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Mesenchymal Stem Cell-Derived Interleukin 1 Receptor Antagonist Promotes Macrophage Polarization and Inhibits B Cell Differentiation

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Key Words. Interleukin 1 receptor antagonist • Mesenchymal stromal cells • Breg • Macrophage • Arthritis

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ABSTRACT

The role of interleukin 1 receptor antagonist (IL1RA) in mediating the immunosuppressive effect of mesenchymal stem/stromal cells (MSCs) has been reported in several studies. However, how MSC-derived IL1RA influences the host response has not been clearly investigated. We therefore derived MSCs from the bone marrow of *IL1RA* knockout mice and evaluated their immunosuppressive effect on different immune cell subsets. *IL1RA* deficient (*IL1RA*$^{-/-}$) or wild type (wt) MSCs inhibited to the same extend the proliferation of T lymphocytes. On the contrary, *IL1RA*$^{-/-}$ MSCs were less effective than wt MSCs to induce in vitro the macrophage polarization from M1 to M2 phenotype secreting IL10 and exerting a suppressive effect on CD4^{+} T cells. Moreover compared with wt MSCs, *IL1RA*$^{-/-}$ MSCs did not efficiently support the survival of quiescent B lymphocytes and block their differentiation toward CD19^{+}CD138^{+} plasmablasts secreting IgG antibodies. The effectiveness of IL1RA secreted by MSCs in controlling inflammation was further shown in vivo using the collagen-induced arthritis murine model. MSCs lacking *IL1RA* expression were unable to protect mice from arthritic progression and even worsened clinical signs, as shown by higher arthritic score and incidence than control arthritic mice. *IL1RA*$^{-/-}$ MSCs were not able to decrease the percentage of Th17 lymphocytes and increase the percentage of Treg cells as well as decreasing the differentiation of B cells toward plasmablasts. Altogether, our results provide evidence of the key role of IL1RA secreted by MSCs to both control the polarization of macrophages toward a M2 phenotype and inhibit B cell differentiation in vivo. STEM CELLS *2016;34:483–492*

SIGNIFICANCE STATEMENT

In the present study, we present evidence that IL1RA is a key mediator of MSC immunosuppressive effect, which could account for the polarization of macrophages toward the M2 phenotype, together with the already described factors PGE2, IL6 and GM-CSF. We also show that MSC-derived IL1RA impacts on the inhibition of B cell differentiation and the induction of Breg-like cells. The novelty of our study is the conclusion that this effect is at least partly mediated by IL1RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a multifactorial immune disease characterized by the pathological comportment of multiple inflammatory cell populations including T lymphocytes, B lymphocytes, macrophages, and neutrophils, all of which contribute to the local cytokine network and chronic synovitis. Together with tumor necrosis factor (TNF)-α, interleukins (IL)-1 and -6 play an important role in initiating and regulating the pathological immune response [1]. Anti-cytokine therapy therefore became the major focus of clinical research during the past decades and currently available biologics for RA principally target monocyte-derived cytokines, including TNFα, IL1β, or IL6 [2]. One of these biologics is Anakinra, the IL1 receptor antagonist (IL1RA). The efficacy of targeting IL1RA to control inflammation has been shown in several preclinical animal models. In the K/BxN serum transfer-induced arthritis model, myeloid cell-derived IL-1RA was reported to control articular inflammation during the acute phase of arthritis [3]. Although the clinical use of IL1RA has been demonstrated in the management of RA and proposed for diverse human disorders [4, 5],

recombinant IL1RA shows weak effect in RA patients while it is more effective in juvenile idiopathic arthritis [6]. IL1RA is produced by monocytes, macrophages, neutrophils, epithelial cells, and fibroblasts as four isoforms by alternative RNA splicing and translation initiation [7]. Only one isoform of 17 kDA called soluble sIL1RA is secreted; the three other intracellular isoforms icIL1RA1, icIL1RA2, and icIL1RA3 are expressed intracellularly [8]. Both sIL1RA and icIL1RA1 bind to and inhibit IL1RI receptor. In normal homeostasis, IL1RA counterbalances the effect of IL1α and IL1β with other natural IL1 inhibitors, such as soluble IL1RI or IL1RII [4]. While IL1α and IL1β bind to the IL1RI and activate the subsequent intracellular signal transduction pathway, IL1RA functions as a competitive receptor antagonist, thereby inhibiting the biological actions of IL1. IL1RA is also secreted by mesenchymal stromal/stem cells (MSC) [9]. MSCs are mainly isolated from bone marrow (BM), adipose tissue or umbilical cord, and are characterized by the capacity to differentiate toward chondrocytes, osteoblasts, and adipocytes. They exert immunosuppressive properties that confer on them the potential to be used for therapeutic applications in inflammatory diseases, including RA [10]. MSCs induce immune tolerance through the inhibition of proinflammatory T lymphocytes and dendritic cells (DCs) [11, 12]. MSCs promote the survival of resting B lymphocytes but inhibit the polyclonal B cells activation in the presence of interferon (IFN)-γ [13]. They inhibit LPS-mediated macrophage activation and induce a shift from proinflammatory M1 into anti-inflammatory M2 macrophage phenotype [14]. Inducible nitric oxyde synthase (iNOS) for murine cells and indoleamine-2,3-dioxygenase (IDO) for human MSCs have been proposed to be some of the key molecules mediating immunosuppression [15]. Other important mediators are IL-6, PDL-1, TSG6, TGF-β1, and prostaglandin E2 (PGE2) [16–19]. A large body of studies has accumulated to describe the therapeutic effect of MSCs in reducing inflammation and suppressing immune cell function in preclinical injury and autoimmune settings [20]. In the collagen-induced arthritis (CIA) model, we and others have shown their protective and curative roles [17, 21, 22].

Since the demonstration that IL1RA secreted by MSCs protect mice from lung injury by inhibiting tumor necrosis factor (tnf)-alpha (TNFα) and IL1α production by macrophages and the recruitment of lymphocytes and neutrophils, a number of studies has reported its role in the anti-inflammatory effect of MSCs [9, 23, 24]. However, how MSC-derived IL1RA modulates the pathological immune response has not been clearly investigated. The aim of this study was to determine whether IL1RA affects the generation, differentiation, and activation of different immune cell subsets involved in the inflammatory response, with a focus on collagen-induced arthritis as an in vivo model.

Materials and Methods

Generation of Mesenchymal Stem Cells

IL1RA knockout C57BL/6 mice were obtained from W. van den Berg (The Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands). The BM from femurs and tibias was flushed out and seeded at 5×10^5 cells per square centimeter in proliferative medium consisting of a modified minimum essential Eagle's medium (αMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza, Levallois-Perret, France), and 2 ng/ml human basic fibroblast growth factor (bFGF) (R&D Systems, Lille, France). At 70% confluence, adherent cells were trypsinized and plated for subsequent culture. Phenotypic analysis was performed when cells were passaged till having a population of cells that was negative for the expression of the hematopoietic marker CD45 (passage 5). Wild type (wt) MSCs from C57BL/6 mice were already described [25]. Cells were used between passages 6 to 10.

Flow Cytometry Analysis

Cells were incubated in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) with antibodies specific for CD19, CD44, CD45, CD73, CD86, CD105, CD138, Sca-1, B220, major histocompatibility complex class II (MHCII), F4/80, ICAM, VCAM, and PDL1 (eBioscience, Paris, France) or the respective isotype controls for 20 minutes at 4°C in the dark (BD-Bioscience, Le Pont-de-Claix, France). For intracellular staining, cells were stimulated with PMA (50 ng/ml), ionomycin (1 μg/ml) and brefeldin A (10 μg/ml) for 4 hours. Cells were stained with antibodies specific for mouse CD4 and CD25 for 30 minutes and permeabilized with the Perm/Fix solution (eBioscience). Intracellular staining with antibodies specific for IL10, IL17, IFN-γ (BD-Bioscience) or FOXP3 (eBioscience) was then performed for 30 minutes in the dark. For apoptosis detection, cells were incubated with the Annexin V-PE apoptosis detection kit following manufacturer's instructions (eBioscience). The labeled cells were analyzed by flow cytometry using a FACSCanto cytometer and the Diva software (BD-Bioscience).

Differentiation Assays

Differentiation of MSCs was induced by culture in specific conditions for 21 days as described previously [17]. Briefly, adipogenesis was induced by culture in complete Dulbecco's modified Eagle's medium (DMEM)-F12 (Lonza) containing 16 μM biotin, 18 μM panthotenic acid, 100 μM ascorbic acid, 5 μg/ml insulin, 0.03 μM dexamethasone, 1 μg/ml transferrin, and 2 ng/ml triiodothyronine (T3) (Sigma-Aldrich, Saint-Quentin Fallavier, France). For osteogenesis induction, cells were plated at 6×10^4 cells per square centimeter in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml ascorbic acid. Chondrogenesis was induced by pelleting 2.5×10^5 cells in 500 μl of DMEM medium containing 10 ng/ml transforming growth factor beta (TGFB)-3 (R&D Systems), 1 mM sodium pyruvate, 170 μM ascorbic-2-phosphate acid, 350 μM proline, and insulin–transferrin–selenic acid (Lonza) in 15 ml conic tubes. Media were changed every 2 days for 21 days before differentiation assessment by quantifying markers specific for each lineage by real-time quantitative polymerase chain reaction (RT-qPCR).

Real-Time Quantitative Polymerase Chain Reaction

RNA was extracted using the RNeasy kit (Qiagen, Courtaboeuf, France) and reverse-transcribed using the M-MLV enzyme (Fisher scientific, Illkirch, France). Primers were designed using the Primer3 software. Sequences were as follows: *IL6* 5′-TGGGACTGATGCTGGTGACA and 3′-TTCCACGATTTCCCAG AGAACA; *IL1RA*, 5′-AGGCCCCACCACCAGCTTTGA and 3′-GGGGC TCTTCCGGTGTGTTGGT; *iNOS*, 5′-CCTTGTTCAGCTACGCCTTC and 3′-GCTTGTCACCACCAGCAGTAGGGGCTCTTCCGGTGTGTTGGT;

Cox2, 5′-GCATTCTTTGCCCAGCACTT and 3′-AGACCAGGCA CCAGACCAAAGA; *TGFB1* 5′-TGCGCTTGCAGAGATTAAAA and 3′-CTGCCGTACAACTCCAGTGA or published previously [26]. PCR reaction was carried out on 25 ng of cDNA samples using 5 μmol/l of each primer and 5 μl 2 × SybrGreen PCR Master Mix (Roche, Meylan, France). The following conditions were used: 95°C for 5 minutes; 40 cycles at 95°C for 15 seconds; 64°C for 10 seconds and 72°C for 20 seconds in a LightCycler 480 system (Roche diagnostics, Meylan, France). All values were normalized to *RPS9* housekeeping gene and expressed as relative expression using the formulae $2^{-\Delta CT}$.

Cytokine and NO Quantification

For cytokine quantification, MSCs were cultured in proliferative medium and activated or not with 20 ng/ml of IFN-γ alone or together with 10 ng/ml of TNFα or IL1β (R&D Systems). Supernatants were recovered after 48 hours. In coculture experiments, immune cells were added to MSCs at indicated ratios for 48 hours before supernatant recovery. Cytokine production by splenocytes or lymph node cells was analyzed after incubation of 2×10^6 cells in MLR medium and stimulation with 25 μg/ml of chicken collagen II (cCII) for 48 hours. MLR medium consisted in IMDM (Life Technologies) containing 10% of heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 mM HEPES, and 50 μM of beta-mercaptoethanol (Life Technologies). Quantification of IL1RA, IL6, IL10, PGE2, and TNFα in culture supernatants was performed by enzyme-linked immunosorbent assay (ELISA) from R&D Systems. TNFα and IL1β were quantified using the specific cytometric bead array (CBA) enhanced sensitivity Flex Set kit (BD-Bioscience) when indicated.

For nitric oxide (NO) production, NO_2 production was quantified using a modified Griess reagent (Sigma-Aldrich).

Proliferation Assay

Splenocytes were obtained after mechanical dissociation of spleens and $CD4^+$ T lymphocytes were isolated using the Dynal CD4 negative isolation kit according to the manufacturer's instructions (Life Technologies, Saint Aubin, France). $CD4^+$ T cells labeled with Cell Trace Violet (CTV; Molecular Probes, Eugene, OR) were activated with 5 μg/ml of concanavalin A (ConA) (Sigma-Aldrich) and cultured in MLR medium. When indicated, CTV $CD4^+$ T cells were cultured in presence of MSCs or supernatants from MSCs:macrophages cocultures. After 72 hours, T cell proliferation was measured by flow cytometry and the CellTiter-Glo luminescent cell viability assay according to the supplier's indications (Promega, Charbonnières-les-Bains, France).

Differentiation of $CD4^+$ T Cells

$CD4^+$ T cells were stimulated with anti-mouse CD3/CD28 Dynabeads (Life Technologies) and cultured alone or with MSCs at the ratio of 1:10 (MSC:T cells). Th17 differentiation was induced with 2.5 ng/ml TGFB1, 50 ng/ml IL6 (R&D Systems), 5 μg/ml of anti-IFN-γ, and anti-IL4 blocking antibodies (BD-Biosciences). Th1 differentiation was obtained by adding 20 ng/ml IL12 (R&D Systems) and 5 μg/ml anti-IL4 antibodies for 4 days.

Isolation and Differentiation of Macrophages

Macrophages (MΦ) were isolated from spleens or BM using the positive selection CD11b kit following manufacturer's instructions (Miltenyi, Paris, France). $CD11b^+$ cells were plated at the density of 2×10^5 cells per square centimeter and cultured in MLR medium containing 20 ng/ml of macrophage colony-stimulating factor (M-CSF) (R&D System). After 5 days of culture, macrophages were activated with 100 ng/ml of lipopolysaccharides (LPS) and cultured alone or with MSCs (1 MSC:10 MΦ) for another 24 hours period. When indicated, recombinant IL1RA (Anakinra; 2 ng/ml) was added in the activating culture medium containing LPS.

Isolation and Differentiation of B Lymphocytes

B lymphocytes were isolated from spleen by negative selection, using the CD43 microbeads kit following supplier's recommendations (Miltenyi). Total B cells were labeled with 2 μM of CTV at 37°C for 10 minutes and cultured in MLR medium without beta-mercaptoethanol in the presence or absence of MSCs at the 1 MSC:5 B cells ratio. For B cell activation, 1 μg/ml of CpG-containing oligodeoxynucleotides (CpG-ODN) 1826 (5′-TCC-ATG-ACG-TTC-CTG-ACG-TT-3′) (Enzo Life Science, Villeurbanne, France), 2.5 μg/ml unconjugated goat F(ab)2 anti-mouse IgM (Jackson Immunoresearch, Suffolk, UK), 25 ng/ml CD40L, and 1000 U of IL2 (R&D Systems) were added to the culture for 3 days. When indicated, 5 ng/ml of IFN-γ was added.

IgG Quantification in Culture Supernatants

Total IgG was quantified by a modified ELISA using goat anti-mouse total IgG microplates (R&D Systems). Briefly, serial dilutions of culture supernatants of B cells were incubated for 1 hour at room temperature (RT). Plates were then washed three times with PBS containing 0.05% Tween 20 and incubated with a goat anti-mouse IgG antibody (BD Bioscience) for 1 hour at RT. Following the incubation with the streptavidin–horseradish peroxidase (R&D Systems) for 15 minutes, the assay was developed with the 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Interchim, Montlucon, France) and read spectrophotometrically at 450 nm.

The cCII-specific immunoglobulins in mouse sera were quantified using alkaline phosphatase-labeled anti-mouse IgG1 or IgG2a antibodies (BD-Biosciences). cCII (5 μg/ml) was incubated in a 96-well plate at 4°C overnight. After three washes, a blocking solution (PBS with 2% bovine serum albumin) was added for 1 hour at RT. Sera were diluted in PBS (1:300 and 1:600 for IgG1 and IgG2a, respectively) and incubated at 4°C overnight. Alkaline phosphatase-conjugated anti-mouse IgG1 and IgG2a antibodies were then added for 1 hour before incubation with p-nitrophenyl phosphate (PnPP) (Sigma Aldrich) and spectrophotometric reading at 450 nm.

Induction of Arthritis

Arthritis was induced in DBA/1 adult male mice (9–10 weeks old) and performed in accordance with the Ethical Committee for animal experimentation of the Languedoc-Roussillon (Approval CEEA-LR-1042). In brief, 2 mg/ml of cCII was diluted in acetic acid 0.05M and emulsified in an equal volume of Freund's complete adjuvant (Thermoscientific, Rockford, IL) in

the presence of 8 mg/ml of *Mycobacterium tuberculosis* (Difco, Le Pont-de-Claix, France). The suspension (100 μl) was injected intradermally at the basis of the tail at day 0. At day 21, a boost with cCII in Freund's incomplete adjuvant was administered. MSCs (1×10^6 cells) were injected intravenously at day 18 and 24. The assessment of clinical signs of arthritis was blinded and performed by two researchers by measuring the paw swelling of the hind paws and evaluating the clinical score using the macroscopic scale as described previously [27]. At euthanasia, blood, draining lymph nodes, and spleens were collected for immune cell analysis by cytometry. Hind limbs were recovered for X-ray microcomputed tomography (μCT) and histological analysis (H&E–safran O staining).

Bone Analysis

The hind paws were fixed in 4% formaldehyde and scanned in a μCT scanner (Skyscan 1172, Bruker microCT, Kontich, Belgium). Image acquisition (image pixel size of 18 μm) required 15 minutes using the following parameters: 50 kV, 500 μA, 0.5 mm aluminum filter, 180° scan. Image reconstruction and analysis was performed using the NRecon software (Skyscan) and required 10 minutes. The bone volume (BV) and bone volume density (bone volume/trabecular bone volume; BV/TV) were calculated using the CTAn software (Skyscan).

Statistical Analysis

All results were expressed as the mean ± SEM. Two way analysis of variance tests were performed to analyze differences between groups and time points. The χ^2 test was used for analyzing the incidence. The Mann–Whitney test was used for comparisons between two groups. The tests used are indicated in the figure legends. *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ were considered statistically significant. Analysis and graphical representation were performed using Graph-Pad Prism software (Graphpad, San Diego, CA).

Results

In Absence of IL1RA, the Secretion of Factors Responsible for the Immunosuppressive Function of MSCs is Stimulated

To investigate the role of IL1RA in the immunosuppressive function of murine MSCs, we isolated MSCs from the BM of *IL1RA* knockout mice. At passage 5 and higher passages, the isolated adherent cells were negative for hematopoietic cell markers (CD45, F4/80) and positive for the MSC surface markers CD44, CD105, and SCA1 while CD73 was not expressed (Supporting Information Fig. 1A). We confirmed that isolated cells were negative for IL1RA both at the mRNA level and protein level (Fig. 1A, 1B). We also quantified the expression of IL1RA after activation with inflammatory mediators known to stimulate the immunosuppressive potential of MSCs. We found that while IFN-γ in conjunction with TNFα had no significant effect on the production of IL1RA by MSCs, IL1RA was significantly increased in wt MSCs but not in *IL1RA*$^{-/-}$ MSCs when IL1β was added with IFN-γ (Fig. 1B). After culture in specific conditions, *IL1RA*$^{-/-}$ cells were shown to differentiate into chondrocytes expressing *Sox9, Col2a1Δ2, ACAN*, adipocytes expressing *Pparg, Lpl, Fabp4*, or osteoblasts expressing *Runx2, Bglap, AP* (Supporting Information Fig. 1B–1D). The degree of differentiation of the *IL1RA*$^{-/-}$ MSCs was however less than that obtained with wt MSCs. All these characteristics confirmed that isolated cells were MSCs.

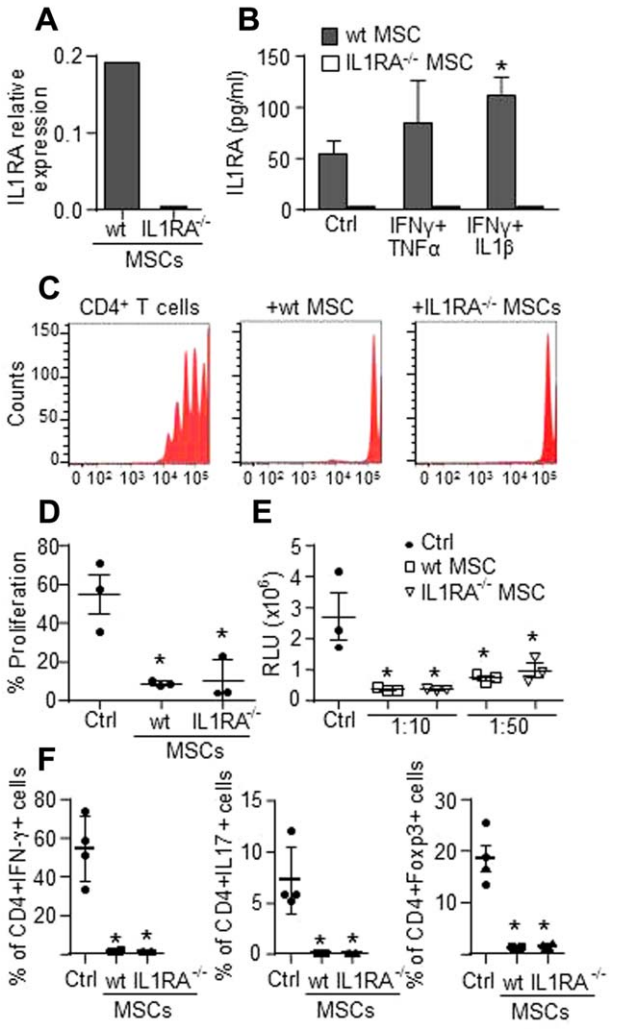
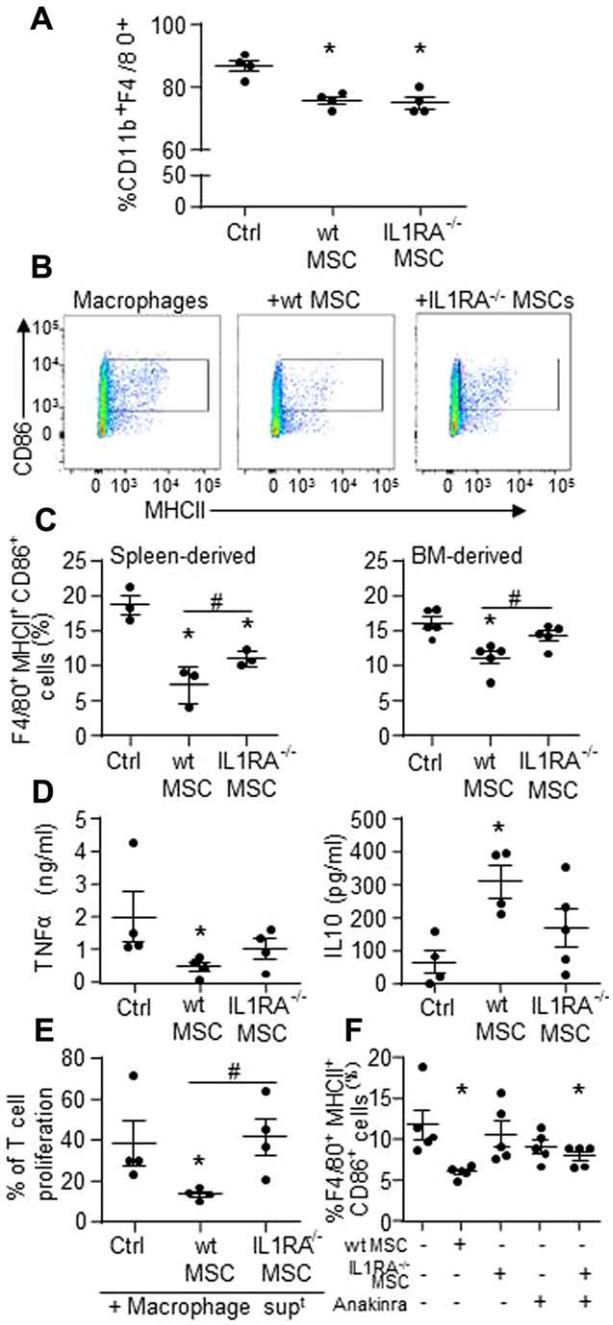


Figure 1. Effect of interleukin 1 receptor antagonist (IL1RA)$^{-/-}$ mesenchymal stem/stromal cells (MSCs) on T cell proliferation and differentiation. **(A)**: Expression of *IL1RA* transcripts in wild type (wt) or *IL1RA*$^{-/-}$ MSCs by real-time quantitative polymerase chain reaction. **(B)**: Quantification of IL1RA in the supernatants of wt or *IL1RA*$^{-/-}$ MSCs in control basal conditions (Ctrl) or after activation with interferon (IFN)-γ and tumor necrosis factor-α or IL1β ($n = 4$). **(C)**: Representative pictures of proliferation of Cell Trace Violet (CTV)-labeled CD4$^+$ T cells after activation with anti-CD3$^+$CD28$^+$ antibodies (left panel) or cocultured with wt or *IL1RA*$^{-/-}$ MSCs (middle and right panels, respectively). **(D)**: Mean proliferation of CTV-labeled CD4$^+$ T cells as described in (C) ($n = 4$). **(E)**: Proliferation of CD4$^+$ T cells expressed in relative luminescent unit (RLU) after Con A activation (Ctrl) or coculture with wt or *IL1RA*$^{-/-}$ MSCs as measured using the viability assay Cell Titer Glo ($n = 4$). **(F)**: Percentage of Th1, Th17, and Treg cells (left, middle and right panels, respectively) induced under in vitro differentiating conditions from naïve T cells (Ctrl) and in coculture with wt or *IL1RA*$^{-/-}$ MSCs ($n = 4$). Statistical analyses used the Mann-Whitney test comparing two groups (*, $p < 0.05$ for treated vs. Ctrl). Abbreviations: IFN, interferon; IL1RA, interleukin 1 receptor antagonist; MSC, mesenchymal stem/stromal cell; wt, wild type.

To further characterize the *IL1RA*$^{-/-}$ MSCs, we determined the production of several factors mediating the immunosuppressive function of activated MSCs. Expression of *Il6, iNos*, and *Cox2* increased upon activation of wt MSCs by IFN-γ alone or IFN-γ plus IL1β or TNFα while *Tgfb1* expression decreased when wt MSCs were activated with IFN-γ plus IL1β or TNFα (Supporting Information Fig. 2A). Interestingly, the expression of *IL6, iNos*, and *Cox2* was significantly higher in *IL1RA*$^{-/-}$ MSCs than wt MSCs in unstimulated control conditions. After MSC stimulation by IFN-$\gamma \pm$ TNFα, *IL6, iNos, Cox2*, and *Tgfb1* expression was significantly increased. Addition of IFN-$\gamma \pm$ IL1β upregulated the expression of all those factors except for *iNos*, which was downregulated. Similar results were obtained when protein levels were quantified. We noticed the upregulation of IL6, PGE2, and NO both in wt and *IL1RA*$^{-/-}$ MSCs after activation with IFN-$\gamma \pm$ TNFα or IL1β (Supporting Information Fig. 2B). The secretion of these factors was however significantly higher in *IL1RA*$^{-/-}$ MSCs. Looking at the expression of markers that are expressed at the cell membrane, we did not find any statistical differences between wt and *IL1RA*$^{-/-}$ MSCs for the expression of PDL1 or VCAM but an increase in ICAM expression in *IL1RA*$^{-/-}$ MSCs in absence of priming. Altogether, a slight increase in the secretion of several immunoregulatory factors has been evidenced for *IL1RA*$^{-/-}$ MSCs.



IL1RA Secreted by MSCs Is Not Responsible for Their Inhibitory Effect on T Lymphocyte Proliferation

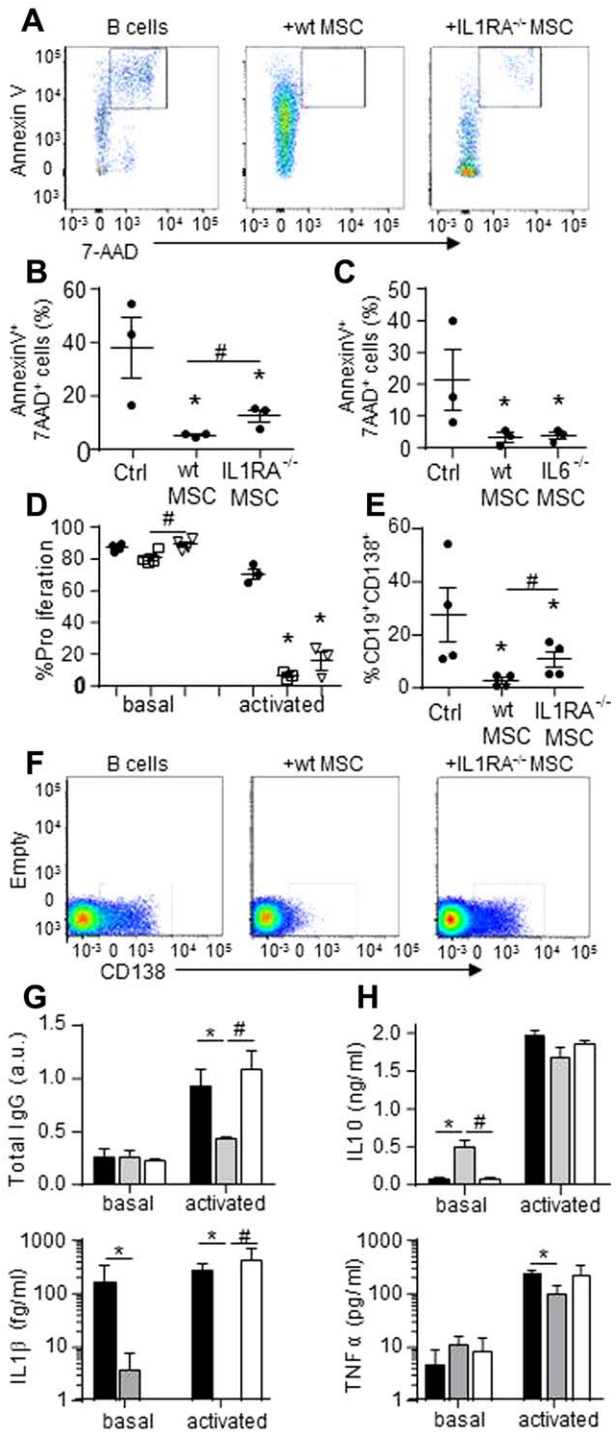
We then investigated the immunosuppressive function of the *IL1RA*$^{-/-}$ MSCs on CTV-labeled CD4$^+$ T cells activated with concanavalin A for 3 days. In coculture, *IL1RA*$^{-/-}$ and wt MSCs inhibited the proliferation of CD4$^+$ T cells to the same extend (Fig. 1C, 1D). This inhibitory effect was confirmed using a viability assay using the same MSCs:CD4$^+$ T cells ratio of 1:10 or a higher ratio of 1:50 (Fig. 1E). Similar results were obtained on ConA-activated T cells using total splenocyte cultures (data not shown). These results indicated that both wt and *IL1RA*$^{-/-}$ MSCs can inhibit CD4$^+$ T cell proliferation. We then determined whether *IL1RA*$^{-/-}$ MSCs could modulate the differentiation potential of naïve CD4$^+$ T cells toward Th1, Th17, or Treg lymphocytes as efficiently as wt MSCs during culture in inductive conditions. CD4$^+$ T cells were cultured with either IL-12 and anti-IL-4 for Th1 differentiation or, TGFB1, IL6, anti-IFN-γ, and anti-IL4 antibodies for Th17 differentiation. In these conditions, *IL1RA*$^{-/-}$ and wt MSCs were equally potent to inhibit the differentiation of naïve CD4$^+$ T cells toward T helper (Fig. 1F). We also observed a equivalent reduction in the number of CD4$^+$Foxp3$^+$ Treg lymphocytes with both wt and *IL1RA*$^{-/-}$ MSCs, demonstrating that IL1RA had no direct effect on T cell differentiation and proliferation.

IL1RA Induces the Differentiation of Macrophages Toward a M2-Like Phenotype

As IL1RA produced by MSCs was not involved in their suppressive function on T cells, we investigated whether *IL1RA*$^{-/-}$

Figure 2. Effect of interleukin 1 receptor antagonist (IL1RA)$^{-/-}$ mesenchymal stem/stromal cells (MSCs) on macrophage differentiation and polarization. **(A)**: Percentage of macrophages generated from bone marrow (BM) progenitors in control inducing conditions (Ctrl) or in coculture wild type (wt) or *IL1RA*$^{-/-}$ MSCs ($n = 4$). **(B)**: Representative pictures of activated macrophage phenotypes (left panel) or after coculture with wt or *IL1RA*$^{-/-}$ MSCs (middle and right panels, respectively). **(C)**: Mean percentage of activated macrophages generated from spleen ($n = 3$) or from BM ($n = 5$) as described in (B). **(D)**: Quantification of tumor necrosis factor-α and IL10 in the supernatants of activated macrophages (Ctrl) or cocultured with wt or *IL1RA*$^{-/-}$ MSCs ($n = 5$). **(E)**: Proliferation of T cells when cultured with the supernatants (sup^t) recovered from activated macrophages (Ctrl) or cocultured with wt or *IL1RA*$^{-/-}$ MSCs ($n = 4$). **(F)**: Addition of IL1RA (Anakinra) to *IL1RA*$^{-/-}$ MSCs restored the inhibitory effect of MSCs on macrophage differentiation ($n = 5$). Statistical analyses used the Mann-Whitney test comparing two groups (*, $p < 0.05$ for treated vs. Ctrl; #, $p < 0.05$ for wt vs. *IL1RA*$^{-/-}$). Abbreviations: BM, bone marrow; IL1RA, interleukin 1 receptor antagonist; MHCII, major histocompatibility complex class II; MSC, mesenchymal stem/stromal cell; wt, wild type.

MSCs could exert reduced immunomodulatory functions on other immune cell types. Because IL1β is primarily produced by monocytes/macrophages, we hypothesized that MSC-derived IL1RA may inhibit the IL1β-induced autocrine activation of these cells [28]. First, we studied the role of IL1RA on the generation of macrophages from BM progenitors. We therefore isolated CD11b$^+$ cells that were cultured with M-CSF for 5 days to induce their differentiation into macrophages. The percentage of CD11b$^+$F4/80$^+$ macrophages was slightly but significantly reduced when progenitors were cultured in presence of wt or *IL1RA*$^{-/-}$ MSCs with no differences between the two cell types (Fig. 2A). We then induced the activation of macrophages and looked at the expression of the activation markers MHCII and CD86 after LPS addition. We found out that the number of MHCII$^+$CD86$^+$ expressing cells among the F4/80$^+$ macrophages was highly decreased in spleen- or BM-derived progenitors in cocultures with wt MSCs. This effect was partially but significantly reversed after culture with *IL1RA*$^{-/-}$ MSCs (Fig. 2B, 2C). The lower number of activated macrophages was associated with a significant decrease in TNFα in presence of wt MSCs but not *IL1RA*$^{-/-}$ MSCs and a significant increase in IL10 levels in presence of wt MSCs (Fig. 2D). Finally, we tested the effect of the supernatants recovered from MSC/macrophage cocultures on the proliferation of activated CD4$^+$ T lymphocytes after 5 days. Under these conditions, the supernatant from wt MSCs/macrophage cocultures, but not from *IL1RA*$^{-/-}$ MSCs, significantly decreased the proliferation of T cells by more than 2-fold (Fig. 2E). The proliferation rate of T cells cultured in presence of supernatant from *IL1RA*$^{-/-}$ MSCs/macrophage cocultures was similar to control. The higher levels of immunomodulatory factors secreted by *IL1RA*$^{-/-}$ MSCs (Supporting Information Fig. 2) did likely not compensate the absence of IL1RA. Finally, we performed a rescue experiment using recombinant IL1RA (Anakinra). When Anakinra was added in the medium for activating macrophages in presence of *IL1RA*$^{-/-}$ MSCs, the inhibition of macrophage differentiation was restored to levels similar to those obtained with wt MSCs (Fig. 2F). These results indicated that IL1RA produced by MSCs had no effect on the differentiation of BM progenitors to macrophages while it promoted the activation of macrophages toward a M2-like macrophage phenotype with immunosuppressive function on CD4$^+$ T cells.



IL1RA Increases the Survival Rate of B Lymphocytes and Inhibits the Maturation of B Lymphocytes

B lymphocytes are also IL1β-producing and -responsive cells. We therefore wanted to determine the effect of wt and *IL1RA*$^{-/-}$ MSCs on the survival and maturation of CD19$^+$ B

Figure 3. Effect of interleukin 1 receptor antagonist (*IL1RA*)$^{-/-}$ mesenchymal stem/stromal cells (MSCs) on B cell survival and maturation. **(A)**: Representative pictures of apoptotic B lymphocytes in control culture conditions (left panel) or after coculture with wild type (wt) or *IL1RA*$^{-/-}$ MSCs (middle and right panels, respectively). **(B)**: Mean percentage of apoptotic B cells as described in (A). **(C)**: Mean percentage of apoptotic B cells in control culture conditions (Ctrl) or with wt or *IL6*$^{-/-}$ MSCs. **(D)**: Proliferation of B cells without ([circf]: Ctrl) or with wt MSCs ([squo]) or *IL1RA*$^{-/-}$ MSCs ([itrio]) in basal or interferon-γ-activated conditions. **(E)**: Percentage of plasmablasts obtained upon activation (Ctrl) or culture with wt or *IL1RA*$^{-/-}$ MSCs. **(F)**: Representative pictures of plasmablasts as described in (E). **(G)**: Quantification of total IgG secreted in supernatants from plasmablasts from (E) and expressed as arbitrary unit (a.u.). **(H)**: Quantification of IL10, IL1β, and tumor necrosis factor-α in the supernatants from plasmablasts from (E). Statistical analyses used the Mann-Whitney test comparing two groups ($n = 4$; *, $p < 0.05$ for treated vs. Ctrl; #, $p < 0.05$ for wt vs. IL1RA$^{-/-}$). Abbreviations: AAD, 7-aminoactinomycine D; IL1RA, interleukin 1 receptor antagonist; MSC, mesenchymal stem/stromal cell; TNF, tumor necrosis factor; wt, wild type.

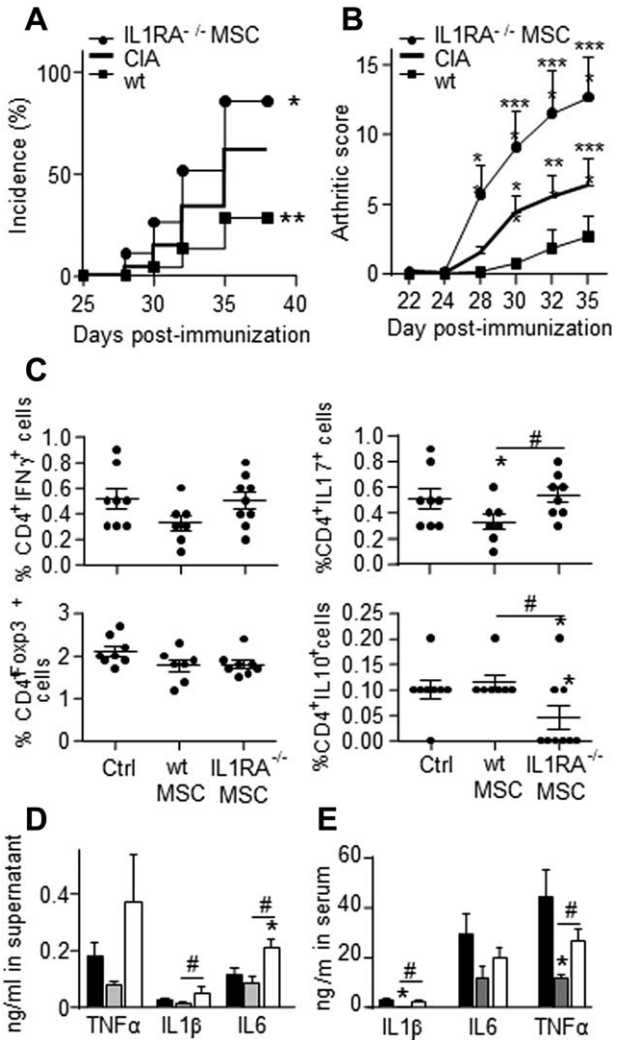


Figure 4. Effect of interleukin 1 receptor antagonist ($IL1RA$)$^{-/-}$ mesenchymal stem/stromal cells (MSCs) in the collagen-induced arthritis model. **(A)**: Incidence of arthritis in mice injected with phosphate-buffered saline (PBS) (Ctrl) or wild type (wt) or $IL1RA^{-/-}$ MSCs. Statistical analysis used the χ[2] test ($n = 18$ from two separate experiments). **(B)**: Arthritic score of mice injected with PBS (Ctrl) or wt or IL1RA$^{-/-}$ MSCs. Statistical analysis used the two way analysis of variance test ($n = 18$ from two separate experiments). **(C)**: Percentage of Th1, Th17, Treg, and Tr1 cells detected in the spleen of Ctrl mice or mice treated with wt or $IL1RA^{-/-}$ MSCs. **(D)**: Quantification of tumor necrosis factor (TNF)-α, IL1β, and IL6 in the supernatants from splenocyte cultures. **(E)**: Quantification of TNF-α, IL1β, and IL6 in the serum of mice. Statistical analysis used the Mann-Whitney test comparing two groups ($n = 8$ for panels (C–E); *, $p < 0.05$ for treated vs. Ctrl; #, $p < 0.05$ for wt vs. $IL1RA^{-/-}$). Abbreviations: CIA, collagen-induced arthritis; IFN, interferon; IL1RA, interleukin 1 receptor antagonist; MSC, mesenchymal stem/stromal cell; TNF, tumor necrosis factor; wt, wild type.

lymphocytes. CD43$^-$ naïve B cells isolated from spleen were cultured for 3 days without stimulation [29]. After 3 days of culture in basal conditions, wt MSCs markedly reduced the percentage of AnnexinV$^+$ 7AAD$^+$ dead B cells while $IL1RA^{-/-}$ MSCs partially but significantly reversed this effect (Fig. 3A, 3B). Since IL6 has been proposed to be an anti-apoptotic/prosurvival factor for B cells, we performed a similar experiment using $IL6^{-/-}$ MSCs. Our results showed that wt and $IL6^{-/-}$ MSCs exerted a similar effect on the survival of B lymphocytes indicating that the anti-apoptotic effect was not mediated by IL6 (Fig. 3C). We therefore demonstrated that IL1RA and not IL6 was partly responsible for the prosurvival role of MSCs on B cells. Looking at the proliferation of B lymphocytes in basal conditions and in presence of wt or $IL1RA^{-/-}$ MSCs, no significant effect of either MSCs was observed (Fig. 3D). By contrast upon IFN-γ stimulation, a substantial impact on their proliferation was observed both with wt and $IL1RA^{-/-}$ MSCs.

Differentiation of B cells toward CD138$^+$ plasmablasts upon CPG-ODN activation was then evaluated. Both wt and $IL1RA^{-/-}$ MSCs significantly inhibited plasmablast differentiation, $IL1RA^{-/-}$ MSCs were however less efficient (Fig. 3E, 3F). Inhibition of plasmablast generation by wt MSCs was confirmed by the low levels of total IgG produced in coculture supernatants while similar levels of total IgG were detected in supernatants from control and $IL1RA^{-/-}$ MSCs (Fig. 3G). Interestingly in basal conditions, IL10 production by B cells was highly upregulated when cocultured with wt MSCs but not with $IL1RA^{-/-}$ MSCs (Fig. 3H). This effect was not observed upon B cell activation. On the contrary, IL1β secretion was significantly downregulated by wt MSCs both in basal and activated conditions but not with $IL1RA^{-/-}$ MSCs in activated conditions. No strong effect of wt or $IL1RA^{-/-}$ MSCs on TNFα secretion was noticed. Altogether, our data suggested that IL1RA plays a role in increasing the survival of B lymphocytes and inhibiting plasmablast differentiation likely through the generation of IL10-producing Breg-like cells.

IL1RA Reduces the Inflammatory Response in Arthritic Mice

We then aimed at investigating the effect of IL1RA in vivo using the inflammatory collagen-induced arthritis (CIA) murine model. In this model, wt MSCs significantly reduced both the incidence and clinical signs of arthritis. Indeed, while a significant increase in the mean arthritic score was observed in the control CIA group from days 30 to 35 as compared to day 22, no significant increase was recorded with wt MSC-treated mice (Fig. 4B). In contrast, $IL1RA^{-/-}$ MSCs were not able to prevent arthritis progression and even worsened the arthritic symptoms as monitored from days 28 to 35 by the clinical score and increased incidence (Fig. 4A, 4B). Histological analysis confirmed the presence of large amounts of inflammatory cells and areas of bone erosion in control and $IL1RA^{-/-}$ MSCs-treated mice and not in wt MSCs-treated group (Supporting Information Fig. 3A). Protection against bone erosion in hind paw by wt MSCs as compared to control or $IL1RA^{-/-}$ MSCs-treated mice was confirmed by μCT analysis (Supporting Information Fig. 3B, 3C). Although not significantly altered, the bone volume and the bone volume density were decreased in several mice in the control and $IL1RA^{-/-}$ MSCs-treated mice (Supporting Information Fig. 3B). We then evaluated the variations of the different T lymphocyte subsets in response to the treatment. We found a decrease in the number of CD4$^+$IFN-γ^+ Th1 and CD4$^+$IL-17$^+$ Th17 lymphocytes in the spleens of wt MSCs-treated mice as compared to CIA or $IL1RA^{-/-}$ MSCs-treated mice (Fig. 4C). Although we did not observe any change in the frequency of CD4$^+$Foxp3$^+$ Treg cells between groups, the percentage of CD4$^+$IL10$^+$ Tr1-like

regulatory cells was significantly decreased in *IL1RA*$^{-/-}$ MSCs-treated mice. Importantly, when we calculated the ratio of the percentage of CD4^{+}IL17^{+} Th17 cells/percentage of CD4^{+}IL10^{+} Tr1 cells in spleens, it was higher in mice injected with *IL1RA*$^{-/-}$ MSCs than with wt MSCs; the ratios were 13 (0.54/0.04) versus 3 (0.32/0.11), respectively. This indicated that the balance between inflammatory and regulatory responses shifted in favor of inflammation when *IL1RA*$^{-/-}$ MSCs were injected. In concordance with these data, lower levels of TNFα, IL1β, and IL6 were measured in the supernatants of Con A-activated splenocytes from wt MSCs-treated mice as compared to *IL1RA*$^{-/-}$ MSCs-treated mice (Fig. 4D). Serum levels of IL1β and TNFα were significantly decreased in wt MSCs-treated mice while they were elevated in *IL1RA*$^{-/-}$ MSCs-treated mice (Fig. 4E).

Looking at the impact of IL1RA on B lymphocytes, we observed a decrease in B220^{+}CD19^{+}CD138^{+} plasmablasts in the blood and spleen of wt MSCs-treated mice as compared to control or *IL1RA*$^{-/-}$ MSCs-treated mice (Fig. 5A–5C). This was related with lower ratios of IgG2a/IgG1 specific for chicken collagen II in wt MSC-treated mice while there was no effect of *IL1RA*$^{-/-}$ MSCs (Fig. 5D). We however did not notice any significant difference in the percentage of CD19^{+}IL10^{+} Breg-like cells in the three groups of mice (Fig. 5E). Finally, we cultured the macrophages isolated from peritoneum and activated them with LPS. While the secretory profile of macrophages was similar in control and wt MSC-treated mice, elevated levels of TNFα were measured in *IL1RA*$^{-/-}$ MSCs-treated mice while NO_2 tended to increase (Fig. 5F). The results confirmed the regulatory role of IL1RA on B lymphocytes and macrophages in vivo in a model of inflammation. The modulation of maturation of these immune cell subsets could impact on the differentiation of Th1 and Th17 lymphocytes and explain the absence of therapeutic effect of *IL1RA*$^{-/-}$ MSCs in arthritis.

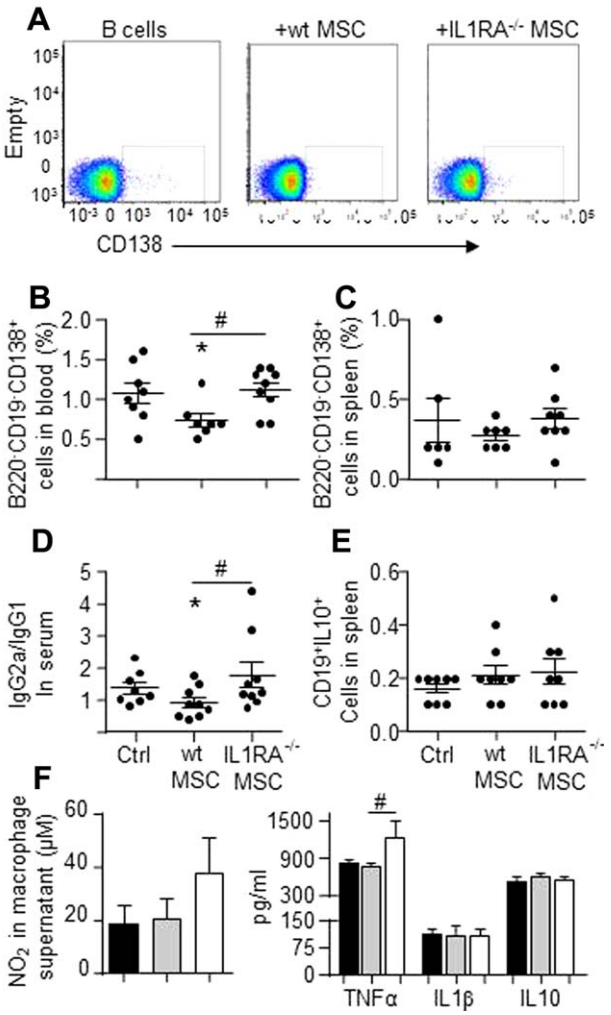


Figure 5. Effect of interleukin 1 receptor antagonist (*IL1RA*)$^{-/-}$ mesenchymal stem/stromal cells (MSCs) on B cells and macrophages in the collagen-induced arthritis model. **(A)**: Representative pictures of plasmablasts in the blood of control mice (left panel) or mice injected with wild type (wt) or *IL1RA*$^{-/-}$ MSCs (middle and right panels, respectively). **(B)**: Mean percentage of plasmablasts in the blood of mice as described in (A). **(C)**: Mean percentage of plasmablasts in spleen of mice. **(D)**: Quantification of collagen II-specific IgG2a and IgG1 antibodies in the serum. **(E)**: Percentage of Breg-like cells in the spleen of mice. **(F)**: Quantification of NO_2, tumor necrosis factor-α, IL1β, and IL10 in the supernatants of macrophages isolated in the peritoneum from arthritic and treated mice. Statistical analyses used the Mann-Whitney test comparing two groups ($n = 8$; *, $p < 0.05$ for treated vs. Ctrl; #, $p < 0.05$ for wt vs. *IL1RA*$^{-/-}$). Abbreviations: IL1RA, interleukin 1 receptor antagonist; MSC, mesenchymal stem/stromal cell; TNF, tumor necrosis factor; wt, wild type.

Discussion

Although IL1RA is proposed to be one of the mediators of the therapeutic effect of MSCs by antagonizing IL1 effects and blocking inflammation, its role on the immune cell function is poorly investigated. Our results indicate that IL1RA secreted by MSCs both acts on macrophages by inducing a polarization toward the anti-inflammatory M2 phenotype and on B lymphocytes by reducing their differentiation toward plasmablasts.

IL1RA is secreted by macrophages, in particular the M2a and M2c subtypes of anti-inflammatory macrophages after stimulation by IL4, IL13, and IL10, respectively [30]. IL1RA was the single gene associated with M2 polarization reported in chronic infectious diseases, where bacterial pathogens have evolved strategies to prevent M1 polarization and escape immune response [31]. Indeed, IL1RA is proposed as a marker of macrophage activation toward a M2 phenotype. However the role of IL1RA on macrophage activation is poorly documented although it has been shown to activate the switch of macrophages toward the M2b signature [32]. Several studies have reported that IL1RA is one mediator contributing to the anti-inflammatory property of MSCs [33–35]. IL1RA produced by MSCs was reported to modulate the inflammatory response by decreasing TNFα produced in vitro by RAW-264.7 macrophages and IL1α gene expression in lung tissues in a model of bleomycin-induced lung injury [9]. However, no direct evidence that IL1RA could induce a M2 phenotype was shown. In this study, we report that IL1RA is not involved in the reduction in macrophage differentiation mediated by MSCs but in the reduction in their maturation as shown by a higher percentage of CD11b^{+}F4/80^{+}MHCII^{+}CD86^{+} macrophages secreting more TNFα and less IL10 in presence of *IL1RA*$^{-/-}$ MSCs versus wt MSCs. More importantly, the supernatant from macrophages cultured in presence of *IL1RA*$^{-/-}$

MSCs was not able to reduce T cell proliferation in a secondary proliferative assay indicating that IL1RA was associated with the polarization of macrophages to suppressive regulatory profile. MSCs have already been shown to skew macrophages toward the M2 lineage [14, 20, 36]. Soluble factors, in particular PGE2, IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) have been reported to mediate the MSC-mediated shift to M2 macrophages [37, 38]. The novelty of our results is that IL1RA secreted by MSCs is a key supplementary soluble factor mediating the polarization of M2 macrophages.

The role of IL1RA on adaptive immune cells is also not fully understood. In this study, we found out that IL1RA secreted by MSCs was not responsible for the inhibition of T cell proliferation in vitro. Although this result seems to be contradictory with previous data where recombinant IL1RA was shown to inhibit the concanavalin A-induced proliferation of T lymphocytes, this effect was observed when IL1RA was added at the high concentration of 10 μg/ml [39]. Under our conditions, the concentration of IL1RA produced by MSCs was far less, in the range of 100 pg/ml. In contrast, IL1RA participated to the prosurvival effect of MSCs on B lymphocytes. This effect was related to the secretion of IL1RA and not of IL6, which is a well-known inducer of B cell survival, since *IL6*$^{-/-}$ and wt MSCs exerted a comparable effect [40]. We also provided evidence that IL1RA contributes to the decreased proliferation of naïve B cells and differentiation into plasma cells. The effect of MSCs on proliferation was primarily observed when B cells were cultured in presence of IFN-γ indicating that activation of T cells is required to prime MSCs to exert their suppressive effect on B cells. These results are in accordance with a recent study using human adipose tissue-derived stem cells [29]. The most striking role of IL1RA produced by IFN-γ-activated MSCs is the inhibition of B cell differentiation. Actually in absence of IL1RA, the levels of total IgG, IL1β, and TNFα are comparable with those observed with plasma cells differentiated with IFN-γ. Importantly, we also observed the upregulation of IL10 in plasmablasts cultured with wt MSCs, which is totally abolished when *IL1RA*$^{-/-}$ MSCs were added. This suggests that Breg-like population could be induced by MSCs in vitro via the production of IL1RA. Similar findings were observed in the collagen-induced arthritis model confirming in vivo our results on the role of IL1RA produced by MSCs on the inhibition of B cell differentiation. However, although the number of Breg-like cells tended to increase in the spleen of mice treated with wt MSCs, it did not reach a significant value. Similarly, the phenotype of murine Breg cells is not clearly identified and we could have missed the corresponding cell subtype. The induction of CD5^{+} IL10-producing Breg cells has been recently reported after MSC administration in patients with refractory graft versus host disease [41]. It would be interesting to look for this population in murine models. Nevertheless, our data point to the notion that in vivo, MSCs can reduce the inflammatory reaction by modulating B cell response, while this is still under debate [42].

Summary

In summary, we present evidence that IL1RA is a novel mediator of MSC immunosuppressive effect, which could account for the polarization of macrophages toward the M2 phenotype, together with the already described factors PGE2, IL6, and GM-CSF. Using BM-derived murine MSCs, we here confirm in vitro and in vivo recent data on the role of human adipose tissue-derived MSCs on the inhibition of B cell differentiation and the induction of Breg-like cells [29]. The novelty of our study is the conclusion that this effect is at least partly mediated by IL1RA.

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Author Contributions

P.L.-C., F.D.: collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; F.D., K.T., C.B., M.F., and M.J.H.: collection and assembly of data, data analysis and interpretation, final approval of the manuscript; C.J.: conception and design of the study, data analysis and interpretation, final approval of the manuscript; D.N.: conception and design of the study, data analysis and interpretation, manuscript writing. P.L-.C., F.D., C.J., and D.N. contributed equally to this article.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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