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Human Adipose-Derived Mesenchymal Stem Cells Modulate Experimental Autoimmune Arthritis by Modifying Early Adaptive T Cell Responses

MERCEDES LOPEZ-SANTALLA,[a] PABLO MANCHEÑO-CORVO,[b] RAMON MENTA,[b] JUAN LOPEZ-BELMONTE,[c] OLGA DELAROSA,[b] JUAN A. BUEREN,[a] WILFRIED DALEMANS,[d] ELEUTERIO LOMBARDO,[b] MARINA I. GARIN[a]

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[a]Unidad Mixta de Terapias Avanzadas, CIEMAT/IIS Fundación Jiménez Díaz/ CIBERER, Madrid, Spain; [b]TiGenix SAU, Madrid, Spain; [c]FARMACROS, Albacete, Spain; [d]TiGenix NV, Leuven, Belgium

Correspondence: Marina I Garin, Ph.D., Division of Hematopoietic Innovative Therapies (HIT) & Advanced Therapy Unit, CIEMAT, Avda. Complutense, 40 Edif. 70, P0. 13, 28040 Madrid, Spain. Telephone: + 34-91-4962525; Fax: + 34-91-3466484; e-mail: marina.garin@ciemat.es

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stromal cells with immunosuppressive properties. They have emerged as a very promising treatment for autoimmunity and inflammatory diseases such as rheumatoid arthritis. Recent data have identified that GM-CSF-expressing CD4 T cells and Th17 cells have critical roles in the pathogenesis of arthritis and other inflammatory diseases. Although many studies have demonstrated that MSCs can either prevent or suppress inflammation, no studies have addressed their modulation on GM-CSF-expressing CD4 T cells and on the plasticity of Th17 cells. To address this, a single dose of human expanded adipose-derived mesenchymal stem cells (eASCs) was administered to mice with established collagen-induced arthritis. A beneficial effect was observed soon after the infusion of the eASCs as shown by a significant decrease in the severity of arthritis. This was accompanied by reduced number of pathogenic GM-CSF^{+}CD4^{+} T cells in the spleen and peripheral blood and by an increase in the number of different subsets of regulatory T cells like FOXP3^{+}CD4^{+} T cells and IL10^{+}IL17^{-}CD4^{+} T cells in the draining lymph nodes (LNs). Interestingly, increased numbers of Th17 cells coexpressing IL10 were also found in draining LNs. These results demonstrate that eASCs ameliorated arthritis after the onset of the disease by reducing the total number of pathogenic GM-CSF^{+}CD4^{+} T and by increasing the number of different subsets of regulatory T cells in draining LNs, including Th17 cells expressing IL10. All these cellular responses, ultimately, lead to the reestablishment of the regulatory/inflammatory balance in the draining LNs. STEM CELLS *2015;33:3493–3503*

SIGNIFICANCE STATEMENT

We identify, for the first time, a novel mechanism by which adipose-derived mesenchymal stem cells modulate ongoing immune responses by promoting an early adaptive T cell signature characterized by decreased levels of pathogenic GM-CSF-secreting CD4+, superscript T cells, increased levels of regulatory T cells and plasticity of effector Th17 cells towards an IL10-driven anti-inflammatory response thus shifting the inflammatory/regulatory balance from GM-CSF inflammatory predominance to IL10 regulatory predominance. Altogether, these data will offer much needed further insight into the mechanisms of action of mesenchymal stem cells for their translation to the clinic.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells that exist in many tissues and contribute to tissue regeneration, among other effects, by modulating inflammation [1–4]. In recent years, stem cell therapy protocols for the treatment of immune-mediated disorders such as graft versus host disease [5], systemic lupus erythematosus [6], inflammatory bowel disease [7–10], autoimmune encephalomyelitis [11, 12], and rheumatoid arthritis (RA) [13, 14] have been proposed. MSCs were initially isolated from bone marrow [2]. Since then, MSCs have been isolated from the stroma of multiple organs and tissues, including adipose tissue [15], tonsils [16], umbilical cord [13, 17], and dental pulp [18–20]. Based on their accessibility, we and others have shown that human adipose-derived MSCs represent a realistic source for cell therapy protocols [15, 21–23].

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RA is an autoimmune disease caused by loss of immunologic self-tolerance that leads to chronic inflammation of the joints followed by subsequent cartilage destruction and bone erosion [24]. Additionally, recent data from murine and human studies have identified that granulocyte macrophage colony-stimulating factor (GM-CSF)-expressing CD4 T cells are instrumental during the induction phase of RA [25–28]. Currently, ongoing clinical trials using monoclonal antibodies against the GM-CSF protein or GM-CSF receptor alpha have shown significant responses in RA patients [29–31].

Th17 cells have previously been described to have critical roles in the pathogenesis of arthritis [32, 33]. The functional plasticity of the T helper lineages has become increasingly evident, particularly during in vivo immune responses [25, 34–40]. Presently, T helper cells are found to be able to express alternative lineage cytokines, such as Th17 cells expressing interferon gamma (IFNg) INFγ or interleukin (IL) IL10 and Th1 cells expressing IL17, GM-CSF, or, even, IL10, defining a more refined phenotype and effector functions of these T cell subsets [35, 38, 40–42]. Accordingly, induction of an alternative lineage cytokine production may potentially have a major impact that leads to the reestablishment of homeostasis as well as an adequate regulatory/inflammatory balance [43]. The presence of Th17 subsets with regulatory functions correlates with their ability to produce IL-10 [35, 38, 41, 44]. This means that Th17 cells are not at the final stage of their differentiation programs and have the potential to be converted into alternative subsets of CD4 T cells depending on the cytokine environment they encounter [36, 39, 40, 45].

Preclinical and clinical studies have shown that cell therapy protocols using MSCs represent a promising tool to treat RA [6, 13, 46–48]. Different immune responses and mechanisms of action have been implicated in the beneficial action of the MSCs. Among those, induction of regulatory T cells [13, 46, 47] and regulatory B cells [49], enhanced secretion of molecules with anti-inflammatory effects such as prostaglandin E2 (PGE2), transforming growth factor beta (TGFβ) [50], and human leukocyte antigen G (HLA G) [51], altered maturation of dendritic cells [52] and generation of macrophages with regulatory phenotype [53] have been documented [54]. However, effects of MSCs on pathogenic GM-CSF-expressing CD4 T cells have not been investigated. At present, limited information is available regarding the effects of MSCs on the functional plasticity of Th17 cells.

The main purpose of our study was to define early T cell responses triggered upon infusion of human expanded adipose-derived MSCs (eASCs) after the onset of collagen-induced arthritis (CIA) in mice. We evaluated the effect of eASCs on pathogenic GM-CSF$^+$CD4$^+$ T cells and Th17 cells, and in different subsets of regulatory T cells. We found that treatment with eASCs reduced the levels of pathogenic GM-CSF$^+$CD4$^+$ T cells and induced plasticity in IL17$^+$CD4$^+$ T cells by inducing the expression of IL10 cytokine. Additionally, increase numbers of different subsets of regulatory T cells in draining lymph nodes (LNs) were observed. All these early T cell responses contribute to the reestablishment of the homeostatic regulatory/inflammatory balance in the periphery.

Materials and Methods

Mice

DBA/1 (H-2q) mice were purchased from Janvier SAS, France. To conduct the experiments, 8 weeks old male mice were used. All experiments were performed in accordance with the corresponding regulations regarding experimental animal welfare (RD 223/1988 and Directive 2010/63/EU protocols) and approved by the Institutional Animal Care and Use Committee at the University of Albacete, Spain.

Generation of Human Expanded Adipose-Derived Stromal Cells

Human samples were obtained after informed consent as approved by the Spanish Ethics Committee of reference for the site of tissue procurement (Clínica de la Luz Hospital, Madrid, Spain). Human adipose tissue aspirates from healthy donors were washed twice with phosphate-buffered saline and digested with 0.075% collagenase (Type I, Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The digested sample was washed with 10% fetal bovine serum (FBS), treated with 160 mM NH_4Cl to eliminate remaining erythrocytes and suspended in culture medium (Dulbecco's modified Eagle's medium with 10% FBS). Cells were seeded ($2–3 \times 10^4$ cells per cm^2) in tissue culture flasks and adherent cells were expanded (37 °C, 5% CO_2) with change of culture medium every 3–4 days [55]. Cells were transferred to a new flask (10^3 cells per cm^2) when they reached 90% confluence. Cells were expanded up to duplication 12–14 and frozen. Experiments were performed with cells from one female adult donor. eASCs were thawed and seeded at least 5 days before their use in vivo. eASCs were characterized by their immunophenotype (positive for CD73 (AD2) and CD90 (5E10) from Becton Dickinson (Franklin Lakes, NJ, http://www.bd.com) and CD105 (43A3) from Biolegend (San Diego, CA, http://www.biolegend.com) and negative for HLA-II (AF6-120.1) from eBiosciences (San Diego, CA, http://www.ebiosciences.com), CD14 (RM052) from Immunotech (Monrovia, CA, http://www.immunotechlab.com), CD19 (J3.119) from Immunotech, CD34 (8G12) from Becton Dickinson), and CD45 (J33) from Beckman Coulter (Brea, CA, http://www.beckmancoulter.com) (Supporting Information Fig. 1) and their trilineage differentiation potential to osteoblasts, adipocytes, and chondroblasts in vitro. All the eASCs used fulfilled the release criteria of identity, purity, and potency needed for their clinical use.

Induction and Evaluation of CIA and Treatment with eASCs

Each mouse was injected subcutaneously in the tail with a first dose of an emulsion of chicken collagen type II (1 mg/ml final concentration, Col II, Chondrex, Redmond, CA, http://www.chondrex.com) in complete Freund's adjuvant (*Mycobacterium Tuberculosis*) in a volume of 0.1 ml per animal. Afterward, 21 days after the first injection of collagen, a second injection of type II collagen was administered to each animal, again subcutaneously in the tail. In this occasion, the collagen suspension was made using incomplete Freund's adjuvant (no *M. tuberculosis*).

Clinical signs of arthritis were evaluated daily after the second immunization to determine clinical evidence of arthritis of the limb joints by macroscopic examination. Treatment with 1 million eASCs (or vehicle) was administered intravenously (tail vein) in Ringer Lactate solution (Grifols Barcelona, Spain, http://www.grifols.com) when an arthritis index score of 2–4 was attained. Control mice were treated with Lactate Ringer solution alone.

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The Arthritis Index Score was conducted until the end of the study. The severity of the arthritis was scored in both, front and hind, paws according to the following arthritis index scoring system: 0, no signs of arthritis; 1 swelling and/or reddening of the paw or 1 digit; 2, two groups of joints inflamed, swelling and/or reddening; 3, more than two groups of swelling and/or reddening joints; 4, inflammation of the whole paw.

At day 7, mice were culled and mononuclear cells were isolated from peripheral blood (PB), spleen, and draining LNs (inguinal and popliteal) using a cell strainer. A Neubauer chamber was used to determine the number of cells. Complete blood counts were obtained using an automated blood cell counter (Abacus, Diatron, Budapest, Hungary, http://www.diatron.com).

Histology and Paw Edema

At day 7, paws were fixed in neutral buffered formalin. Three to four sections were obtained from each paw and stained with hematoxylin and eosin. The degree of inflammation on each section was assessed according to the following scoring system; 0, normal joint structure; 1 slight changes, synovitis and pannus with few discrete erosions of the cartilage; 2, moderate changes, loss of large areas of cartilage, eroded pannus, and synovial hyperplasia with infiltration by mononuclear and polymorphonuclear cells; 3, severe synovitis, erosion of cartilage and bone; 4, total destruction of the joint architecture. Paw edema was assessed daily as the volume of both, hind and front, paws by the use of a plethysmometer. The average for each paw was calculated and in turns the average for all four paws for each animal to reach a single histological score per animal.

Flow Cytometry Analysis

Isolated mononuclear cells from PB, spleen, and draining LNs were surface-stained with antibodies directed against mouse CD45-Peridinin chlorophyll (*PerCP*) (30F11.1), CD3-R-Phycoerythrin (PE)-Vio770 (145-2C11), and CD49b-Allophycocyanin (APC) (DX5) from Miltenyi Biotech (Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), CD4-Fluorescein isothiocyanate (FITC) (H129.19), CD25-PE (PC61) and isotype-matched control IgG from Becton Dickinson Pharmingen (San Diego, http://www.bdbiosciences.com/index[lowen]us.shtml), CD4-Brilliant^{TM} Violet 711 (BV711) (RM4-5) from Biolegend), and CD223-PerCP-eFluor710 (Lag3, eC9B7W) from eBiosciences. For Foxp3-APC antibody (FJK-16s, eBiosciences), intracellular staining with Foxp3/Transcription factor staining buffer set (eBiosciences) was performed according to the manufacturer's instructions. Cells were collected on a BD LSRFortessa flow cytometer (Becton Dickinson). At least 10,000 events were acquired. Data were analyzed using FlowJo software.

Cytokine Analysis

For intracellular analysis of cytokine expression, mononuclear cells were stimulated in vitro with 5 ng/ml phorbol myristate acetate (PMA), in figure legends 2, 3 and 5 is used. (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 hours. GolgiStop and GolgiPlug (BD Pharmingen) were added after 1 hour. Cells were fixed and stained according to manufacturer instructions (Cytokine BD kit, BD Pharmingen). For intracellular staining, the following antibodies were used; IL10-FITC (JES5-16E3) and IL17A-PE (TC11-18H10) from BD Pharmingen and GM-CSF-FITC (MP1-22E9) from eBiosciences.

Statistical Analysis

Arthritis score was presented as the interquartile range (p75 upper edge, p25 lower edge, p50 midline), p95 (line above the box), and p5 (line below the box). Dots represent the outliers. The rest of the variables were presented as the mean and SEM. Normal distribution was analyzed by the Shapiro Wilks test. Nonparametric techniques (Mann–Whitney *U* test) were used. Analysis was performed using the software Stata 11 (StataCorp, Texas, USA, http://www.stata.com).

Results

Intravenous Administration of a Single Dose of eASCs in Established CIA Strongly Reduces the Severity of Experimental Arthritis

In this study, we investigated in vivo the early immunomodulatory role of eASCs in the well-established model of CIA that shares number of clinical, histologic, and immunologic features with RA [46, 56]. Therapeutic infusion of eASCs after the onset of the disease has not been finely studied. In this model, CIA was induced into DBA/1 mice by subcutaneous administration of Col II. Once the immunized mice reached a 2–4 score of arthritis, a single dose of 1×10^6 eASCs per mouse was administered intravenously (day 0 of the experiment). In a preliminary experiment in which the mice were monitored for up to 14 days, we observed a significant delay in the progression of the disease as observed by reduced arthritis severity scores following the injection of eASCs for up to 9 days (data not shown). From day 9 onward, the differences observed were lost, suggesting a transient immunomodulatory activity exerted by the single dose of eASCs infused. According to this, it was decided to conduct the study for up to 7 days following the infusion of the eASCs, a time frame in which the modulatory effects of the eASCs were most relevant. As shown in Figure 1A, from day 1 after the infusion of the eASCs, the arthritis score of eASC-treated CIA mice was significantly lower than the arthritis score of mice that were immunized with Col II and were not treated with eASCs. These observations were further confirmed by measuring paw edema (Fig. 1B) and by histology analysis (Fig. 1C, 1D) of the whole ankle joints, at day 7, that showed a significantly reduced degree of synovitis, pannus formation, and destruction of bone and cartilage in eASC-treated CIA mice compared to CIA mice. These results demonstrated that a single dose of eASCs can modulate CIA after the onset of the disease.

eASC Treatment Reduces the Levels of Pathogenic GM-CSF$^+$CD4$^+$ T Cells

GM-CSF production by pathogenic T cells has been recently associated to several inflammatory autoimmune diseases including RA where this T cell population is instrumental during the induction phase of the disease [25, 27, 57]. To analyze if eASCs exert an effect on pathogenic GM-CSF$^+$CD4$^+$ T cells, we quantified the number of GM-CSF$^+$CD4$^+$ T cells in spleens, PB, and draining LNs in the different groups of mice by staining intracellularly for the expression of GM-CSF within the CD4$^+$ T cells. As expected, in CIA mice we found increased numbers of

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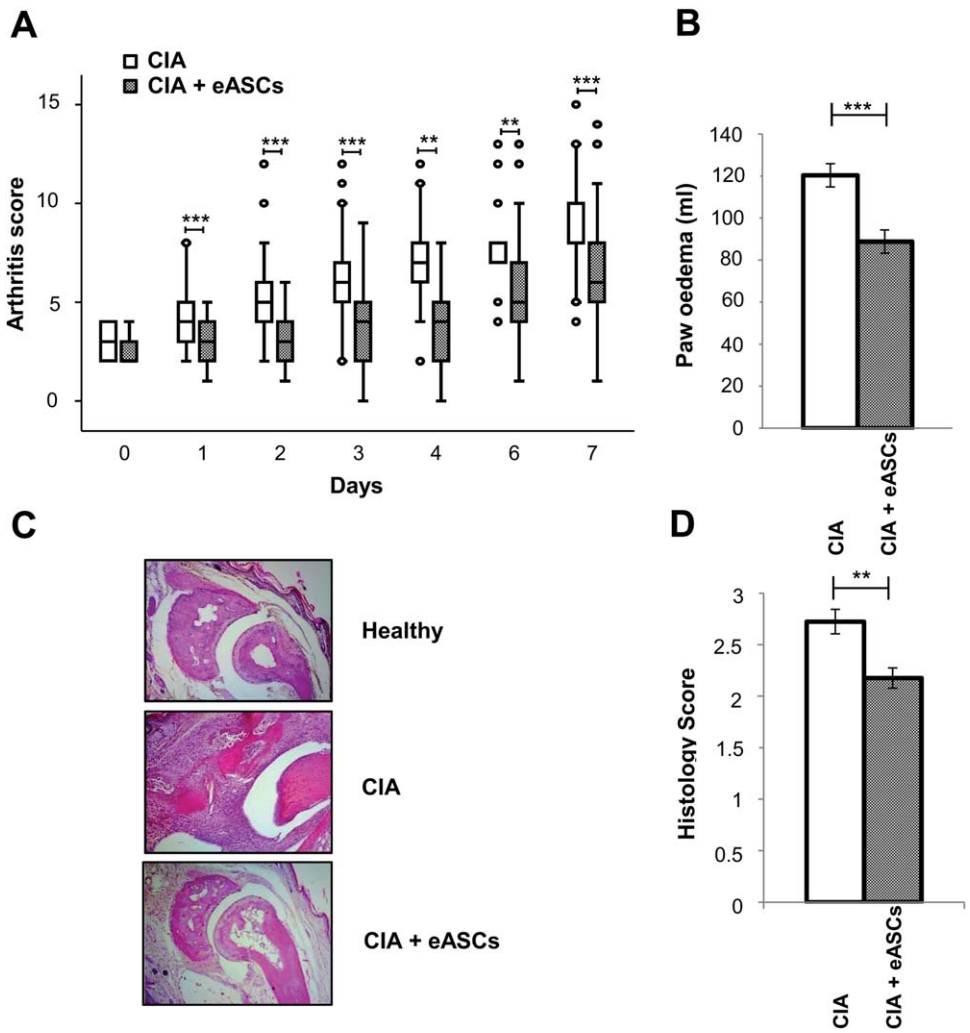

Figure 1. Arthritis status of mice measured by arthritis score, paw edema, and histology. Arthritis score **(A)**, based on the number of swollen paws (front and hind) from the infusion of the eASCs, was evaluated daily for 7 days in the CIA ($n = 46$) and eASC-treated CIA ($n = 64$) mice. Data are presented as the interquartile range (p75 upper edge, p25 lower edge, p50 midline), p95 (line above the box), and p5 (line below the box) of arthritis score. Dots represent the outliers. **(B)**: Paw edema measured at day 7 by a plethysmometer in CIA ($n = 24$) and eASC-treated ($n = 32$) mice. **(C)**: Representative sections showing examples of paw swelling in healthy, CIA, and eASC-treated CIA mice. Scale bars = 200 μm. Original magnification, $\times$ 40. **(D)**: Histology score based on joint structure, synovitis, cell infiltration, and erosion *of pannus*, cartilage, and bone evaluated at day 7 in the CIA ($n = 12$) and eASC-treated CIA ($n = 20$) mice. Data are presented as mean and the SEM of the paw edema and histology score. Significance was analyzed by the Mann–Whitney *U* test and represented by **, $p < .01$; ***, $p < .001$. Results represent four experiments. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells.

GM-CSF-expressing $CD4^+$ T cells in spleen and draining LNs, but not in PB, when compared to healthy mice. Treatment with eASCs significantly reduced the number of GM-CSF^+CD4^+ inflammatory T cells in spleen and PB but not in the draining LNs compared to CIA mice (Fig. 2). To our knowledge, this is the first time to show that pathogenic GM-CSF^+CD4^+ T cells are targeted by eASCs in an ongoing inflammation.

Increased Levels of Regulatory $FOXP3^+CD4^+$ T Cells, $IL10^+IL17^-CD4^+$ T Cells, and $Lag3^+IL10^+CD49b^+CD4^+$ T Cells in Draining LNs of eASC-Treated CIA Mice

The numbers of GM-CSF^+ $CD4^+$ T cells were reduced in spleen and PB and were similar in the draining LNs in the eASC-treated CIA mice in comparison to untreated CIA mice. We questioned if there were alternative mechanisms taking place in the draining LNs that would explain the modulation of the inflammation in the joints in the eASC-treated CIA mice (Fig. 1). Several studies have demonstrated that regulatory T cells are implicated in the cellular responses that lead to the modulatory effects of the eASCs [13, 46, 47]. We next investigated in vivo the effects of eASCs on regulatory T cell subsets defined as $FOXP3^+CD4^+$ T cells (Tregs), $IL10^+IL17^-CD4^+$ T cells (Tr1) and, the recently identified, $Lag3^+IL10^+CD49b^+CD4^+$ T cells ($Lag3^+$Tr1) [58]. These analyses were performed in total cells isolated from spleens, PB, and draining LNs, within the $CD4^+$ T cell population. In healthy mice, among the regulatory T cell subsets, the predominant population in spleen, PB, and draining LNs corresponds to the Tregs, and to a lesser extent to Tr1 cells. $Lag3^+$Tr1 cells are, in fact, included within the Tr1 cells and may correspond to a rather differentiated subtype of regulatory Tr1 cells (Fig. 3). It is known that only a small subset

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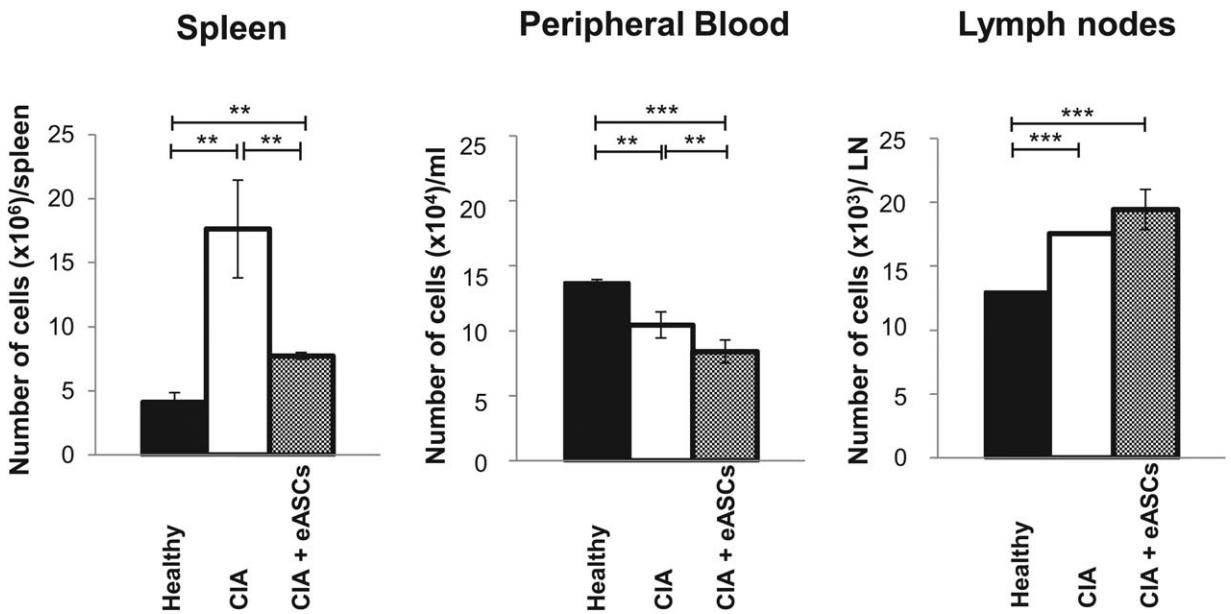

Figure 2. Quantification of GM-CSF$^+$CD4$^+$ T cells in spleen, peripheral blood, and draining lymph nodes at day 7. Cells were activated with PMA and ionomycin for 4 hours in the presence of GolgiStop and GolgiPlug. After incubation, cells were harvested and stained on their surface with anti-CD4 monoclonal antibody. For intracellular staining, cells were fixed, permeabilized, and stained with anti-GM-CSF monoclonal antibody. Data are represented by the mean and the SEM. **, $p < .01$ and ***, $p < .001$ represent the significance analyzed by the Mann–Whitney *U* test. (Healthy, $n = 17$; CIA, $n = 18$; and eASC-treated CIA mice, $n = 36$ for spleen and peripheral blood and Healthy, $n = 5$; CIA, $n = 7$ and eASC-treated CIA mice, $n = 13$ for draining lymph nodes). Results represent four experiments. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells.

of Tregs can express IL10 [59]. In our experiments, the majority of the Tregs did not coexpress IL10 (data not shown).

At day 7, the three subsets of regulatory T cells (Tregs, Tr1, and Lag3$^+$Tr1) were significantly increased in the spleen and decreased in the PB of mice affected by CIA in comparison to the healthy mice (Fig. 3). In draining LNs, Tregs and Lag3$^+$Tr1 cells were increased whereas Tr1 cells were decreased in CIA mice compared with healthy mice. In eASC-treated CIA mice, the total number of regulatory T cells in spleen was clearly reduced when compared to mice with CIA. In sharp contrast, the numbers of Tregs, Tr1, and Lag3$^+$Tr1 cells were significantly increased in the draining LNs of eASC-treated CIA mice with respect to CIA mice and, strikingly high respect to healthy mice in the case of Tregs and Lag3$^+$Tr1. In PB, only the Tr1 cells were significantly increased in CIA mice treated with eASCs reaching similar levels to healthy mice whereas the levels of Tregs and Lag3$^+$Tr1 cells were lower in eASC-treated CIA mice than in CIA and healthy mice. Taken together, these results suggest that the inflammatory environment of the CIA mice favors the induction of regulatory T cells in the spleen and that the treatment with eASCs enhances regulatory T cell responses in the draining LNs where the inflammation is taking place, most likely, at the expense of reducing their numbers in the spleens.

eASCs in Established CIA can Shift the Immune Response from Inflammation to Tolerance by Increasing the Regulatory/Inflammatory Balance in PB and Draining LNs

As shown previously, the levels of the inflammatory GM-CSF$^+$CD4$^+$ T cells and different subsets of regulatory T cells were greatly altered in secondary lymphoid organs in CIA and in CIA mice treated with eASCs respect to healthy mice. Surprisingly, pathogenic GM-CSF$^+$ CD4$^+$ T cells were not decreased in the draining LNs in eASC-treated CIA mice compared to CIA mice whereas there was a concomitant modulation of the inflammation in the joints in the eASC-treated mice. To clarify this, we analyzed the regulatory/inflammatory ratio in spleen, PB, and draining LNs since alterations in the regulatory/inflammatory balance are associated with inflammatory and autoimmune diseases [43]. We calculated the regulatory CD4$^+$ T cell pool by estimating the Tregs and Tr1 CD4$^+$ T cell populations only. The Lag3$^+$Tr1 T cells are, in fact, a subset of T cells included within the Tr1 cell population. The(Tregs + Tr1)/GM-CSF$^+$CD4$^+$ ratio was significantly increased in PB and in draining LNs in eASC-treated CIA mice respect to CIA mice (Fig. 4). On the contrary, in the spleen, this regulatory/inflammatory balance was lower in the eASC-treated mice than in the CIA mice. These results may suggest that in response to CIA the numbers of regulatory T cells are increased in spleen and that, upon infusion of the eASCs, there is a rapid mobilization of the regulatory T cells toward the inflamed tissues where they rapidly counter-balance the ongoing inflammation.

Increased Plasticity of Th17 Cells in eASC-Treated CIA Mice

At present, the functional plasticity of the T helper lineages has become increasingly evident during in vivo immune responses [36, 39, 40] and the plasticity of effector T cells should be considered when analyzing the modulation of immune responses. Previous studies have shown that the Th17 cells are not at the

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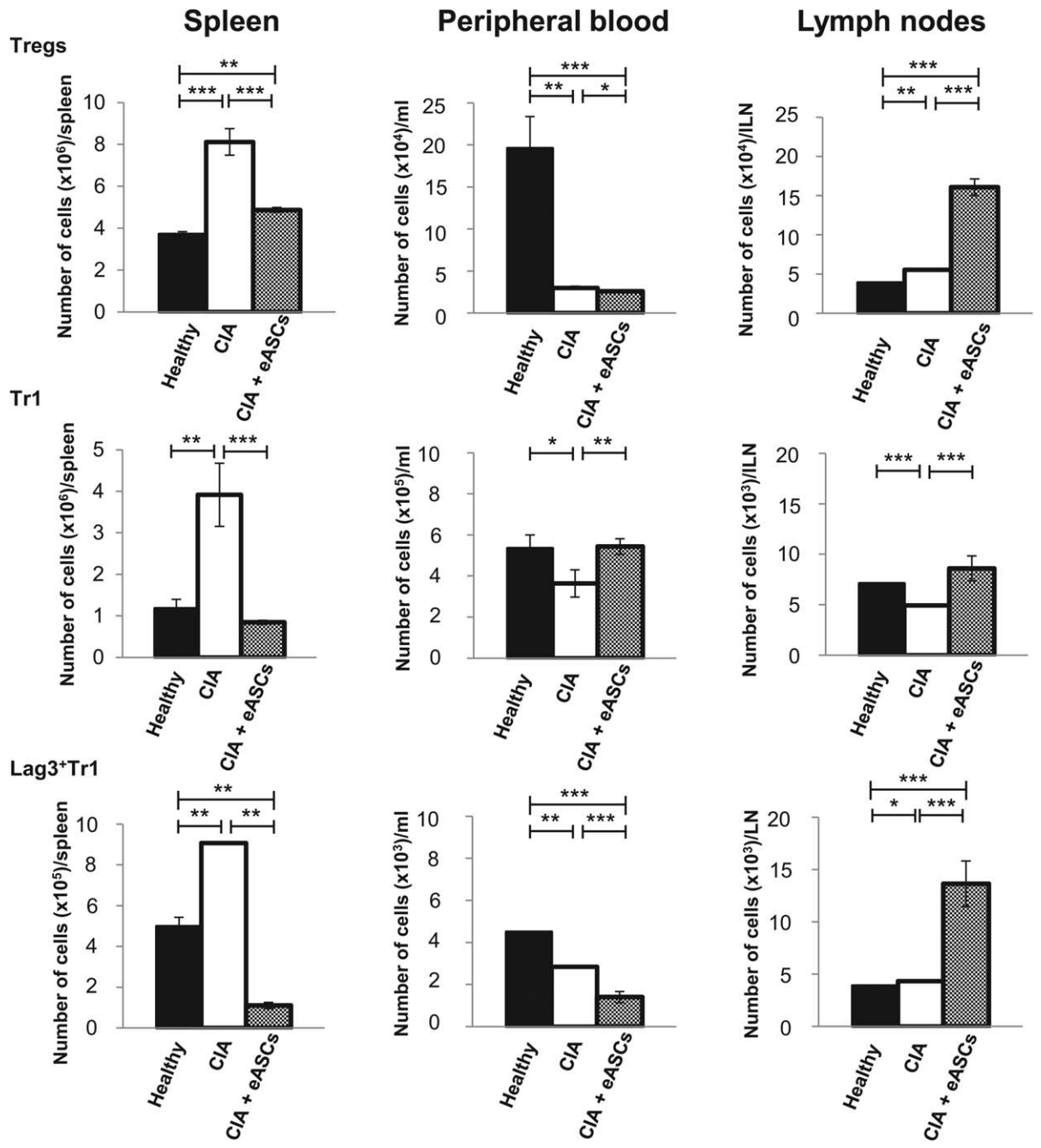

Figure 3. Quantification of regulatory T cell populations identified as $FOXP3^+CD4^+$ (Tregs), $IL10^+IL17^-CD4^+$, and $Lag3^+$ $IL10^+CD49b^+CD4^+$ ($Lag3^+Tr1$) in spleen, peripheral blood, and draining lymph nodes at day 7. Cells were activated with PMA and ionomycin for 4 hours in the presence of GolgiStop and GolgiPlug. After incubation, cells were harvested and stained on their surface with anti-CD4, anti-Lag3, and anti-CD49b monoclonal antibodies. For intracellular staining, cells were fixed, permeabilized and stained with anti-IL17, anti-IL10, and anti-FOXP3 monoclonal antibodies. Data are represented by the mean and the SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$ represent the significance analyzed by the Mann–Whitney *U* test (healthy, $n = 17$; CIA, $n = 18$; CIA+eASCs, $n = 35$ for spleen and peripheral blood and healthy $n = 7$; CIA, $n = 7$; CIA+eASCs, $n = 14$ for draining lymph nodes). Results represent four experiments. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells.

final stage of their differentiation program. In general, the presence of Th17 subsets with regulatory functions correlates with their ability to secrete IL-10 [35, 38, 41, 45]. Also, their effector functions and migratory capacity are coregulated during T helper cell differentiation [34, 60]. Regarding these recent findings, we investigated the generation of Th17 cells with regulatory functions. For this purpose, we analyzed in vivo the coexpression of both IL17 and IL10 cytokines in $CD4^+$ T cells obtained from spleen, PB, and draining LNs in CIA mice treated or not with eASCs. Figure 5 shows the total numbers of $IL10^+IL17^+CD4^+$ T cells and $IL10^-IL17^+CD4^+$ T cells in the spleen, PB, and draining LNs. CIA mice had increased

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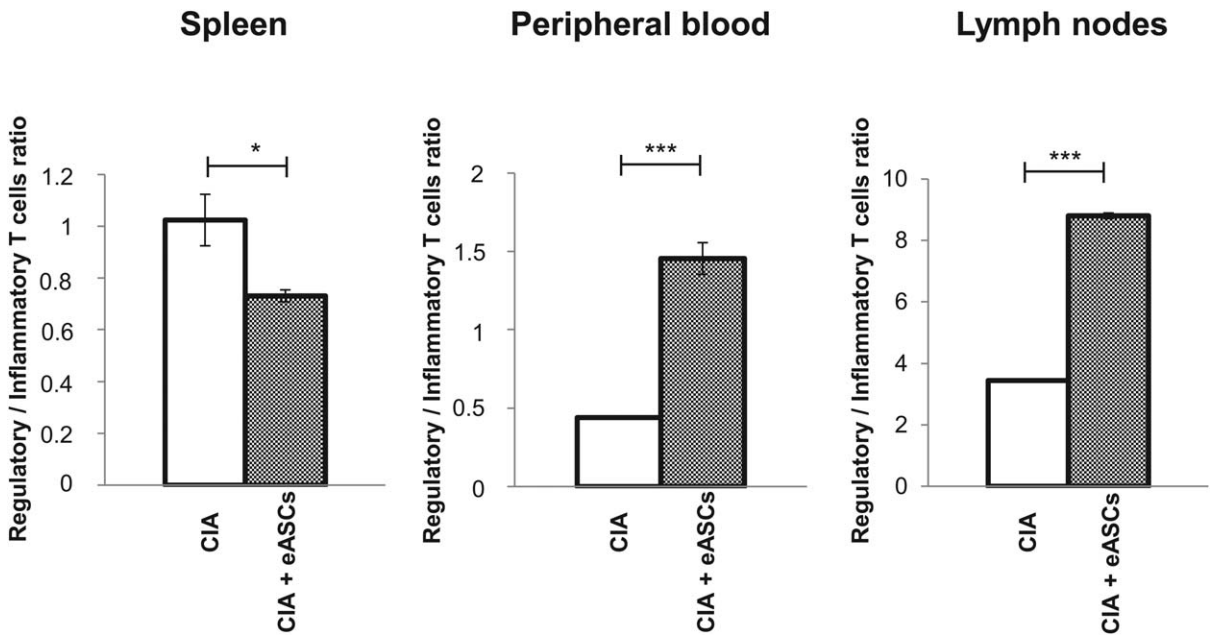

Figure 4. Regulatory/inflammatory balance evaluated in spleen, peripheral blood and draining lymph nodes at day 7. Regulatory/inflammatory balance was calculated on the basis of (Tregs + Tr1)/GM-CSF$^+$CD4$^+$ T cells. Data are represented by the mean and SEM. *, $p < .05$; ***, $p < .001$ represent the significance analyzed by the Mann–Whitney *U* test. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells

numbers of IL10$^+$IL17$^+$CD4$^+$ T cells and IL10$^-$IL17$^+$CD4$^+$ T cells in spleen and LNs compared to healthy mice whereas no differences were found in PB. After infusion of eASCs, IL10$^+$IL17$^+$CD4$^+$ T cells and IL10$^-$IL17$^+$CD4$^+$ T cells were significantly decreased in the spleen and PB and were increased in the draining LNs compared to both CIA and healthy mice.

Taken together, these results suggest that cell therapy treatment with eASCs of mice with established CIA favors the trafficking of both, IL10$^+$IL17$^+$CD4$^+$ T cells and IL10$^-$IL17$^+$CD4$^+$ T cells, from the spleen to the draining LNs. Analyzing the IL10$^+$IL17$^+$CD4$^+$/IL10$^-$IL17$^+$CD4$^+$ balance in these tissues (Fig. 6), we observed that, in eASC-treated CIA mice, this ratio was significantly increased in spleen and in the draining LNs with respect to CIA mice. In contrast, no differences were found in the PB samples in CIA treated or not with eASCs. These results indicated that theIL10$^+$IL17$^+$CD4$^+$ T cell population was increased respect to the IL10$^-$IL17$^+$CD4$^+$ T cell population in the spleen and in the draining LNs, so that, eASCs not only affect the migration of the T cells but also favors their plasticity toward a regulatory phenotype in CIA mice.

Discussion

Targeting pathogenic autoreactive T cells is of critical importance in RA treatment because deregulated adaptive immunity contributes to the pathology of the disease [24, 61]. Recent data from human and murine studies have identified T cells as a key source of GM-CSF cytokine and suggest that GM-CSF is instrumental during the induction phase of autoimmune diseases such as RA [25–27]. Ongoing clinical trials using monoclonal antibodies against the GM-CSF protein or GM-CSF receptor alpha have shown beneficial responses in RA patients and in CIA mice [30, 31]. Several studies have shown that eASCs can either prevent or restrain autoimmune diseases including CIA by a variety of immunomodulatory mechanisms [13, 46, 47, 62]. So far, it is unknown whether the eASCs could target pathogenic GM-CSF-producing T cells.

Although compiling data have demonstrated that mouse and human eASCs differ in some of their mechanisms of immunomodulation, studies in preclinical models of arthritis using human eASCs allow to dissect pathways shared by the human and mouse systems. Moreover, we have recently shown that eASCs are short-live after in vivo administration [63], even when used in a syngeneic setting [64] indicating that eASCs, regardless the MHC context, can prime host immune cells through an array of not fully understood specific molecular mechanism, which, in turn, adopt a regulatory phenotype.

In our model, collagenII immunization induced the classical CIA with ascending inflammation of the joints accompanied by increased infiltration of pathogenic GM-CSF$^+$ CD4$^+$ T cells both systemically (spleen and PB) and in the draining LNs. Surprisingly, eASC-treated CIA mice had decreased numbers of the pathogenic GM-CSF$^+$ CD4$^+$ T cells in spleen and PB but not in the draining LNs. To our knowledge, this is the first time that in vivo treatment with eASCs can target pathogenic GM-CSF$^+$CD4$^+$ T cells. eASCs did not decrease GM-CSF$^+$ CD4$^+$ T cells in the draining LNs so we questioned whether there were alternative mechanisms taking place in the draining LNs that would explain the modulation of the inflammation in the joints. Interestingly, as a consequence of the inflammation, CIA mice had increased numbers of Tregs, Tr1, and Lag3$^+$Tr1 in the spleen. Upon treatment with eASCs, the numbers of these regulatory T cell populations increased in the draining LNs, which may account for the observed delay in the progression of the CIA, at the expense of reducing their

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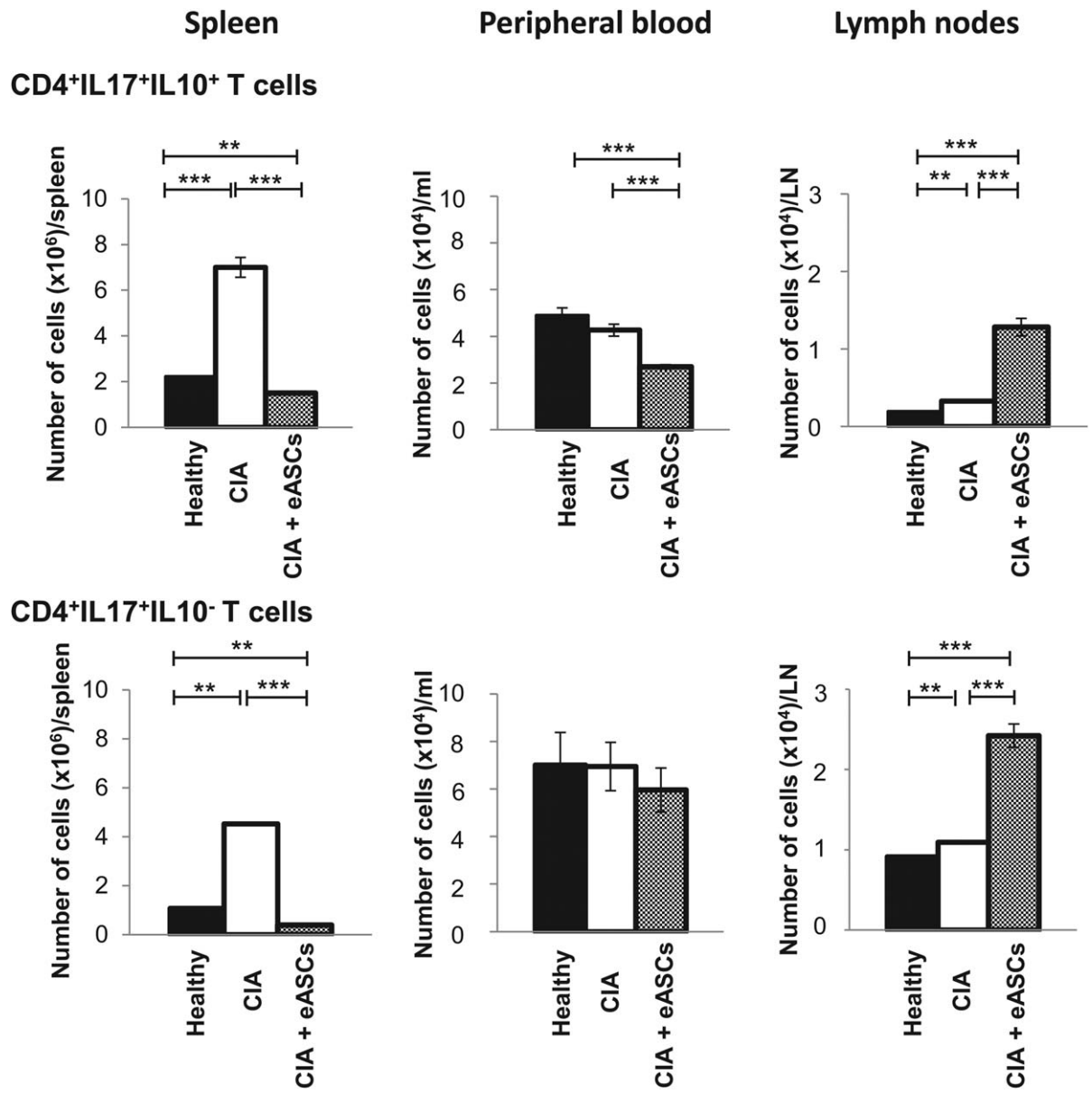

Figure 5. Expression of IL10 in Th17 cells in spleen, peripheral blood and draining lymph nodes at day 7. $IL10^{+}IL17^{+}CD4^{+}$ T cells and $IL10^{-}IL17^{+}CD4^{+}$ T cells were measured in spleen, peripheral blood, and draining lymph nodes at day 7. Cells were activated with PMA and ionomycin for 4 hours in the presence of GolgiStop and GolgiPlug. After incubation, cells were harvested and stained on their surface with anti-CD4 monoclonal antibody. For intracellular staining, cells were fixed, permeabilized, and stained with anti-IL17 and anti-IL10 monoclonal antibodies. Data are represented by the mean and the SEM. Healthy, $n = 5$; CIA, $n = 6$; CIA+eASCs, $n = 14$ for spleen and draining lymph nodes. Healthy, $n = 17$; CIA, $n = 26$; CIA+eASCs, $n = 43$ for peripheral blood. **, $p < .01$; ***, $p < .001$ represent the significance analyzed by the Mann–Whitney *U* test. Results represent four experiments. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells.

numbers in the spleens. As a consequence, the regulatory/inflammatory balance in PB and in the draining LNs was reestablished, thus contributing to the amelioration of CIA. Taken together, these results suggest that soon after the infusion of the eASCs into the arthritic mice, the early modulation observed in the eASC-treated CIA mice could be explained by either rapid mobilization of the regulatory T cells from the spleen to the inflamed tissues or to a rapid induction in the draining LNs. Both mechanisms could be responsible for the increase regulatory T cell responses in the draining LNs where the inflammation is taking place and are in agreement to previous reports where these effects have been observed separately [34, 44].

Th17 cells have been described previously to have a critical role during the pathogenesis of arthritis [32, 33]. In addition to this, Th1, Th2, Th17, and regulatory T cell subsets were once deemed highly stable and their characteristic cytokine profiles were thought to be inhibitory for the differentiation program of the other subsets. Nowadays, T helper cells are found to be able to express alternative lineage cytokines, such as Th17 cells expressing INFγ [35, 38, 40, 41, 44, 45]. At present, the functional plasticity of the T helper lineages has become increasingly evident during in vivo immune responses [36, 39, 40] and plasticity of effector T cells should be considered when analyzing the modulation of immune responses. At

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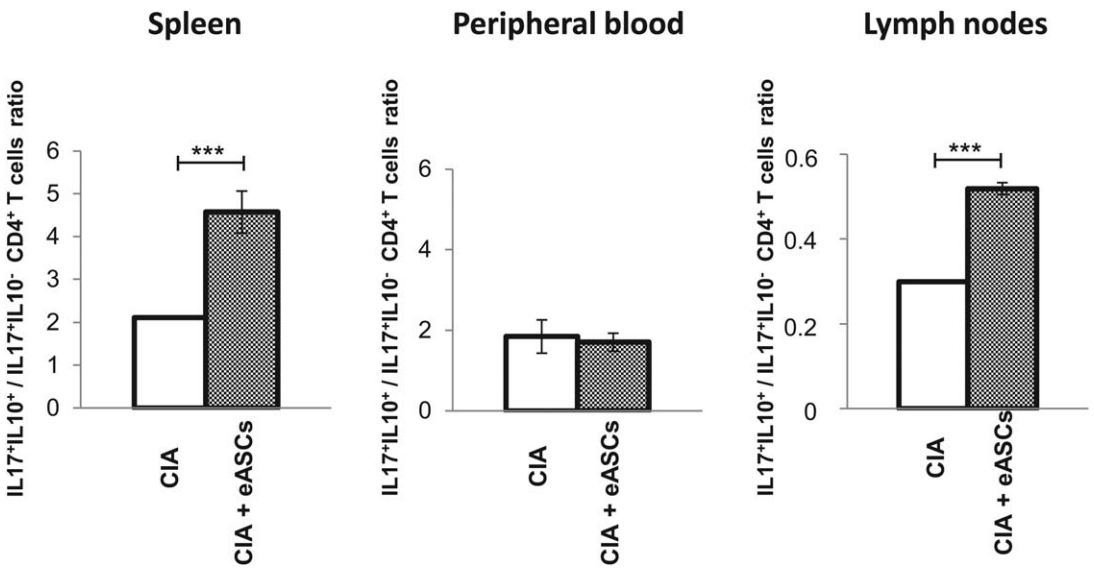

Figure 6. $IL10^{+}IL17^{+}CD4^{+}/IL10^{-}IL17^{+}CD4^{+}$ T cell ratio in spleen, peripheral blood, and draining lymph nodes at day 7. Data are represented by the mean and SEM. ***, $p < .001$ represent the significance analyzed by the Mann–Whitney *U* test. Abbreviations: CIA, collagen induced arthritis; eASCs, expanded adipose-derived mesenchymal stem cells

the moment, limited information exists on the capacity of MSCs to induce plasticity in Th17 cells toward a regulatory phenotype. We observed that as a consequence of CIA, the plasticity of the Th17 cells toward IL10-expressing Th17 was increased both in spleen and in draining LNs when compared to healthy mice. Strikingly, in eASC-treated CIA mice, the IL10-expressing Th17 cells were significantly reduced in the spleen and PB when compared to CIA and healthy mice. However, these IL10-producing Th17 cells were mainly found in the draining LNs. Our data suggest that in response to the ongoing inflammation the plasticity of the Th17 cells toward IL10-producing cells is induced and that the treatment with the eASCs seemed to have an impact on their trafficking favoring their migration from the spleen to the draining LNs. These results may also suggest the induction of expression of homing receptors on effector T cells favoring their trafficking toward inflamed tissues where the majority of inflammatory and regulatory T cell populations were found. Additional studies need to be conducted to clarify these observations.

At present, the reactive LNs are the object of growing attention when monitoring patients with chronic inflammatory diseases [65]. We analyzed, for the first time, T cell responses in spleens, PB, and draining LNs in parallel. The results obtained showed that PB may not reflect in a reliable manner the progression of the inflammatory disease. These observations also suggest that when patients are monitored to study the progression of the disease based on PB samples only rather biased information is obtained and this should be taken into consideration.

Conclusion

Altogether, our results identify, for the first time, a novel mechanism by which eASCs modulate ongoing immune responses by promoting an early adaptive regulatory T cell signature characterized by decreased levels of pathogenic GM-CSF-secreting T cells, increased levels of regulatory T cells and plasticity of effector Th17 cells toward an IL10-driven anti-inflammatory response thus restoring the regulatory/inflammatory balance following the onset of the disease.

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Author Contributions

M.L.-S. and M.I.G.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; P.M.-C., R.M.: collection and/or assembly of data; J.L.-B.: conception and design and collection and/or assembly of data; O.D.: conception and design and revised manuscript for important intellectual content; J.A.B.: revised manuscript for important intellectual content; W.D.: data analysis and interpretation and revised manuscript for important intellectual content; E.L.: conception and design and data analysis and interpretation.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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