Journal of Autoimmunity 65 (2015) 82–89

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Myeloid-derived suppressor cells contribute to bone erosion in collagen-induced arthritis by differentiating to osteoclasts

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ARTICLE INFO

Article history:
Received 31 March 2015
Received in revised form
14 August 2015
Accepted 18 August 2015
Available online 28 August 2015

Keywords:
MDSC
Osteoclast precursor
Osteoclast
Bone destruction
Collagen-induced arthritis and rheumatoid arthritis

ABSTRACT

Bone erosion is a sign of severe rheumatoid arthritis and osteoclasts play a major role in the bone resorption. Recently, myeloid-derived suppressor cells (MDSC) has been reported to be increased in collagen-induced arthritis (CIA). The number of circulating MDSCs is shown to correlate with rheumatoid arthritis. These findings suggest that MDSCs are precursor cells involved in bone erosion. In this study, MDSCs isolated from mice with CIA stimulated with M-CSF and RANKL in vitro expressed osteoclast markers and acquired osteoclast bone resorption function. MDSCs sorted from CIA mice were transferred into the tibia of normal DBA/1J mice and bones were subjected to histological and Micro CT analyses. The transferred CIA-MDSCs were shown to differentiate into $TRAP^+$ osteoclasts that were capable of bone resorption in vivo. MDSCs isolated from normal mice had more potent suppressor activity and much less capability to differentiate to osteoclast. Additional experiments showed that NF-κB inhibitor Bay 11−7082 or IκB inhibitor peptide blocked the differentiation of MDSCs to osteoclast and bone resorption. IL-1Ra also blocked this differentiation. In contrast, the addition of IL-1α further enhanced osteoclast differentiation and bone resorption. These results suggest that MDSCs are a source of osteoclast precursors and inflammatory cytokines such as IL-1, contributing significantly to erosive changes seen in rheumatoid arthritis and related disorders.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammatory synovitis and subsequent cartilage and bone destruction (erosive changes) leading to severe disabilities [1,2]. Bone erosions have been reported to be present in 45% of patients with early rheumatoid arthritis [3]. Bone erosion involves osteoclastic bone resorption [4]. Osteoclasts are multinucleated polykaryons commonly derived from monocyte/macrophage lineage. Their major function is bone absorption [5]. Monocytes/macrophages in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) differentiate into osteoclasts [6]. Over production of osteoclast or increased osteoclast activity often leads to cartilage and bone destruction [7]. In RA, a large number of osteoclasts accumulate in the synovial tissue, causing resorption pits and local bone destruction in the joint [8]. However, the origin of the precursors of these osteoclasts remains obscure [9].

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells with immunosuppressive functions. They accumulate

Abbreviations: MDSC, Myeloid-derived suppressor cell; RA, Rheumatoid arthritis; M-CSF, Macrophage Colony-stimulating Factor; RANKL, Receptor activator of NF-κB ligand; CIA, Collagen-induced arthritis; CtsK, Cathepsin-K; IL-1Ra, IL-1 receptor antagonist; TRAP, tartrate-resistant acid phosphatase; ECL, enhanced chemiluminescence; MRI, Magnetic resonance imaging.

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http://dx.doi.org/10.1016/j.jaut.2015.08.010
0896-8411/

in the spleen and bone marrow in a variety of pathologic conditions [10]. In mice, MDSCs are characterized by the expression of cell surface markers CD11b and Gr-1 and are further defined into $CD11b^{+}Ly6C^{high}Ly6G^{-}$ or $CD11b^{+}Ly6C^{low}Ly6G^{+}$ subset [11]. The number of MDSCs was reported to increase in autoimmune diseases [12–15]. Recent reports from us and other investigators [15,16] showed that MDSCs are increased in CIA and that the depletion of MDSC diminished joint swelling and bony destruction. In RA patients, there was a correlation between DAS 28 and the number of circulating MDSC. These observations suggest that MDSCs act as osteoclast precursors in both CIA and rheumatoid arthritis. In this study, MDSCs isolated from mice with CIA were shown to differentiate to osteoclasts with the ability to cause bone resorption both in vitro and in vivo.

2. Materials and methods

2.1. Mice

Eight week old male DBA/1J mice were purchased from SLAC Laboratory Animal Centre (Shanghai, China) and kept in specific pathogen-free conditions in Sun Yat-sen University Animal Center. Chow and water were supplied *ad libitum*. All animal procedures were approved by the ethical committee of the First Affiliated Hospital, Sun Yat-sen University and performed in accordance with the guidelines provided by the National Institute of Health Guide for Care and Use of Animals.

2.2. Collagen-induced arthritis

Collagen-induced arthritis (CIA) was carried out as previously described [17]. Bovine type II collagen (CII, Chondrex, USA) was emulsified with Freund's complete adjuvant (Chondrex, USA) at an equal volume. 100 μl emulsion containing 100 μg of CII was injected into mice intradermally at the base of the tail on day 0. The mice received a booster challenge of CII emulsified with Freund's incomplete adjuvant on day 21. Mice were monitored by two blinded examiners every two days for signs of arthritis onset and for arthritis scoring.

2.3. Single cell suspension preparation

Mice were sacrificed by cardiac puncture after they were anaesthetized with chloral hydrate. Long bones and spleens were collected. PBS (3 × 1 ml) was injected into the cavities of long bones to flush out the marrow content. Collected cell suspensions were filtered through a nylon filter. Spleens were teased and cell suspensions were collected after filtering through a nylon mesh. Red cells were lysed by red cell lysing buffer (Sigma, USA).

2.4. Immunosuppressive assay

Bone marrow cells isolated from CIA mice on day 35 after the first immunization were stained with FITC-anti-Gr-1 and PE-anti-CD11b antibodies. $CD11b^{+}Gr\text{-}1^{+}$ MDSCs from bone marrow cells of CIA or normal mice were isolated by flow cytometry (BD influx, USA). The purity of cells was confirmed >95% by flow analysis. Isolated splenocytes labeled with 5, 6-carboxyfluorescein diacetatesuccinimidyl ester (CFSE) (Invitrogen, USA) according to the manufacture's instruction. 5×10^5 splenocytes were co-cultured with sorted MDSCs in 96-well culture plates in the presence of 1 μg/ml of anti-CD3/CD28 antibodies (Biolegend, USA) at a ratio of 1:1. After 72 h of stimulation, cells were collected and stained with APC-anti-CD4 antibody (BD Pharmingen, USA). The proliferation of $CD4^{+}$ T cells was calculated according to the dilution of CFSE [18].

2.5. Osteoclast differentiation

$CD11^{+}bGr\text{-}1^{+}$ MDSCs from mice with CIA (35 days after the first immunization) or normal mice were sorted by flow cytometry (BD influx, USA). The purification was confirmed by flow cytometry (>95%). 2×10^5 MDSCs were seeded into 48-well culture plates with or without coverslips in α-MEM (Gibco, USA), 10% heat-inactivated FCS (Hyclone, USA), 50 ng/ml of M-CSF and 100 ng/ml of RANKL (Both from Peprotech, USA). This culture media is referred to as osteoclast differentiation media. In some experiments, 10 ng/ml of IL-1α (Peprotech, USA), 300 ng/ml of IL-1 receptor antagonist (IL-1Ra, Prospec, Israel), 2.5 μM of Bay 11–7082 (Sigma, USA) or 200 μM IκB kinase inhibitor peptide (Calbiochem, USA) were included. Culture media was replaced every two days.

Bone marrow derived macrophages that are classical osteoclast precursors were used as positive control. To prepare bone marrow derived macrophages, cells were collected from the long bones of normal DBA/1J mice. Cells were washed twice and let cells to adhere in the dishes. Non-adherent bone marrow cells were collected and cultured in α-MEM containing 10 ng/ml of M-CSF (Peprotech, USA). Cells cultured in M-CSF for 2 days were used as bone marrow derived macrophages. Thereafter, cells were cultured with medium with 50 ng/ml of M-CSF and 100 ng/ml of RANKL [19].

The differentiation of MDSCs to osteoclasts was detected by staining for tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (Sigma, USA) according to the manufacturer's instructions.

2.6. F-actin and bone resorption assays

MDSCs were sorted from CIA bone marrow cells on day 35 and 2×10^5 cells were cultured under osteoclast differentiation conditions for up to 12 days. Culture media was replaced every two days. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min, washed with PBS twice and stained with FITC-phalloidin (Sigma, USA) at 37 °C for 30 min to detect F-actin. For the bone resorption assay [20], bovine cortical bone slides were layered at the bottom of culture plates. 2×10^5 of sorted MDSCs were seeded onto the slides and cultured in the osteoclast differentiation media for up to 15 days. Resorption pits on the slides were shown by staining with toluidine blue.

2.7. qPCR

Sorted $CD11b^{+}Gr\text{-}1^{+}$ MDSC cells were cultured under osteoclast differentiation conditions for up to 12 days. Cells were collected from day 6 for the detection of MMP-9, Cathepsin-k (Ctsk) and TRAP by qPCR. The PCR primers were MMP-9 (forward 5′-CTTCTTCTCTGGACGTCAAATG-3′, reverse 5′-CATTTTGGAAACTCACACGCC-3′), Ctsk (forward 5′-GATGCTTACCCATATGTGGGC-3′, reverse 5′-CATATCCTTTGTTTCCCCAGC-3′), TRAP (forward 5′-GCCAAGATGGATTCATGGGTGG-3′, reverse 5′-CAGAGACATGATGAAGTCAGCG-3′) and GAPDH (forward 5′-ACATCATCCCTGCATCCACTG-3′, reverse 5′-TCATTGAGAGCAATGCCAGC-3′). RNA was reverse-transcribed into cDNA using a reverse transcript kit (Fermentas, USA) according to the manufacturer's instructions. cDNA was amplified by using recombinant Taq DNA polymerase (Fermentas, USA). SYBR green-based quantitative real-time PCR was performed using a Bio-Rad IQ5 (Bio-Rad, USA). The amplification was performed using the following conditions: preheating at 95 °C for 10 min and then 35 cycles of denaturing at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Results of comparative real-time PCR were analyzed using IQ5 Software (Bio-Rad, USA). GAPDH was used as a control.