error bars represent SD. Similar results were obtained in three independent experiments. B) After challenge (day 0), the ear thickness of mice adoptively transferred with $1.5\ x10^5$ $CD4^+CD49b^+$ (n=6) or $CD4^+CD49b^-$ (n=5) T cells, were compared with control mice (n=2). Data are expressed as mean differences between the hapten-and vehicle-challenged ears. Error

bars represent SD. Differences were analyzed with the nonparametric Mann-Whitney test (* = $p < 0.05$ with a 95% confidence interval).

Fig. 5. Adoptive transfer of $CD4^+CD49b^+$ T decreases severity of established arthritis. A) Severity scores of arthritic mice adoptively transferred with 10^5 purified $CD4^+CD49b^+$ (n=7) or $CD4^+CD49b^-$ T cells (n= 6) at days 28 after immunization. Each paw was scored from 0 to 4 according to the severity of arthritis, with a maximal score of 16 per mouse. Values represent the mean ± SEM of each group. Results are representative of two independent experiments. B) Histological sections of hind paws from control, $CD4^+CD49b^+$ or $CD4^+CD49b^-$ injected mice were stained with hematoxylin and eosin (left) or for TRAP activity (middle), and area of erosion and number of osteoclast (right) were quantitively assessed. C) Severity scores of arthritic mice adoptively transfered with 10^5 purified $CD4^+CD49b^+$ at day 28 (n=8) or repetitively injected with 0.5 x 10^6 iDC (n=8) at days 21, 23 and 25 after immunization. Results are representative of two independent experiments. D) Anti-bCII IgG1 and IgG2a Ab titers were measured on days 30, 37 and 58 after immunization in sera of mice. Values represent the mean ± SEM of each group. Differences were analyzed with the nonparametric Mann-Whitney test (* = $p < 0.05$ with a 95% confidence interval).