

ORIGINAL RESEARCH

Adipose-derived mesenchymal stromal cells modulate experimental autoimmune arthritis by inducing an early regulatory innate cell signature

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Abstract

Modulation of innate immune responses in rheumatoid arthritis and other immune-mediated disorders is of critical importance in the clinic since a growing body of information has shown the key contribution of dysregulated innate responses in the progression of the disease. Mesenchymal stromal cells (MSCs) are the focus of intensive efforts worldwide due to their key role in tissue regeneration and modulation of inflammation. In this study, we define innate immune responses occurring during the early course of treatment with a single dose of expanded adipose-derived MSCs (eASCs) in established collagen-induced arthritis. eASCs delay the progression of the disease during the early phase of the disease. This is accompanied by a transient induction of Ly6C⁺ monocytes that differentiate into IL10⁺F4/80⁺ cells in arthritic mice. Strikingly, the induced IL10⁺F4/80⁺ myeloid cells preferentially accumulated in the draining lymph nodes. This effect was accompanied with a concomitant declining of their frequencies in the spleens. Our results show that eASCs attenuate the arthritic process by inducing an early innate cell signature that involves a transient induction of Ly6C⁺ monocytes in periphery that differentiate into IL10⁺F4/80⁺ macrophages. Our findings demonstrate that early regulatory innate cell responses, involving the monocyte compartment, are targeted by the eASCs during the onset of collagen-induced inflammation.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology that is characterized by synovial hyperplasia and cartilage/bone destruction with systemic

comorbidities. Accumulating data show that CD4 T cells, especially IL-17-producing T helper (Th17), and neutrophils play a significant role during the chronic inflammation [1, 2]. In recent years, myeloid-derived suppressor cells (MDSCs) have also attracted considerable attention by their

increase in RA patients [3–5] and experimental models of arthritis [4–8]. In mice, they are defined as Gr1⁺ CD11b⁺ cells with a suppressive effector function. Based on the expression of Ly6G and Ly6C molecules, two subsets of MDSCs have been described, i.e., the granulocytic MDSCs defined as Ly6G⁺Ly6C^{low} CD11b⁺ cells and the monocytic MDSCs defined as Ly6G⁻Ly6C^{hi}CD11b⁺ cells [9, 10]. At present, disagreements exist on the role played by the MDSCs in RA [3–8]. Their anti-inflammatory function in RA has been claimed by several groups [3, 6–8], while other reports have shown their proinflammatory role during the progression of experimental arthritis as well as in patients with RA [4, 5].

Despite major progress in the understanding of pathogenesis of RA, strong unmet medical need remains [11]. New approaches are, therefore, necessary and mesenchymal stem cells (MSCs) could represent a valuable therapeutic strategy for RA [12–15]. The use of MSCs in the clinical field has gathered tremendous momentum over the last decade, advanced by varying levels of success in clinical trials [13, 16–19] and by the progress in our understanding of their mechanisms of action [20–22]. Preclinical and clinical studies have demonstrated that MSCs attenuate inflammatory response by induction of regulatory T cells [13, 23–25], secretion of molecules with anti-inflammatory effects [26], inhibition of dendritic cell maturation [27], and generation of macrophages with regulatory phenotype [28–33], among others. Number of studies have demonstrated that MSCs, either *in vitro* and *in vivo*, can induce MDSCs [29–34] and these populations are responsible for the beneficial effects of the MSCs in modulating the inflammation [29, 30, 32–35].

The majority of the *in vivo* studies with eASCs for preventing collagen-induced arthritis used multiple doses of eASCs before the onset of the disease [36–38]. Several studies have demonstrated that multiple doses of eASCs can have a sustained beneficial effect in a therapeutic protocol [23, 37]. The sustained effect observed when multiple doses of eASCs are used might be the result of a very complex response which may not be easily explained by direct interaction with the eASCs. We have recently demonstrated that a single dose of eASCs during the onset of the disease significantly decrease the severity of the arthritis and this was accompanied by the induction of different subsets of regulatory T cells and IL10-producing Th17 cells [25]. In this context, we hypothesized whether cell therapy with eASCs also induces early innate responses that would contribute to the reestablishment of the regulatory/inflammatory balance during the ongoing inflammation. Our findings demonstrate that an early regulatory innate response, involving the monocyte compartment, is induced soon after the infusion the

eASCs which may lead to an effective modulation of the ongoing inflammation.

Results

eASC treatment delayed the progression of established disease in experimental arthritis

To study early innate cell responses induced by administration of eASCs, we first evaluated the progression of inflammation in a collagen-induced mice model (CIA) treated or not with eASCs. A single dose of 1×10^6 eASCs/mouse was administered intravenously (day 0) at the onset of the detectable disease signs (arthritis score between 2 and 4). As shown in Figure 1A, from day 1, 24 h after the infusion of the eASCs, there was a statistically significant delay in the progression of the disease in eASC-treated CIA mice compared to the CIA mice. From day 9 onwards, the differences observed were lost. The fact that the effect of the eASCs was transient was somewhat expected as previous studies have demonstrated that multiple doses of eASCs can have a sustained beneficial effect when used in a therapeutic protocol [23, 37]. According to this kinetic, the analyses of cell responses were conducted from day 0 to 7 when the modulatory effects of the eASCs were clearly distinguished. The beneficial effects of eASCs were further confirmed by a reduction in paw edema at days 3 and 7 in the eASC-treated CIA mice (Fig. 1B) and by histology analysis (Fig. 1C and D) of the whole ankle joints. At day 7, the modulation of inflammation was also confirmed by analysis of inflammatory myeloid populations in the spleen. As shown in Figure 1E, the frequencies of myeloid cells expressing GM-CSF, TNF α , and INF γ were significantly reduced in eASC-treated CIA mice compared to CIA mice. The GM-CSF-expressing myeloid cells also co-expressed INF γ and TNF α although to a lesser extent than the GM-CSF cytokine (data not shown).

eASC treatment induced a transient increase in monocytes in peripheral blood of arthritic mice

The progression of inflammation was monitored by hematological analysis of peripheral blood (PB) samples. As expected, mice with CIA had a marked granulocytosis in circulation. Treatment with eASCs had no influence in the granulocytes (Fig. 2A). Surprisingly, at day 3, there was an increase in the percentage of monocytes in the eASC-treated CIA mice compared to CIA mice. At day 7, the percentage of monocytes in eASC-treated CIA mice did not differ from those of CIA and healthy mice (Fig. 2B). These results suggested that, as a consequence of the infusion of the eASCs in CIA mice, there was a transient increase in monocytes in circulation. This observation indicated

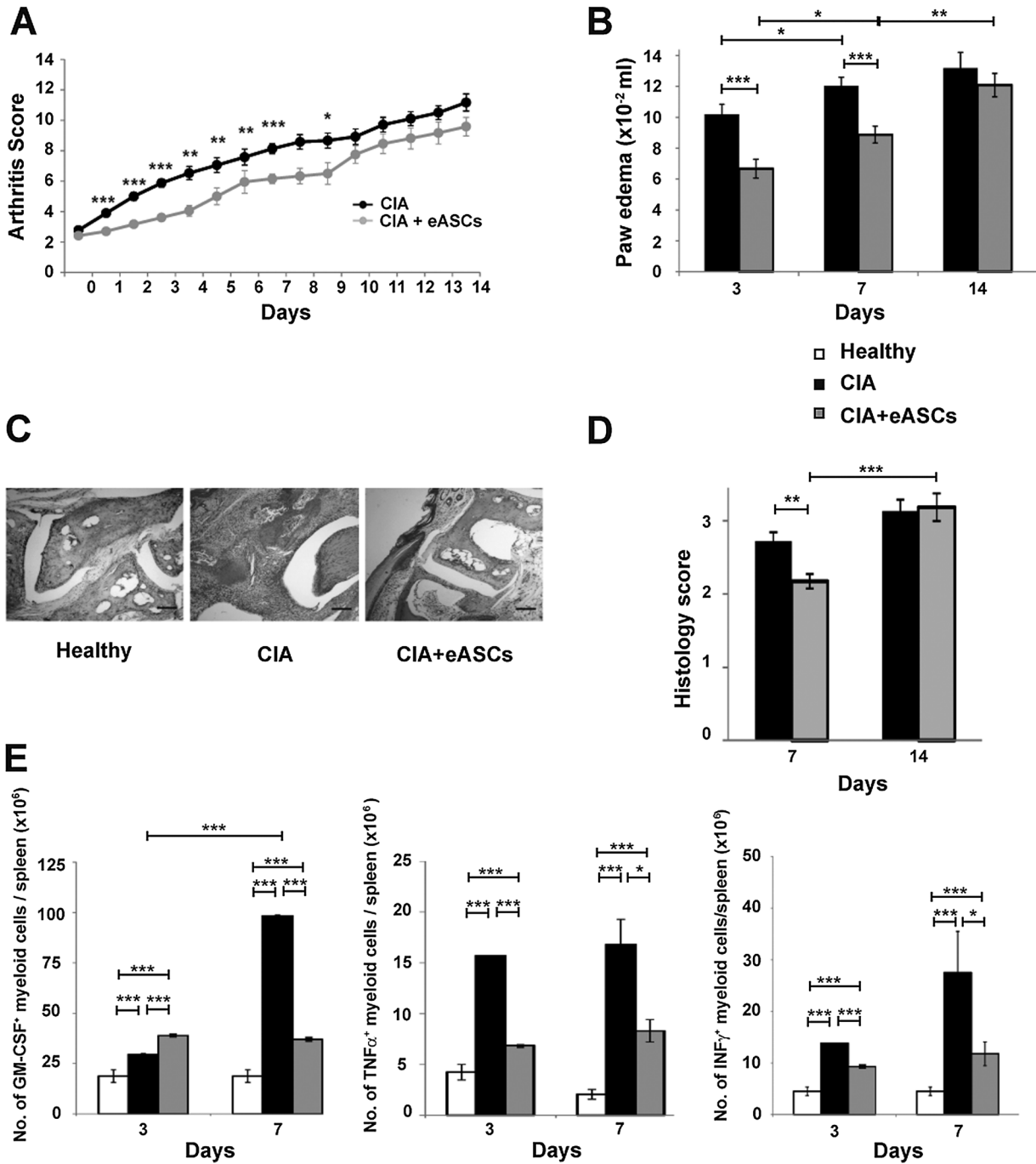


Figure 1. Arthritis status of mice measured by arthritis score, paw edema, histology, and number of inflammatory myeloid cells. Arthritis score (A), based on the number of swollen paws (front and hind), was evaluated daily for 14 days in the CIA ($n = 49$) and eASC-treated CIA ($n = 67$) mice. (B) Paw edema is measured at day 3, 7, and 14 by a plethysmometer in CIA ($n = 34$) and eASC-treated CIA ($n = 52$) 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 synovitis and cell infiltration evaluated at days 7 and 14 in the CIA ($n = 12$) and eASC-treated CIA ($n = 20$) mice. (E) Quantification of GM-CSF⁺, TNF α ⁺, and INF γ ⁺ inflammatory myeloid cells in spleen by flow cytometry. Cells were activated with PMA and ionomycin for 4 h in the presence of GolgiStop and GolgiPlug. After incubation, cells were harvested and stained on their surface. For intracellular staining, cells were fixed permeabilized and stained. Percentages of GM-CSF⁺, TNF α ⁺, and INF γ ⁺ inflammatory myeloid cells in the spleen at day 3 and 7 of healthy, CIA, and eASC-treated CIA mice were shown. Healthy $n = 24$, CIA $n = 25$, and eASC-treated CIA $n = 42$ for GM-CSF⁺ and TNF α ⁺ myeloid cells and healthy, $n = 24$; CIA, $n = 13$, and eASC-treated CIA, $n = 22$ for INF γ ⁺ myeloid cells. Data are presented as mean and the standard error of the mean of the arthritis score, paw edema, histology score, and number of inflammatory myeloid cells. Significance was analyzed by the Mann–Whitney test and represented by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Results represent four experiments.

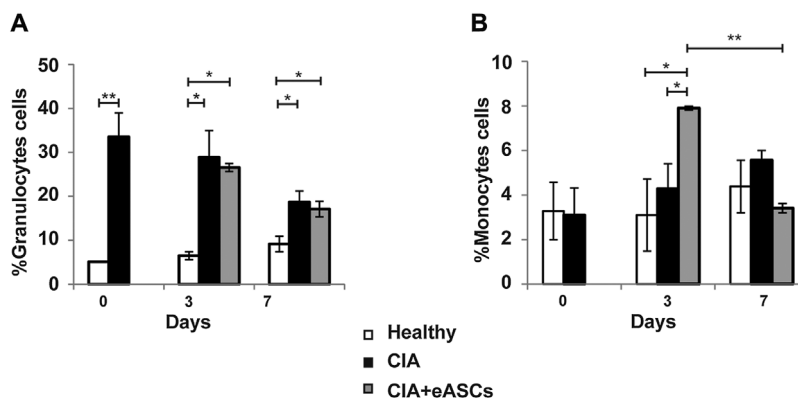


Figure 2. Hematological analysis of peripheral blood samples. Peripheral blood was analyzed by an automated blood cell-counter. (A) Percentage of granulocytes cells and (B) percentage of monocytes cells of healthy, CIA, and eASC-treated CIA mice at day 0, 3, and 7. Data are represented by the mean and the standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ represent the significance analysed by the Mann-Whitney test (healthy, $n = 9$; CIA, $n = 14$; and eASC-treated CIA, $n = 12$). Data correspond to one representative out of four experiments.

that early innate responses, within the monocyte compartment, were actively participating in the cell responses induced by the eASCs.

The transient increase of monocytes in PB was due to a rapid induction of Gr1⁺CD11b⁺ myeloid cells

Peripheral blood samples were analyzed by flow cytometry in order to identify the myeloid populations that were induced by the eASCs. Gr1⁺CD11b⁺ myeloid cells were increased in CIA mice when compared to healthy mice. The infusion of eASCs led to a transient induction of Gr1⁺CD11b⁺ myeloid cells in the eASC-treated CIA mice compared to CIA mice. These differences were not observed at day 7 (Fig. 3A and B).

The granulocyte receptor-1 (Gr1, detected by the clone RB6-8C5 monoclonal antibody) was initially believed to be expressed only by mature granulocytes. Later on, studies have demonstrated that the RB6-8C5 antibody reacts with the structurally glycosylated phosphatidylinositol (GPI)-anchored proteins; Ly-6G (21–25 kDa), expressed by granulocytes, and Ly-6C (14–16 kDa), expressed by several subsets of monocytes/macrophages as well as dendritic cells and lymphocytes. To further define the subsets of myeloid populations that were induced by the eASCs, PB samples were labeled with specific monoclonal antibodies to Ly6C (clone 1G7, G10) and Ly6G (clone 1A8) antigens. As shown in Figure 3C and Supplementary Figure S1A, Ly6C⁺CD11b⁺ cells were increased in CIA mice compared to healthy mice. Strikingly, upon treatment with eASCs, Ly6C⁺CD11b⁺ cells were transiently increased at day 3 compared to CIA mice. At day 7, the levels of Ly6C⁺CD11b⁺ cells in circulation decreased in both CIA mice, treated or not with eASCs (Figure 3C and Supplementary Figure S1A).

On the other hand, the frequencies of Ly6G⁺CD11b⁺ cells were significantly increased in both CIA and eASC-treated CIA mice, respectively to healthy mice (Fig. 3D and Supplementary Figure S1B). Altogether, these data suggested that the transient increase of Gr1⁺CD11b⁺ myeloid cells observed in the eASC-treated CIA mice was due to the contribution of the Ly6C⁺CD11b⁺ myeloid cells and not to the Ly6G⁺CD11b⁺ cells.

eASCs primed Ly6C⁺ monocytes to a regulatory IL10⁺F4/80⁺ phenotype favoring their trafficking to dLNs

Ly6C⁺ monocytes play multiple roles upon tissue recruitment and the differentiation and effector functions of these cells can be shaped according to the environment, both at steady state and during inflammation [39, 40]. To explore the possibility as to whether the eASCs can prime Ly6C⁺ monocytes for regulatory function, we analyzed the expression of IL10 within the Ly6C⁺ monocytes in PB. Interestingly, at day 7, eASC-treated CIA mice had a significant increase in the proportion of Ly6C⁺CD11b⁺ myeloid cells co-expressing IL10 compared to CIA mice. In CIA mice, these populations were also induced when compared to healthy mice, most likely as a consequence of the ongoing inflammation (Fig. 4A and B). This observation may suggest that the induction of the IL10⁺Ly6C⁺CD11b⁺ myeloid cells occurred in CIA as a consequence of the disease and the treatment with the eASCs amplified this regulatory innate response induced by the ongoing inflammation.

At day 7, there was a significant increase in the number of IL10⁺F4/80⁺ cells within the Ly6C⁺ myeloid cells in eASC-treated CIA mice, compared to CIA mice, indicating that these populations actually differentiated into regulatory macrophages in circulation (Fig. 4C and D). This population

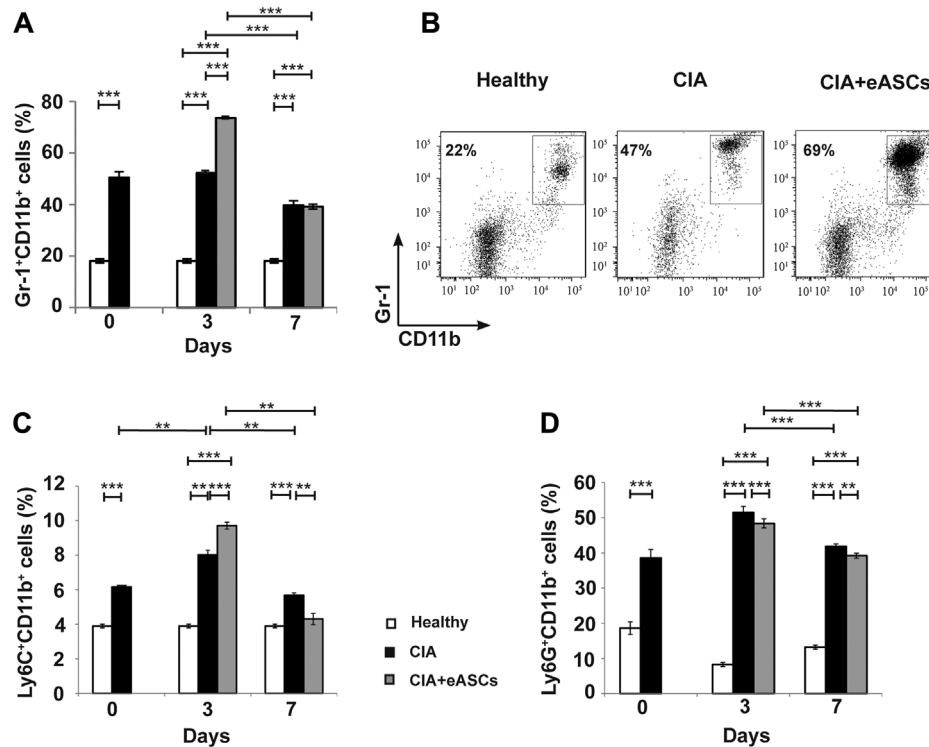


Figure 3. Quantification of Gr-1⁺CD11b⁺, Ly6C⁺CD11b⁺, and Ly6G⁺CD11b⁺ myeloid cell populations in peripheral blood by flow cytometry. Cells were stained on their surface with anti-CD11b, anti-Gr-1, anti-Ly6C, and anti-Ly6G monoclonal antibodies. (A) Percentage of Gr-1⁺CD11b⁺ myeloid cells in peripheral blood at day 0, 3, and 7, (B) representative dot plot of Gr-1⁺CD11b⁺ myeloid cell population in peripheral blood at day 3, (C) percentage of Ly6C⁺CD11b⁺ myeloid cells at day 0, 3, and 7 in peripheral blood, and (D) percentage of Ly6G⁺CD11b⁺ myeloid cells at day 0, 3, and 7 in peripheral blood of healthy, CIA, and eASC-treated CIA mice. Data are represented by the mean and the standard error of the mean. ***P* < 0.01 and ****P* < 0.001 represent the significance analyzed by the Mann–Whitney test (healthy, *n* = 20; CIA, *n* = 19; and eASC-treated CIA, *n* = 23). Results represent four experiments.

was also greatly increased in the dLNs of CIA mice treated with eASCs. In sharp contrast, the number of IL10⁺F4/80⁺ cells within the Ly6C⁺ myeloid cells was dramatically reduced in the spleen of eASC-treated CIA mice compared to CIA and healthy mice (Fig. 5). These findings may indicate that the eASC treatment induced the skewing of Ly6C⁺ monocytes to a regulatory phenotype and their accumulation to the dLNs where the inflammation was taking place.

Discussion

Here, we show that cell therapy with eASCs, among other effects, modulate the inflammatory innate cell responses with a concomitant delay in the progression of the arthritis. Modulation of innate immune responses in RA is of critical importance in the clinic since a growing body of evidence has shown the key contribution of dysregulated innate responses during the progression of the disease [1, 41]. The fact that the effect of the eASCs was transient was somewhat expected. The majority of the *in vivo* studies with eASCs for preventing collagen-induced arthritis used multiple doses of MSCs

before the onset of the disease [36–38]. Gonzalez MA and collaborators demonstrated that three doses of eASCs can have a sustained beneficial effect when used in a therapeutic protocol [23]. In the present study, we have used a single dose of eASCs for treating arthritic mice when the score of arthritis was between 2 and 4. This experimental strategy allowed us to dissect in great detail, the cell responses induced upon the infusion of the eASCs in an inflammatory environment. The sustained effect observed when multiple doses of eASCs are used for treating arthritic mice might be the result of a very complex response which may not be easily explained by direct interaction with the eASCs.

In our CIA experiments, arthritic mice rapidly developed a strong innate response soon after the challenge with the collagen II antigen. In the periphery, mice with CIA had a marked granulocytosis. The inflammatory nature of the granulocytes was confirmed by the expression of GM-CSF, TNF α , and INF γ cytokines in myeloid cells. The modulatory effects of the eASCs during the onset of the disease had a major impact on these populations as CIA mice treated with eASCs had reduced number of inflammatory myeloid cells

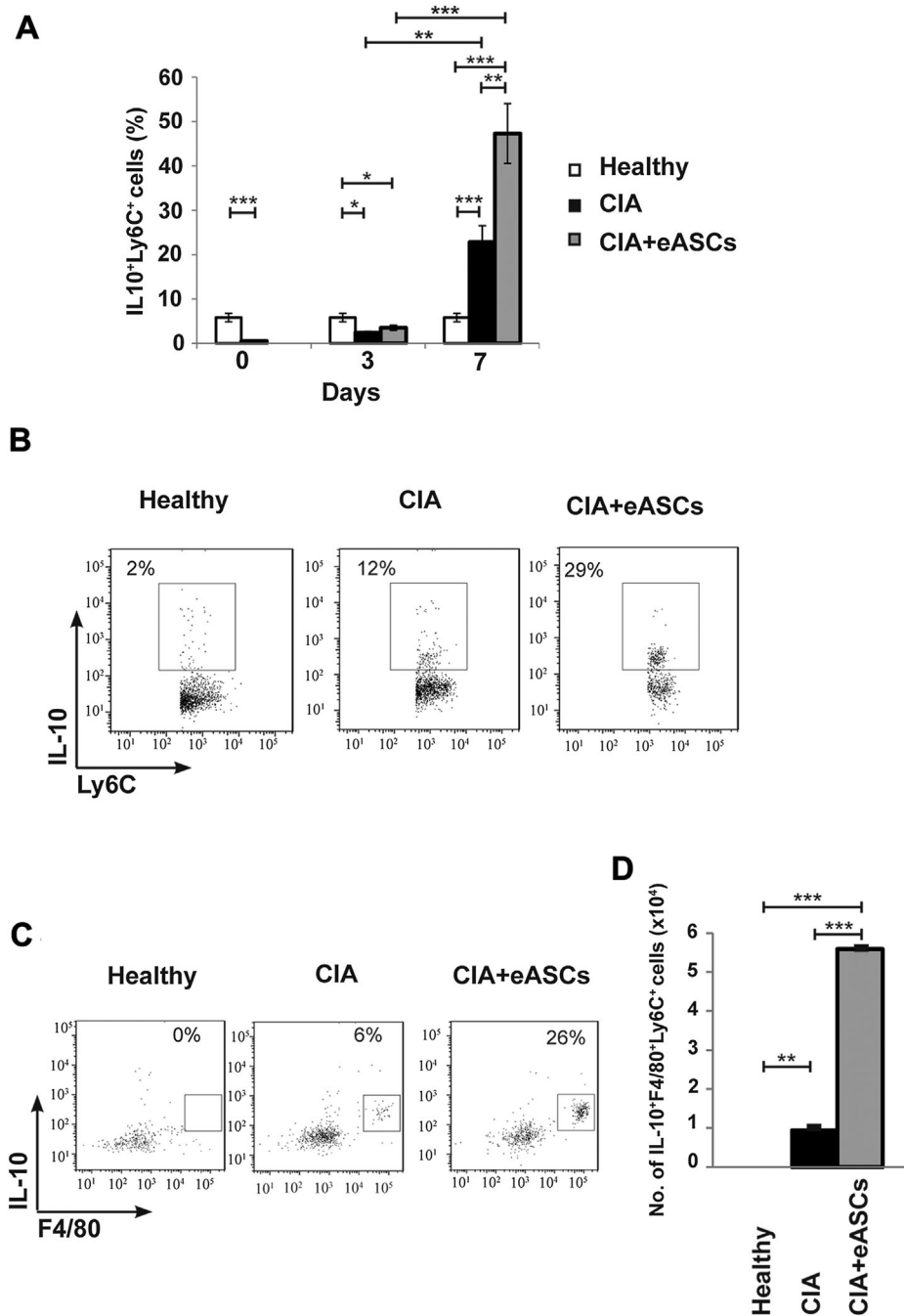
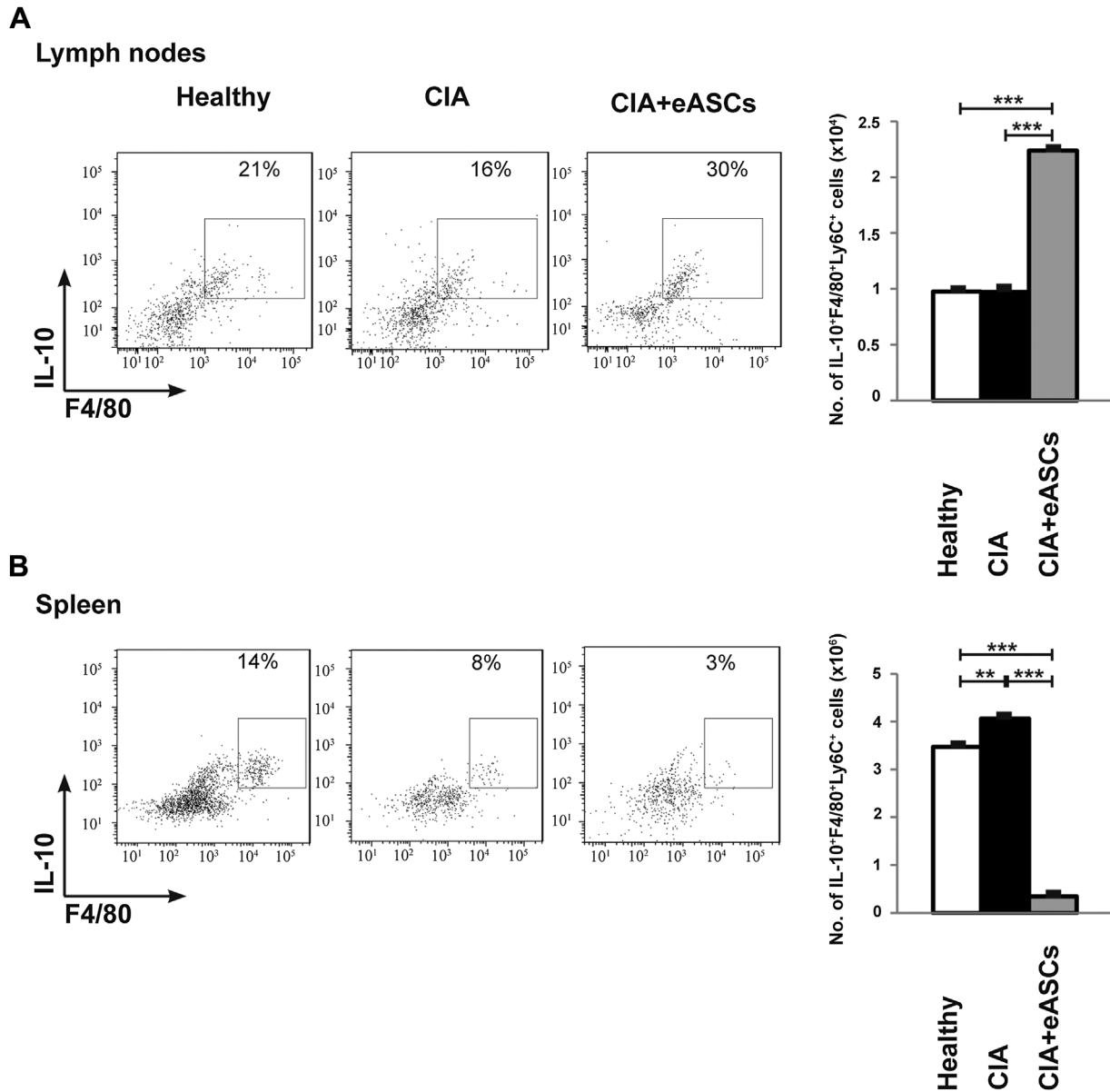


Figure 4. Quantification of IL-10⁺Ly6C⁺ and IL10⁺F4/80⁺ within Ly6C⁺ myeloid cell populations in peripheral blood by flow cytometry. Cells were activated with PMA and ionomycin for 4 h in the presence of GolgiStop and GolgiPlug. After incubation, cells were harvested and stained on their surface with anti-Ly6C and anti-F4/80 monoclonal antibodies. For intracellular staining, cells were fixed, permeabilized, and stained with anti-IL-10 monoclonal antibody. (A) Percentage of IL-10⁺Ly6C⁺ myeloid cell populations in peripheral blood at day 0, 3, and 7, (B) representative plot of IL-10⁺Ly6C⁺ myeloid cells at day 7 in peripheral blood, (C) representative plot of IL10⁺F4/80⁺ myeloid cell within the Ly6C⁺ myeloid cells at day 7 in peripheral blood, and (D) number of IL-10⁺F4/80⁺Ly6C⁺ myeloid cells in peripheral blood at day 7 of healthy, CIA, and eASC-treated CIA mice. Data are represented by the mean and the standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ represent the significance analyzed by the Mann-Whitney test (healthy, $n = 12$; CIA, $n = 13$; and eASC-treated CIA, $n = 14$). Results represent four experiments.



(Fig. 1E). This is a very interesting effect of the eASCs since the majority of therapeutic approaches mediate their beneficial action through cytokine blockade such as TNF α , IL6, IL1, and GM-CSF [2, 11, 42–45] rather than targeting the inflammatory cells themselves. Currently, ongoing clinical trials using monoclonal antibodies against the GM-CSF protein or GM-CSF receptor α have shown

significant responses in RA patients [43–45]. This would imply that the cell therapy with eASCs can target the underlying cause of the disease and not the inflammation itself.

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-lived after *in vivo* administration [46], even when used in a syngeneic setting [47], 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.

Of interest in this study, the hematological analysis revealed a transient increase in the frequencies of monocytes in circulation in CIA mice treated with eASCs (Fig. 2). By flow cytometry, the phenotype of these cells corresponded to Gr1⁺CD11b⁺ myeloid cells (Fig. 3). Our data are in accordance with previous *in vitro* and *in vivo* studies where it has been shown that MSCs favored the induction of Gr1⁺CD11b⁺ myeloid cells with a regulatory phenotype [29, 30, 33, 35, 48]. These cells have been described as heterogeneous populations of myeloid cells with immunoregulatory properties also known as myeloid derived suppressor cells (MDSCs) [9, 10]. It is well known that MDSCs accumulate in RA patients [3–5] and in experimental inflammation including CIA [4–6].

Gr1⁺CD11b⁺ regulatory myeloid cells are able to modulate adaptive immune responses through induction of different populations of regulatory T cells *in vitro* [48, 49]. Recently, we have demonstrated that eASCs modulate ongoing immune responses by promoting an early adaptive regulatory T cell signature. In that study, we found increased levels of regulatory T cells and plasticity of effector Th17 cells toward an IL10-driven anti-inflammatory response. Ultimately, these cell responses led to restoring the regulatory/inflammatory balance following the onset of the disease [25]. These observations allowed us to hypothesize whether the eASCs orchestrate the adaptive regulatory T cell responses through a mechanism involving innate responses.

The role of MDSCs during the development of RA is controversial. Gr1⁺CD11b⁺ cells are composed by two different subsets of cells based on the expression of Ly6C and Ly6G molecules. Our data showed that the transient increase in the Gr1⁺CD11b⁺ cells in the eASC-treated CIA mice was mainly due to the contribution of the Ly6C⁺CD11b⁺ cells. In eASC-treated CIA mice, the expression of IL10 within Ly6C⁺CD11b⁺ cells was higher than in CIA mice. Furthermore, one week after the infusion of the eASCs, the majority of IL10⁺Ly6C⁺CD11b⁺ cells expressed the F4/80 macrophage marker. These data are in agreement with Nemeth and collaborators where, in a model of sepsis, increase frequencies of Gr1⁺F4/80⁺ myeloid cells expressing IL10 upon treatment with MSCs were observed. Neutralisation of IL10 or the IL10 receptor clearly reduced the protective effects of the MSCs [29]. Lee *et al.* [33], using a mouse model of experimental autoimmune uveitis, observed that intravenous infusion of MSCs led to an accumulation of

Ly6C⁺CD11b⁺ cells in draining lymph nodes and that depletion of the Gr-1⁺ cells, by neutralizing antibody against Gr-1, abrogated the effects of the MSCs. In line with these observations, in a model of solid organ transplantation, Obermajer *et al.*, demonstrated the induction of F4/80⁺Gr1⁺CD11b⁺ MDSCs by the MSCs. Ultimately, the MDSCs induced the plasticity of the Th17 cells toward ex-IL17 Foxp3⁺ regulatory T cells [32]. In support of this link, the increase of regulatory myeloid cells in the dLNs found in this study is accompanied by the increase of regulatory T cells as described in our previous report [25].

Different reports endorsed that the immunomodulatory capacity of MSCs takes place both by direct cell-to-cell contact and by means of soluble factors. Gonzalez-Rey *et al.* [24] proposed that hASC–monocyte cocultures produced high amounts of IL10 through a cell to cell contact-dependent mechanism. It has been demonstrated that bone marrow MSCs (BM-MSCs) induce monocytes and dendritic cells in a cell contact-dependent manner to secrete high IL10 amounts [50]. Nemeth *et al.* proposed that both mechanisms, both direct contact and secreted factors, induced the secretion of IL10 by LPS-stimulated macrophages in co-culture experiments with MSCs; although the levels are significantly higher when the LPS-macrophages were in direct contact with MSCs than when they were cultured in transwell plates without a direct contact with the MSCs or exposed to MSC-conditioned medium [29].

Other groups claimed that secreted factors produced by the MSCs are responsible for the skewing of monocytes toward an anti-inflammatory function. In this sense, [33, 51, 52] Yen *et al.* [53] suggested that the expansion of Gr-1⁺CD11b⁺ MDSCs occurs through the secretion of hepatocyte growth factor (HGF) secreted by the MSCs that involves c-Met (the HGF receptor) and downstream phosphorylation of STAT3, a key factor that favors the expansion of MDSC. Melief *et al.* [31] proposed that the factor implicated in the skewing of the monocytes to a regulatory phenotype is via IL6. In line with these observations, Campioni *et al.* [54] proposed that the production of soluble HLA-G by the MSCs is a key molecule mediating their interaction with monocytes.

Interestingly, the IL10⁺F4/80⁺Ly6C⁺ myeloid cells accumulated in the PB and dLNs whereas they were reduced in the spleens in eASC-treated CIA mice. These data suggested that eASCs could favor their migration from spleen to dLNs which could be an additional effect leading to a delayed progression of CIA. In a recent study, Lee *et al.* have shown that MSCs modulate immune responses by a mechanism that involves the recruitment of the Ly6C⁺Ly6G⁻CD11b⁺ MDSCs to the dLNs in a CCL2-dependent manner, thus, reducing the number of Th1 and Th17 cells in periphery [33]. At present, the reactive dLNs are the object of growing attention when monitoring patients with chronic

inflammatory diseases being a relevant tool to investigate immunological changes in the early phases and during the progression of arthritis [55].

In summary, our data demonstrate that eASCs modulate ongoing inflammatory innate responses *in vivo* by a transient increase of Ly6C⁺ monocytes in PB. By day 7, increased frequencies of IL10⁺F4/80⁺Ly6C⁺ regulatory monocytes were found in periphery in eASC-treated CIA mice (Supplementary Figure S2). The presence of the eASCs during the onset of inflammation may have an impact on their trafficking as shown by their preferential accumulation in the dLNs. These results open new avenues for treating RA and other inflammatory-mediated diseases where early innate responses could favor sustained adaptive regulatory T cell responses at the inflamed tissues.

Materials and Methods

Mice

DBA/1 male mice of 6–8 weeks of age were obtained from Janvier SAS, France. 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 processed as described elsewhere [25]. All the eASCs used fulfilled the release criteria of identity, purity, and potency needed for their clinical use.

Induction and evaluation of collagen-induced arthritis (CIA) and treatment with eASCs

Mice were injected subcutaneously in the tail with an emulsion of Chicken Collagen Type II (1 mg/mL, Chondrex, Redmond, CA). A second injection was administered 21 days after the first injection. Clinical signs of arthritis were evaluated daily after the second immunization to determine clinical evidence of arthritis as described [25].

Treatment with one million eASCs was administered intravenously (tail vein) (Grifols, Madrid, Spain) when an arthritis index score of 2–4 was attained [36, 46, 56]. The Arthritis Index Score was conducted until the end of the study according to Ref. [25].

At days 0, 3, and 7 cells were isolated from PB. Complete blood counts were obtained using an automated blood cell-counter (Abacus, Diatron, Budapest, Hungary). At days 3 and 7, mice were culled and mononuclear cells were isolated from spleen and dLNs (inguinal and popliteal) using a cell strainer. A Neubauer chamber was used to determine the number of cells.

Histology and paw edema analysis

At days 7 and 14, paws were fixed in neutral buffered formalin; 3–4 sections were obtained from each paw and stained with hematoxylin and eosin. The degree of inflammation in each section was assessed according to Ref. [25]. Paw edema was assessed daily as the volume of both hind and front paws by the use of a plethysmometer.

Flow cytometry analysis

Isolated mononuclear cells from PB, spleen and dLNs were surface stained with antibodies directed against mouse CD45PerCP (30F11.1), Ly6C-FITC (1G7.G10), and Ly6G-PerCPVio770 (1A8) from Miltenyi Biotech (Bergisch Gladbach, Germany), F4/80-PECy5 (BM8) from eBiosciences (San Diego, CA), streptavidin-BV650 from Biolegend (San Diego, CA), and CD11b-biotin (M1/70), Gr1-biotin (RB6-8C5), Ly6G-BV421 (1A8), and isotype-matched control IgG from Becton Dickinson Pharmingen (Franklin Lakes, NJ). Cells were collected on a BD LSR Fortessa flow cytometer (Becton Dickinson). At least 10,000 events were acquired. Data were analyzed using FlowJo software (Ashland, OR).

Cytokine analysis

For intracellular analysis of cytokine expression, mononuclear cells were stimulated *in vitro* with 5 ng/mL phorbol myristate acetate (PMA; Sigma–Aldrich) and 500 ng/mL ionomycin (Sigma–Aldrich) for 4 h. GolgiStop and GolgiPlug (BD Pharmingen) were added after 1 h. Cells were fixed and stained according to manufacturer instructions (Cytokine BD kit, BD Pharmingen). For intracellular staining, GM-CSF-FITC (MP1-22E9), TNF α (MP6-XT22), and INF γ (XMG1.2) from eBiosciences and IL10-FITC (JES5-16E3) from BD Pharmingen were used.

Statistical analysis

Data are presented as the mean and the standard error of the mean. Normal distribution was analysed by the Shapiro–Wilks test. Non-parametric techniques (Mann–Whitney *U*-test) were used. Analyses were performed using the software Stata 11 (StataCorp, College Station, TX).

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Conflicts of Interest

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Quantification of Ly6C⁺CD11b⁺ and Ly6G⁺CD11b⁺ myeloid cell populations in peripheral blood by flow cytometry.

Figure S2. Schematic model for early innate responses induced by the expanded adipose-derived stem cells (eASCs) in established CIA.