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Dentritic cells and the battle against arthritis

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Chapter 4

Immature Dendritic Cells Suppress
Collagen-Induced Arthritis By In Vivo
Expansion Of CD49b+ Regulatory T
Cells

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Immature Dendritic Cells Suppress Collagen-Induced Arthritis by In Vivo Expansion of CD49b⁺ Regulatory T Cells¹

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Dendritic cells (DCs) are specialized APCs with an important role in the initiation and regulation of immune responses. Immature DCs (iDCs) reportedly mediate tolerance in the absence of maturation/inflammatory stimuli, presumably by the induction of regulatory T cells. In this study, we show for the first time that repetitive iDC injections trigger the expansion of a novel regulatory population with high immunomodulatory properties, able to protect mice from collagen-induced arthritis. These regulatory T cells are characterized by the expression of the CD49b molecule and correspond to a CD4⁺ α -galactosylceramide/CD1d-nonrestricted T cell population producing IL-10. Adoptive transfer of $<10^5$ TCR β^+ CD49b⁺ cells isolated from the liver of iDCs-vaccinated mice, conferred a complete protection against arthritis. This protection was associated with an attenuation of the B and T cell response associated with a local secretion of IL-10. Thus, together these data demonstrate that iDCs can expand and activate a novel regulatory population of CD49b⁺ T cells, with high immunosuppressive potential able to mediate protection against a systemic autoimmune disease. *The Journal of Immunology*, 2006, 177: 3806–3813.

Dendritic cells (DCs)⁴ are professional APCs that play a crucial role as initiators and modulators of adaptive immune responses. Although DC-based vaccines have been used successfully to generate cytolytic T cell activity against tumor Ags, evidence has accumulated that DCs also have a potent ability to tolerize T cells in an Ag-specific manner (1). Therefore, current and prospective strategies to promote the inherent tolerogenic potential of DCs are a rational approach for the therapy of autoimmune diseases such as rheumatoid arthritis (RA) (2).

Recent experimental evidence both in human and mouse experimental models has demonstrated that immature DCs (iDCs) can mediate tolerance, presumably by the induction of regulatory T cells. Repetitive in vitro stimulations of human cord blood-derived T cells with allogeneic iDCs was shown to result in the induction of nonproliferating, IL-10-producing T cells (3). Dhodapkar et al. (4) reported the Ag-specific inhibition of CD8⁺ T cell cytotoxic activity and the

appearance of peptide-specific IL-10-producing T cells in humans following in vivo injection of autologous iDC. Both studies associated the appearance of DC-induced regulatory T cells with the production of IL-10 and highlighted the importance of immune regulation rather than deletion of effector T cells. Nonetheless, the nature of these regulatory T cells was not addressed.

The relevance of using not fully mature DCs (mDC) for the induction of Ag-specific tolerance has also been tested in animal models of autoimmune disease (5, 6). Injection of TNF-treated DCs, exposed ex vivo to Ag, induced peptide-specific IL-10-producing T cells and prevented experimental allergic encephalomyelitis (EAE) (6). Similarly, we have shown that the vaccination of DBA/1 mice with semimature TNF-treated DCs pulsed with bovine collagen type II (bCII), protected mice from collagen-induced arthritis (CIA) (7). The protection was dependent on DC-bCII loading and correlated with a shift from Ag-specific Th1- to Th2-immunity. Use of iDC has been validated as the injections of iDC reduced the incidence of diabetes in prediabetic NOD mice (8). Altogether, these observations indicate that DC vaccination can be used for the design of novel interventions for the treatment of systemic autoimmune diseases.

In this study, we show that repetitive injections of iDCs, pulsed in medium without bCII, are also highly efficient in treating CIA by preventing arthritis incidence in >50% of the animals. Repetitive injections of DCs cause expansion of a CD49b⁺ T cell population in the liver and spleen of treated mice. Remarkably, adoptive transfer of the CD49b⁺ T cells isolated from iDC-treated animals, but not from TNF- or LPS-DC-treated mice, could efficiently transfer protection. Taken together, these results show that iDC can expand and activate a population of CD49b⁺ T cells with high regulatory potential that can mediate protection against a systemic autoimmune disease.

Materials and Methods

Animals

DBA/1 mice (Harlan) were bred in our facilities, and C57BL/6 mice were purchased from Janvier Laboratories. Experiments with animals were conducted in accordance with the national guidelines for animal care.

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⁴Abbreviations used in this paper: DC, dendritic cell; RA, rheumatoid arthritis; iDC, immature DC; mDC, mature DC; EAE, experimental allergic encephalomyelitis; bCII, bovine collagen type II; CIA, collagen-induced arthritis; α -GalCer, α -galactosylceramide; ICS, intracellular cytokine staining; PPD, purified protein derivative; LPS-DC, LPS-treated DC.

Generation and injections of DCs

DCs were generated as described previously (9, 10). Briefly, bone marrow cells were harvested from the femur and tibiae of DBA/1 mice and washed in RPMI 1640 following lysis of RBC. T and B cells were depleted using mouse pan T and pan B Dynabeads (DynaL Biotech), and monocytes were removed by adhesion in RPMI 1640 5% FCS. Remaining cells were cultured in complete medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamin, 5×10^{-5} M 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, essential amino acids, and 1 mM sodium pyruvate) with 1,000 IU/ml recombinant murine GM-CSF (R&D Systems) and 1,000 IU/ml recombinant murine IL-4 (R&D Systems) at 5×10^5 cells/ml in a 24-well plate. Culture medium was renewed at day 2 and 4. DCs were pulsed or not with dialyzed-bCII (2 μ g/ml) at day 6. To induce maturation of DCs, DCs were harvested at day 6, and incubated for 24 h with 50 ng/ml TNF (R&D Systems) or 1 μ g/ml LPS (Sigma-Aldrich) in presence of dialyzed-bCII. For *in vivo* experiments, DCs were harvested and washed twice. Mice were injected i.p. with 0.5×10^6 DCs in 100 μ l PBS, at 7, 5, and 3 days before the bCII immunization.

Abs and FACS analysis

DCs were identified using PE-anti-CD11c (HL3) and FITC-anti-MHC class II Abs (M5/114.15.2). CD11c/MHC class II double-positive cells revealed an average of 70% of DCs at day 6. DC maturation was determined based on the relative levels of FITC-MHC class II and PE-anti-CD40 (3 of 23), PE-anti-CD80 (16-10A1), and PE-anti-CD86 (GL1) expression. All Abs were obtained from BD Pharmingen, except for the MHC class II Ab (Miltenyi Biotec). DCs were incubated for 20 min in the dark with the appropriate Abs. After a washing step, the cells were fixed in 1% paraformaldehyde before analysis when needed.

Cell suspensions from blood, spleen, and lymph nodes were prepared according to standard procedures. Lymphocytes from liver were obtained as reported previously (11). Briefly, the liver was pressed through a 100- μ m cell strainer and suspended in PBS. After treatment with red cell lysis buffer, cells were washed three times with PBS. Mononuclear cells were isolated after centrifugation in isotonic 33.8% Percoll (Amersham Biosciences) for 12 min at $693 \times g$. Recovered leukocytes were washed before labeling. To avoid nonspecific binding of Abs to FcR γ , cells were preincubated with anti-mouse CD16/32 (2.4G2) mAb before staining with PE-conjugated anti-CD49b (clone DX5), FITC-conjugated anti-TCR β (H57-597), PE-Cy7-conjugated NK-1.1 (PK136), or PE-Cy5-conjugated CD4 (RM4-5). The PE-conjugated-CD11d tetramer loaded with α -galactosylceramide (α -GalCer) was provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA) and used as described previously (12). For the FoxP3 labeling with the FITC-conjugated FJK-16s Ab (eBioscience), enrichment of DX5-positive cells was performed using DX5 beads (Miltenyi Biotec) following the manufacturer's protocol. After washing with PBS, the stained cells were analyzed on a FACScan (BD Biosciences), and the data were processed with the CellQuest program (BD Biosciences).

Intracellular cytokine staining (ICS)

For ICS, perfused livers of two BALB/c mice per group were pooled for analysis. Liver mononuclear cells (0.5×10^6 cells/well in 96-wells plate) were stimulated overnight at 37°C with syngenic 0.1×10^6 iDC/well or 0.5×10^6 anti-CD3/anti-CD28 Ab-coated Dynabeads/well (DynaL Biotech). During the last 3 h of stimulation, 10 μ g/ml brefeldin A (Sigma-Aldrich) was added. Subsequently, cells were stained with PE-conjugated anti-CD49b (clone DX5) and PerCP-conjugated anti-CD4 (RM4-5) Abs (20 min, on ice). After washing step with PBA (PBS containing 0.5% BSA, 0.02% azide), cells were fixed in 4% paraformaldehyde for 5 min on ice. Cells were subsequently permeabilized in PBA containing 0.1% saponin (PBA-sap) supplemented with 10% FCS for 10 min on ice. ICS was performed with allophycocyanin-conjugated anti-IFN- γ (XMG1.2), anti-IL-5 (TRFK5), anti-IL-10 (JES5-16E3) Abs, or allophycocyanin-conjugated isotypes controls (R3-34 and A95-1) diluted in PBA-sap for 20 min on ice. Cells were washed with PBA and fixed in 1% paraformaldehyde. Data acquisition and analysis were performed on a FACSCalibur using CellQuestPro software (BD Biosciences).

TCR β^+ CD49b $^+$ cell isolation and adoptive cell transfer experiments

Mononuclear cells from the liver were isolated after centrifugation in isotonic Percoll as described above. Recovered leukocytes were washed before staining with anti-TCR β and anti-CD49b-conjugated Abs. Cell sorting was performed on FACS Vantage SE (BD Biosciences). After cell sorting, cells were washed, and 6×10^4 cells were injected i.v. in the tail vein of

DBA/1 mice, 12 h before arthritis induction. For adoptive cell transfer experiment with splenocytes of DC-vaccinated mice, DX5-positive cells were isolated with DX5 beads (Miltenyi Biotec) according to manufacturer's procedure. Purified 60×10^4 cells were injected i.v. in the tail vein of DBA/1 mice, 12 h before arthritis induction.

Induction and clinical evaluation of arthritis

Male 8-wk-old DBA/1 mice were immunized with bCII (BD Biosciences) as described previously (7). From day 21, the thickness of each paw was measured with a caliper every other day, 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 0.2-mm increase in joint diameter; 2 = significant swelling and redness with 0.2- to 0.3-mm increase in joint thickness; 3 = severe swelling and redness from joint to digit with 0.3- to 0.5-mm increase in joint thickness; and 4 = maximal swelling, deformity, and ankylosis with an increase in paw swelling above 0.5 mm. Each limb was graded, resulting in a maximal clinical score of 16 per animal and expressed as the mean score on a given day.

Radiological and histological examination

Radiological lesions of each paw were scored from 0 to 3 according to the presence of demineralization, narrowing of joint space, erosion, and loss of joint integrity. The hindpaws from freshly dissected mice were immersion-fixed in 4% paraformaldehyde for at least 24 h, decalcified, embedded in paraffin, and 5- μ m sections were stained with hematoxylin/eosin/safranin O. Paw sections were examined by two independent observers blind, and two different areas of each paw (mid- and hindfoot joints) were scored on four successive sections as described previously (13).

Measurement of serum anti-bCII and anti-PPD Ab levels

Serum level of Ab against bCII or purified protein derivative (PPD) was measured by a standard ELISA as described previously (7, 14). Ab units for bCII and PPD were determined using a reference serum created from pooled sera of arthritic mice and assigned an arbitrary level of Ag-specific Abs.

Analysis of cytokine production by lymph node cells

Popliteal and inguinal lymph node cells as well as spleen cells from the various mice were collected and cultured at 2×10^6 cells/well as published previously (7). Medium alone, 50 μ g/ml heat-denatured bCII or Con A (5 μ g/ml) were added. Supernatants were harvested at 24 and 48 h and stored at -20°C until tested. Murine IFN- γ , IL-10, and IL-4 in the supernatant were assayed by ELISA (R&D Systems).

Statistical analysis

Statistical difference in the incidence of arthritis between groups of mice was determined using exact Fisher's test. All others statistical analyses were performed using the nonparametric Mann-Whitney *U* test or Student's paired *t* test as appropriate according to data distribution.

Results

Immunoregulatory properties of iDCs

To examine the immunomodulatory role of immature myeloid DCs, iDCs were generated *in vitro* by culturing DBA/1 mouse

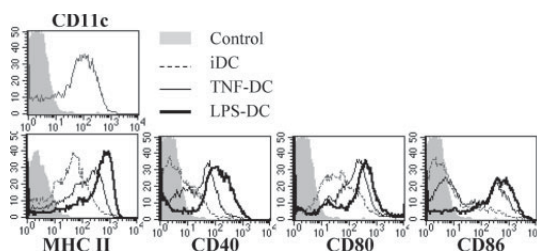
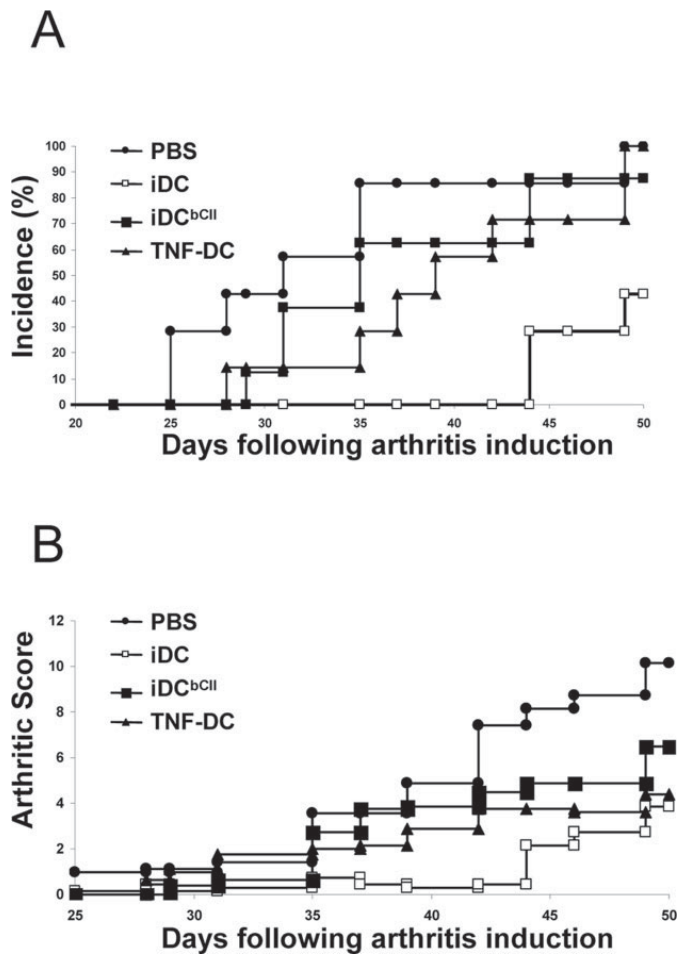


FIGURE 1. Phenotype of bone marrow-derived DCs. DCs were generated from DBA/1 mice after 6 days of culture of bone marrow-derived progenitor cells in the presence of GM-CSF and IL-4. At day 6, maturation was induced either by adding TNF- α (50 ng/ml) or LPS (1 μ g/ml) during 18 h. Double staining with anti-MHC class II and anti-CD11c, or anti-CD40, anti-CD80, and anti-CD86 mAbs was performed on iDCs, semi-mDCs (TNF-DC), and fully mDCs (LPS-DC), and cells were analyzed by flow cytometry.

FIGURE 2. Immunoregulatory properties of iDC. DBA/1 mice were injected with PBS or with 5×10^5 bCII-pulsed or -unpulsed iDCs, or bCII-pulsed TNF-DCs at days -7, -5, and -3 before arthritis induction. Clinical features of arthritis were determined until day 50 ($n = 7$ mice/group). **A.** Percentage of arthritic mice in each group during the course of the experiment. The incidence of arthritis differed significantly between the medium-pulsed iDC-treated group of mice and the PBS control group. **B.** Mean of the severity scores. Similar results were obtained in three independent experiments. p values were calculated for each day, using individual score data. The severity scores for the mice treated with unpulsed iDC differed from those treated with PBS from day 35 ($p < 0.016$) to day 50 ($p < 0.003$). Compared with PBS-treated mice, scores differed from day 44 ($p < 0.0314$) to day 49 ($p < 0.0013$) for mice treated with TNF-treated DC and at day 50 ($p < 0.017$) for bCII-pulsed iDC.



bone marrow precursors in the presence of GM-CSF and IL-4. We compared the therapeutic potential of repetitive injections of bCII-pulsed vs medium-pulsed iDCs before arthritis induction with collagen. We also included bCII-pulsed semi-mDCs obtained after addition of TNF- α , as we have shown previously that the latter cells protect against CIA in an Ag-dependent manner.

Table I. Clinical efficiency of iDC or semi-DC injections in CIA

	Onset ^a (days)	Amax ^b (mm)	Radiological ^c Score	Histological ^d Score
PBS	31.7 \pm 3.3	2.67 \pm 0.14	3.3 \pm 0.7	4.1 \pm 0.9
iDC	45.6 \pm 1.6*	2.16 \pm 0.08*	1.4 \pm 0.5*	1.4 \pm 0.5*
iDC ^{bCII}	35.6 \pm 2.3	2.45 \pm 0.13	2.8 \pm 0.7	2.9 \pm 0.6
TNF-DC	39.8 \pm 2.8	2.42 \pm 0.11	2.9 \pm 0.6	2.4 \pm 0.6

^a Day of clinical occurrence of arthritis observed in any of the hindpaws. Values represent the mean \pm SEM of all arthritic mice. The * indicates statistical significance ($p = 0.031$) using Student's t test.

^b Average of the maximal paw swelling reached by each paw during the course of the disease. Values represent the mean \pm SEM of all mice. The * indicates statistical significance ($p = 0.045$) using Student's t test.

^{c,d} Radiological and histological scores reached by each mouse. Each paw was graded from 0 to 3 according to the severity of arthritis. Values represent the mean \pm SEM of all mice. The * indicates statistical significance ($p = 0.019$) using Student's t test.

Complete characterization of DCs surface markers was performed by double-color flow cytometry (Fig. 1). Approximately 70% of cells were positive for both CD11c and MHC class II after 6 days of culture. DC maturation was monitored by the relative expression levels of MHC class II and costimulatory molecules such as CD40, CD80, or CD86. LPS-treated DCs (LPS-DCs) were used as control of full DC maturation. TNF-DCs displayed an intermediate expression level of costimulatory and MHC class II molecules consistent with our previous results (7). The iDCs showed a characteristic profile with low expression of MHC class II and CD80 molecules and weak expression of the CD40 and CD86 cell surface molecules. This phenotype was unchanged after loading the DCs with bCII (data not shown). Compared with TNF- or LPS-treated DCs, the weak ability of the iDCs to stimulate proliferative T cell responses was confirmed by MLR and was not altered by the bCII loading (results not shown).

Mice vaccinated with medium-pulsed iDCs showed significant delayed onset, lower incidence, and decreased severity of CIA, in comparison with bCII-pulsed iDCs, TNF-DCs, and the PBS-treated group, as assessed by paw swelling and radiological and histological analysis (Fig. 2 and Table I). The incidence of the disease in mice treated with medium-pulsed iDCs was 43% on day 50, whereas 100% of mice developed clinical signs of arthritis in PBS-treated mice (Fig. 2A).

Similarly, mice vaccinated with medium-pulsed iDCs displayed significantly less severe disease compared with PBS-treated mice (Fig. 2*B*), which was correlated with a significant decrease of the average maximal paw swelling (Table I). This reduction paralleled a complete abrogation of many characteristics of CIA, like inflammation of synovial tissue, infiltration of mononuclear cells into the joint cavity, and synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion in >80% of the mice, as determined by histopathological analyses (Table I). As described previously, treatment with bCII-pulsed TNF-DCs decreased the severity of arthritis (7). A similar protective effect was observed in this study after injections of bCII-pulsed iDCs. Remarkably, repetitive injections of medium-pulsed-iDCs offered the best protection against disease.

Expansion of $TCR\beta^+CD49b^+$ cells by repetitive injections of DCs

To investigate the possible mechanism responsible for dampening disease outcome after vaccination with iDCs, we monitored the increase of several potential regulatory cells, such as $CD4^+CD25^+$ T cells, NK cells, and NKT cells. Peripheral blood, liver, spleen, and lymph nodes from mice that had received iDCs were collected and analyzed by flow cytometry for the presence of the specific markers. No significant variation in the presence of the $CD4^+CD25^+$ T cell population was detected in all tissues tested. However, a significant increase of the

$TCR\beta^+DX5^+$ cells was measured in both liver (Fig. 3) and spleen. The DX5 Ab has been described to recognize a cell surface molecule expressed on conventional NK cells, identified as CD49b (α_2 integrin), but also stains 2–4% of $CD4^+$ T cells, which were initially thought to correspond to NKT cells. In the spleen, the frequency of $TCR\beta^+CD49b^+$ cells increased in a statistically significant manner from 1.1% in PBS-injected mice to 1.6% in iDCs and 1.9% in TNF-DC-treated mice ($p < 0.001$). The $TCR\beta^+CD49b^+$ population was barely modified in the spleen of LPS-DC-injected mice. In contrast, in the liver, as shown in Fig. 3*A*, the $TCR\beta^+CD49b^+$ population was significantly increased in all DC-injected groups ($p < 0.001$). This population accounted for 4–5% of the control liver lymphocytes and increased to 9–14% of liver lymphocytes from DCs vaccinated mice, independently of the maturation and the bCII loading of the DCs. The $TCR\beta^+CD49b^+$ cells expressed slightly lower $TCR\beta$ level than mature $\alpha\beta$ T cells and less DX5 than $TCR\alpha\beta^-$ NK cells (Fig. 3*A*). This population was also characterized by the expression of CD4 and CD18 (data not shown). Because the DBA/1 strain is NK1.1 negative, we investigated the effect of repetitive iDC injections on the $CD49b^+$ vs $NK1.1^+$ populations in the C57BL/6 mice that are positive for the NK1.1 marker, to obtain a better characterization of the phenotype of these cells (Fig. 3*B*). A clear increase of the $CD4^+CD49b^+$ population in the liver of animals injected with

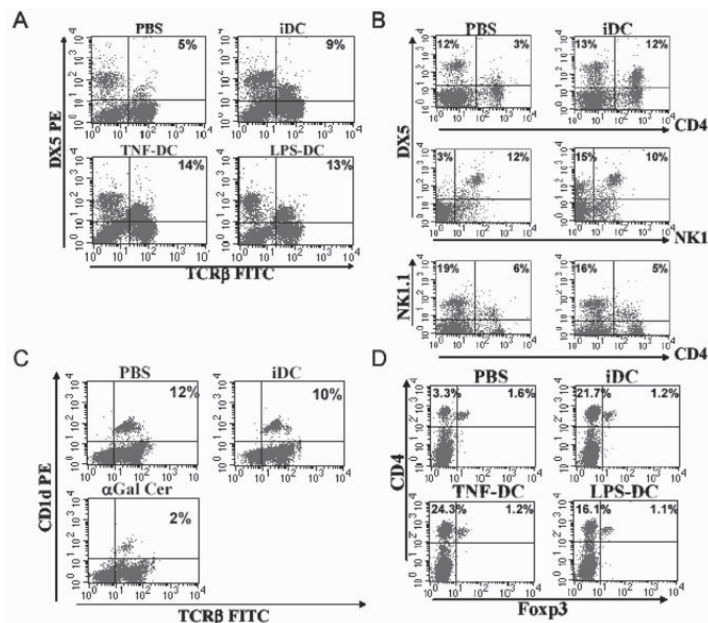


FIGURE 3. Expansion of a CD1d-independent $CD49b^+$ T cell population induced by repetitive injections of DCs. *A*, Expansion of $TCR\beta^+DX5^+$ cells in the liver of DC-vaccinated mice. DBA/1 mice were killed after three injections with PBS, iDC, semi-mDC (TNF-DC), or fully mDCs (LPS-DC). Mononuclear cells isolated from the liver were harvested from DC-injected or PBS-injected mice and stained for the expression of the $TCR\beta$ -chain ($TCR\beta$ FITC) and CD49b (DX5 PE) for FACS analysis. The means of the percentage of double-positive $TCR\beta^+DX5^+$ cells are indicated ($n = 15$ mice/group). Similar results were obtained in four independent experiments. *B*, Phenotypic analysis of the expanded population in a NK1.1-positive strain. C57BL/6 mice were killed after three injections of PBS or iDCs. Mononuclear cells from the liver were analyzed by immunostaining and flow cytometry for expression of the NK1.1 (NK1.1 PE-Cy7), CD49b (DX5 PE), and CD4 (CD4 FITC) markers. *C*, The population of $DX5^+$ cells represent CD1d-independent T cells. Mononuclear cells from the liver of PBS- or iDC-injected mice were stained with PE-conjugated CD1d tetramers loaded with α -GalCer (CD1d PE) and $TCR\beta$ -chain ($TCR\beta$ FITC). As a positive control, mice were injected with $4 \mu\text{g}$ of α -GalCer i.v. 2 h before euthanasia (α -GalCer). Data are representative of six mice per group. *D*, $DX5^+$ cells were purified by magnetic beads from pooled mononuclear cells isolated from the liver of DC-injected mice ($n = 11$ mice in each group). Purified $DX5^+$ cells were stained with CD4 (PE6-Cy7) and subjected to intracellular staining for the detection of FoxP3 (FoxP3-FITC). Percentage of positive cells are indicated in the upper quadrants.

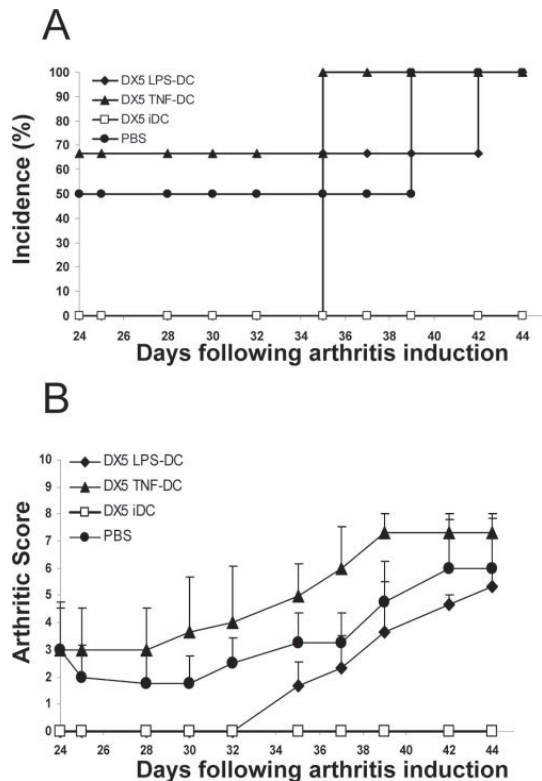


FIGURE 4. Adoptive cell transfer experiments with TCR β^+ CD49b⁺ from the iDC-vaccinated mice prevents arthritis. DBA/1 mice were killed after three injections of iDC, semi-mDC (TNF-DC), and fully mDCs (LPS-DC). Mononuclear cells were isolated from the liver of the various DC-injected groups ($n = 11$ mice/group), and the TCR β^+ CD49b⁺ cells were sorted by FACS, named iDCDX5, TNF-DCDX5, and LPS-DCDX5, respectively. Purified cells (6×10^4) were injected i.v. to age-matched male DBA/1 mice 1 day before arthritis induction. *A*, Percentage of arthritic mice in each group. *B*, Severity scores of arthritis. 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 \pm SEM of each mouse. Data are from at least three mice per group. Results are representative of two independent experiments.

iDCs was observed compared with those treated with PBS only. However, these CD49b⁺ cells do not express NK1.1, suggesting that the TCR β^+ CD49b⁺ cells are not classical type I NKT cells (15). To support this result, we stained this population with the α -GalCer/CD1d-tetramer, which is the most specific NKT cell marker available (12, 16). No variation of tetramer-positive cells was found in the liver of iDC-injected mice (Fig. 3C), whereas a clear decrease of the tetramer-positive cells was observed in the liver of mice following NKT cell activation after i.v. injection of α -GalCer. These results further indicate that the TCR β^+ CD49b⁺ population, expanded after injections of iDCs corresponded to α -GalCer/CD1d-nonreactive T cells.

Because the expression of the forkhead transcription factor (FoxP3) is specific for regulatory T cells (17), mononuclear cells from the liver of iDC-injected mice were purified by magnetic sorting with DX5-coated beads and analyzed by flow cytometry. A small subset of the CD4⁺CD49b⁺ cells was positive

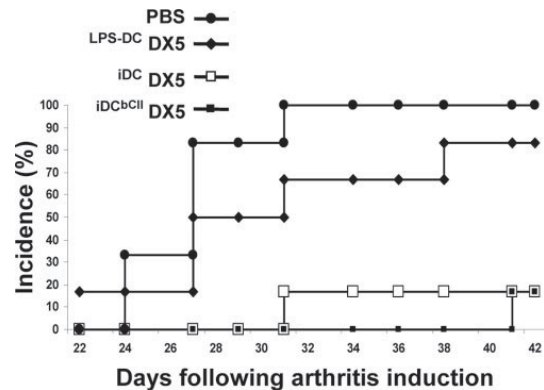


FIGURE 5. Adoptive cell transfer experiment with DX5⁺ cells isolated from the spleen of DC-treated animals. Splenocytes were isolated from DBA/1 female mice ($n = 12$ mice/group) after repetitive injections of bCII-loaded iDCs, medium-pulsed iDCs, or LPS-DCs. DX5⁺ cells were purified by magnetic beads and injected i.v. to age-matched male DBA/1 mice 1 day before arthritis induction. Percentage of arthritic mice in each group are shown. The incidence of arthritis differed significantly between the iDC-treated group and the PBS control group ($p < 0.05$). Data are from six mice per group.

for the expression of FoxP3 (Fig. 3D). However, the expansion of this population after iDCs injection was not correlated with an increase of FoxP3⁺ cells, suggesting that the increased CD4⁺CD49b⁺ population is essentially not expressing FoxP3 and is different from the population of CD4⁺CD25⁺ natural suppressor T cells. Together, these data indicate that repetitive injections of iDCs expanded in the liver and spleen of injected mice a α -GalCer/CD1d-independent T cell population that is positive for the CD49b marker.

Transfer of the protection by the TCR β^+ CD49b⁺ population isolated from iDC-vaccinated mice

Because DX5⁺ NKT/T cells have been shown to mediate protection in models for EAE and diabetes (18–21), we next evaluated the protective role of the DX5⁺ T cell population that had been induced by DC injection in adoptive cell transfer experiments in CIA. The TCR β^+ CD49b⁺ cells were purified from the liver lymphocytes of mice that had been injected with unpulsed iDCs, TNF-DCs, or LPS-DCs. Subsequently, 6×10^4 isolated cells were injected i.v. to naive DBA/1 males, 1 day before arthritis induction. Mice injected with TCR β^+ CD49b⁺ cells, isolated from the liver of iDC-vaccinated group, were completely protected against arthritis, as indicated by the low level of incidence and paw swelling severity, until at least 44 days after disease induction (Fig. 4). In contrast, mice injected with TCR β^+ CD49b⁺ cells isolated from either TNF-DC- or LPS-DC-treated groups showed incidence and severity of CIA comparable to PBS-treated mice. Clinical observations did correlate with radiological and histological analysis of the paws (data not shown). Similarly, transfer of DX5⁺ cells that had been purified by magnetic sorting from the spleen of bCII-pulsed and -unpulsed iDC-injected mice conferred protection against arthritis (Fig. 5). Thus, the CD49b⁺CD4⁺ T cells expanded after repetitive injections of iDCs exhibit high protective properties, whereas the TCR β^+ CD49b⁺ population induced by semimature or mDCs had no effect on the development of the disease.

Protection associated with attenuation of the B and T cell responses, and a local secretion of IL-10 by activated lymph node cells

We next investigated the mechanisms underlying the protection against CIA following the adoptive transfer of TCR β^+ CD49b $^+$ T cells isolated from iDC-injected mice (i DC DX5). High levels of autoreactive IgG2a Abs directed against bCII are associated with the development of CIA and correlate with disease severity. To investigate whether serum levels of bCII-specific Abs were altered after infusion of TCR β^+ CD49b $^+$ T cells, mice sera were analyzed for the presence of anti-bCII IgG2a and IgG1 isotypes at various times during the disease course. High levels of bCII-specific IgG Abs were measured in the PBS-treated mice, as well as in mice injected with TCR β^+ CD49b $^+$ cells, isolated from mice treated with either TNF-DC or LPS-DC. However, a significant decrease in the levels of both anti-bCII IgG isotypes was observed in the i DC DX5-treated group (Fig. 6A). Because CIA is primarily an Ab-driven disease, these results are significant and suggest that the amelioration of the disease, mediated by TCR β^+ CD49b $^+$ T cells, is due to the reduction of the Ab response. To investigate whether the reduction in IgG titer was Ag-specific, we also analyzed the Ab response against PPD. Because PPD is a component of CFA, mice vaccinated with bCII in CFA will also mount an anti-PPD response. A similar decrease in serum levels of anti-PPD IgG1 and IgG2a was observed (our unpublished data), thereby confirming the bCII-nonspecific inhibition of the humoral response after injection of TCR β^+ CD49b $^+$ cells purified from iDC-treated mice.

Because CIA is considered as a Th1-mediated autoimmune disorder, and because Th2-derived cytokines have been shown to ameliorate the disease, we analyzed the effect of adoptive TCR β^+ CD49b $^+$ cell transfer on the Th1 and Th2 cytokine pro-

duction by cells from spleen and draining lymph nodes. The cytokine secretion profile of splenocytes isolated from mice protected by the adoptive transfer of i DC DX5 T cells was partially modified with a decrease in IFN- γ secretion compared with controls (Fig. 6B). The protection was also associated with a 4-fold decrease in IFN- γ secretion and a slight decrease in IL-4 secretion by activated draining lymph node cells, as compared with other groups. Interestingly, a striking up-regulation of IL-10 secretion was measured in the supernatant of cells from draining lymph node after stimulation with Con A (Fig. 6C). In all these experiments, polyclonal stimulation of spleen cells and lymph nodes cells was necessary to induce a sizeable amount of cytokine secretion because bCII-stimulation was below detection level, except for the control group. These results can be explained either by the fact that the frequency of bCII-specific T cells are below detection level due to the fact that the T cell proliferation assays were performed at euthanasia >40 days after the first immunization, or by the non-Ag-specificity of the T cells. Altogether, these results suggest that the protective effect induced by adoptive transfer of the i DC DX5 $^+$ cells primarily occurs in the lymph nodes, draining the site of the vaccination because the prominent up-regulation of IL-10-production is predominantly found at this site.

Induction of IL-10-producing CD4 $^+$ CD49b $^+$ cells by iDC vaccination

To characterize the regulatory T cell population that is specifically induced by iDCs, we compared the cytokine production profile of the CD4 $^+$ CD49b $^+$ cells expanded in the liver after immature vs mDC vaccination by performing IFN- γ , IL-5, and IL-10 ICS (Fig. 7). Compared with control mice that received three injections of PBS, both iDC- and LPS-DC-vaccinated mice showed a significant

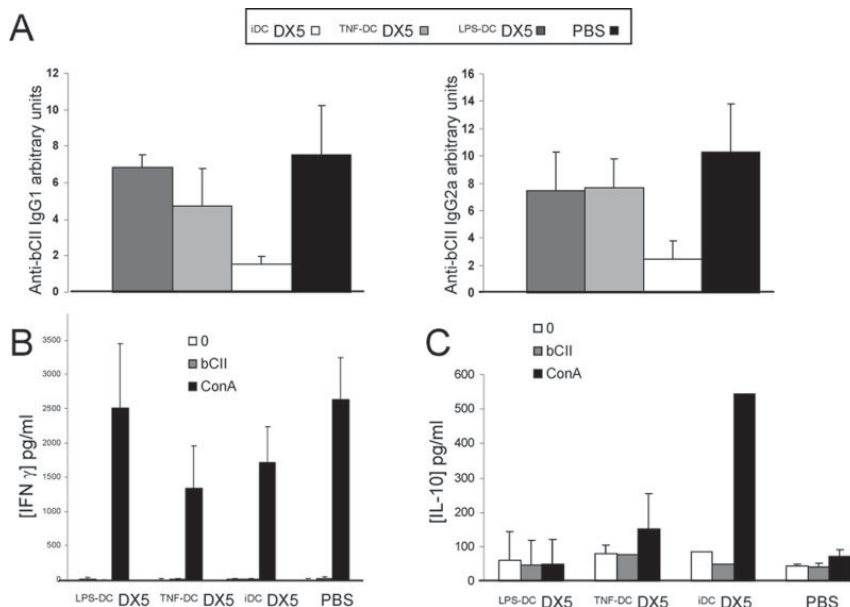


FIGURE 6. CD49b $^+$ T cells expanded by iDCs decreased bCII-specific IgG levels and increased local IL-10 secretion. *A*, bCII-specific serum IgG1 and IgG2a levels collected 30 days after primary immunization with bCII. Values represent the mean \pm SEM of arbitrary units (3–4 mice/group). *B*, IFN- γ level in culture supernatant from splenocytes after 24-h stimulation with 5 μ g/ml Con A or 50 μ g/ml denatured bCII. *C*, IL-10 level assayed by ELISA in culture supernatants from draining lymph nodes after 24-h stimulation with 5 μ g/ml Con A or 50 μ g/ml denatured bCII. Cells from lymph nodes were counted for each animal and analyzed individually, except for the protected mice by adoptive cell transfer of i DC DX5. Due to the small amount of recovered cells, a pool of the four mice was analyzed for IL-10 secretion. Results are representative of two independent experiment.

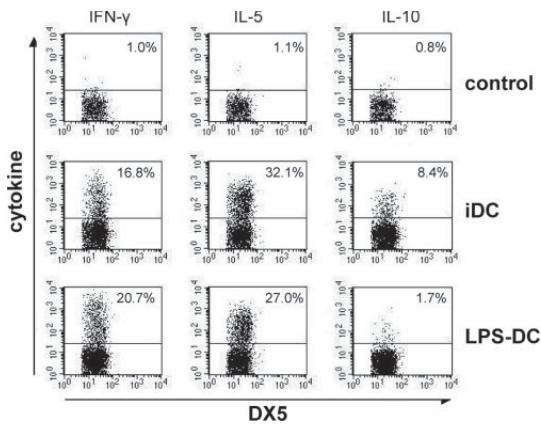


FIGURE 7. Induction of IL-10-producing CD4⁺CD49b⁺ cells by iDC vaccination. Mice were injected three times (days -7, -5, and -3) with PBS (control) or 0.5×10^6 iDCs or LPS-DCs. At day 0, the cytokine production of CD4⁺CD49b⁺ cells from the liver was analyzed ($n = 2/\text{group}$). Liver mononuclear cells were incubated overnight with iDC, and ICS was performed for IFN- γ , IL-5, and IL-10. Plots were gated on CD4⁺DX5⁺ cells. The percentages of cytokine-producing CD4⁺CD49b⁺ cells are indicated. One of two experiments giving similar results is shown.

increase in IFN- γ - and IL-5-producing CD4⁺CD49b⁺ cells after *in vitro* stimulation with iDC. Similar results were obtained when the cell population was stimulated with anti-CD3/anti-CD28 Ab-coated beads (data not shown). Interestingly, only in the CD4⁺CD49b⁺ population isolated from iDC-vaccinated mice, a significant portion of cells produced IL-10 in response to stimulation with iDC. Indeed, CD4⁺CD49b⁺ cells isolated from control and LPS-DC-vaccinated mice showed no significant or marginal IL-10 production. All of these results indicate that CD49b⁺ T cells are activated following repetitive injections of DCs, but that only iDC vaccination induces specifically the expansion of CD4⁺CD49b⁺ cells that are able to produce the regulating cytokine IL-10.

Discussion

In this study, we show for the first time, following iDC injections, the expansion of a novel regulatory population with high immunomodulatory properties, able to protect mice from an experimental model of autoimmune disease. The TCR β ⁺CD49b⁺ cells accounted for 4–5% of the control liver lymphocytes and increased to 8–14% in the liver lymphocytes of mice that received DC injections. Adoptive transfer of $<10^5$ TCR β ⁺CD49b⁺ cells, isolated from the liver of DC-vaccinated mice, was able to confer a complete protection against arthritis, only when injected DCs were immature. This protection was associated with an attenuation of the B and T cell response and secretion of IL-10 by CD49b⁺ T cells from liver as well as by activated cells isolated from lymph nodes draining the site of immunization.

The induction of Ag-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. Although the underlying mechanisms are not fully elucidated, generation of regulatory T cells by tolerogenic/regulatory DC represents the subject of intensive investigations. As it was previously demonstrated in diabetes (5), we show in this study that bone marrow-derived iDCs generated in the presence of GM-CSF and IL-4 are potent immunomodulatory cells able to induce protection against arthritis. In both studies, Ag pulsing of the iDC did not

improve their ability to prevent disease, suggesting either that DCs may process and present autoantigens to T cells *in vivo* or are able to mediate a non-Ag-specific therapeutic effect. In this study, we show that iDC can induce tolerance and/or modulate the outcome of autoimmunity by the induction of a CD49b⁺ regulatory T cell population, which represents a novel mechanism that is used by DC to steer the outcome of an immune response. We previously reported that the repetitive injections of mice with bCII-pulsed TNF-treated DC were also able to protect mice from arthritis by skewing the T cell response to a Th2-phenotype with a high secretion of IL-4 and IL-5 (7). These results highlight the various regulatory mechanisms established by differentially modulated DCs to achieve tolerance.

Both in the case of immature and mature regulatory DCs, DC-derived IL-10 has a crucial role in the development of adaptive regulatory T cells, although various other soluble or membrane-bound molecules are likely to be involved (22–24). In our study, the induction of CD49b⁺ regulatory T cells by iDC seems to be IL-10 independent because the myeloid bone marrow-derived DC generated in presence of GM-CSF and IL-4 do not secrete IL-10 even after maturation (data not shown). We showed in this study that the CD49b⁺ T cells expanded following repetitive injections of semimature or fully mDC did not protect mice against arthritis. These results suggest that the suboptimal Ag presentation and/or costimulation exerted by DC seems to play a crucial role in the outcome of reactivity mediated by these TCR β ⁺CD49b⁺ regulatory T cells. Although it is currently not known how the TCR β ⁺CD49b⁺ T cells are activated by iDC, our observations are in line with the hypothesis that steady-state DCs act as guardians that defend the integrity of tissues by tempering undesired immune responses. Our data indicate that the activation and skewing of TCR β ⁺CD49b⁺ T cells contribute to this protective function of iDC.

The regulatory T cells induced following DC-vaccination are characterized by the expression of the CD49b molecule, the integrin α_2 chain. We showed that they are CD4⁺, α -GalCer/CD1d-nonrestricted, and do not express the FoxP3 transcription factor, demonstrating that these regulatory T cells are different from the CD4⁺CD25⁺ natural suppressor T cells. To gain more insight into the unique protective properties of the regulatory T cell population that is specifically induced by iDCs, we compared the cytokine production profile of the CD4⁺CD49b⁺ cells expanded after iDC vs mDC vaccination. We demonstrated that CD4⁺CD49b⁺ cells isolated from the liver of iDC- and mDC-vaccinated mice were able to produce IFN- γ and IL-5 after overnight stimulation with either iDC or anti-CD3/CD28-coated beads. In contrast, CD4⁺CD49b⁺ cells isolated from PBS-treated mice displayed no comparable expression of these cytokines, indicating that a differential program is induced in the latter CD49b⁺ cells compared with cells activated by DC. More importantly, we demonstrated that iDC vaccination induces specifically the expansion of CD4⁺CD49b⁺ cells that are able to produce the regulating cytokine IL-10. Although the mode of action of these CD49b⁺ T cells needs further investigation to understand their unique protective properties, these results suggest an activation of the CD49b⁺ T cells after injections of DCs compared with PBS-treated mice and a specific expansion of IL-10-producing cells by iDC vaccination.

Recently, it has been described that, following activation by adjuvant, CD49b⁺ NK cells are recruited in lymph nodes draining the immunization site (25). Although, the TCR β ⁺CD49b⁺ cells that are induced after vaccination with iDC are, most likely, not classical CD49b⁺ NK cells because they express a $\alpha\beta$ TCR, it is tempting to speculate that a similar mechanism may attract CD49b⁺ T cells to the draining lymph nodes, accounting for an

attenuation of the humoral immune response against immunizing Ags (bCII and PPD).

Very few studies have specifically examined the *in vivo* function of CD49b⁺ α -GalCer-CD1d-independent T cells. The immunosuppressive capabilities of these cells were first evidenced in a TCR transgenic NOD mouse model of type 1 diabetes (18). In that study, blockade of IL-10 or TGF- β partially impeded the protection mediated by DX5-expressing cells. The TCR β ⁺CD49b⁺ T cells that are expanded following injections of iDCs seem to share many characteristics with these previously described cells. Repetitive stimulations of the CD49b⁺ T cells may favor the development of their regulatory properties as recently demonstrated for the NKT cells after repetitive stimulation with α -GalCer (26). It will be important to investigate whether the TCR β ⁺CD49b⁺ T cells represent a particular lineage of regulatory cells, as well as to understand their specificity and molecular targets to get a full appreciation of their protective properties after expansion by iDC. To our knowledge, this is the first report defining a clear *in vivo* correlation between injection of iDCs and expansion of a CD49b⁺ T cell population with high immunosuppressive properties in a murine model of autoimmune disease.

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Disclosures

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