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IFN-γ and IL-33 modulate mesenchymal stem cells function targeting Th1/Th17 axis in a murine skin transplantation model

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ABSTRACT

The immune regulatory properties of IL-33 have indicated that this cytokine has the capacity to target several immune cells under a variety of immunological responses, including overt inflammation and tolerance. Due to its versatile mechanistics, we sought to investigate the role of IL-33 on mesenchymal stem cells (MSC), a population of cells with recognizable modulatory functions. Our data indicates that IL-33 does not affect the expression of classical MSC markers such as CD29, CD44 and CD73, or the lack of CD45, CD11b and CD117. Also, we found that IL-33 greatly induces iNOS expression and stimulates the secretion of TGF-β and IL-6. Next, we decided to test IFN-γ/IL-33-treated MSC using a skin transplantation model. Our data indicate that allogeneic skin-grafted animals treated with IFN-γ/IL-33-modulated MSC reject as controls. Complementing this finding, we observed that *ex vivo* re-stimulated draining lymph nodes (dLN) cells from these mice secrete lower amounts of IFN-γ and a slightly higher amount of IL-17. Beside a reduction in CD4+ and CD8+ T cells number, we preliminarily found an increment in the frequencies of CD4+Foxp3+IL-17+ T cells. Altogether, our data propose that IL-33 and IFN-γ modulate MSC phenotype and function, most likely targeting Th1/Th17 axis.

1. Introduction

Mesenchymal stem cells (MSC) correspond to a cell population that could be isolated from various tissues such as bone marrow, umbilical cord or peripheral blood, and adipose tissue, among others [1–3]. In accordance with the International Society of Cellular Therapy (ISCT), there are minimal criteria that allow for identification of these cells, including their adherence to plastic when in cell culture, their self-renewal capacity and multipotency due to the ability to differentiate toward diverse lineages (osteoblast, chondrocyte and adipocyte) [4]. In terms of phenotype, there is no specific marker for MSC, thus a panel of molecules is considered, which includes the presence of CD29, CD44, CD73 and Sca-1, and the absence of CD45 and CD11b [5]. The first studies on MSC were focused in their regenerative potential, but now it is well accepted their function in the immune system. According to the literature, MSC are able to modulate innate and adaptive immunity, but to exert this effect they must be "conditioned" or "licensed" by inflammatory cytokines. For example, IFN-γ is one of the cytokines that itself is able to ease MSC's immune regulatory properties, but it may also act in synergy with TNF-α, IL-1α or IL-1β [6,7]. Among the immune regulatory capabilities described for MSC, several mechanisms have been studied, including the expression/release of soluble factors, such as cytokines (IL-6, TGF-β), growth and differentiation factors, chemokines, enzymes (inducible Nitric Oxide Synthase [iNOS], Arginase 1 [Arg1], Indoleamine 2,3-dioxygenase [IDO]) and metabolites, although a cell-to-cell mechanism has also been described [8–10]. Altogether, MSC drive the recruitment of distinct immune cell populations, and the inhibition in cell proliferation, activation and differentiation may result in immune tolerance, depending on the circumstances [2].

IL-33 was described in 2005 as a new member of the IL-1 family, corresponding to a ligand of the orphan receptor ST2. Physiologically, IL-33 is expressed abundantly and constitutively as a pro-protein in the nucleus of endothelial and epithelial cells [11], and it is released as a mature form in response to tissue injury and infections, activating cells from the innate and adaptive immune system. Due to this role, IL-33 was coined as *alarmin*. As other members of the IL-1 family, IL-33 was initially associated with a Th2-type response since it triggered the production of IL-4, IL-5 and IL-13 from other cells [12]. Furthermore, it has been described that IL-33 is able to act on immune cells such as mast cells, basophils and eosinophils [13,14], enhancing the secretion of Th2-type cytokines. Due to these actions, IL-33 was first associated with allergic responses, but it is currently established that IL-33 plays a role in Th1 and Th17-type profiles as well, as indicated in studies

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involving Experimental Autoimmune Encephalomyelitis (EAE), Collagen-induced Arthritis (CIA) and asthma models [15–19]. Even more, recent reports have demonstrated that the administration of IL-33 in transplanted animals drives immune tolerance by increasing the number and the *de novo* differentiation of Foxp3 + Tregs [20–22]. To date, there are no studies describing a potential direct role of IL-33 on MSC, or a putative impact on T cell-mediated immunity via IL-33-treated MSC. Thus, the purpose of this study was to evaluate whether IL-33 may affect MSC biology (including phenotype, cytokine secretion and expression of relevant modulatory genes) *in vitro* and *in vivo* using a skin transplantation model. Even though our results show that IFN-γ/IL-33 triggers changes in some aspects of MSC biology, such as cytokine production and expression of iNOS, no positive effect on transplant tolerance was achieved. Together with the impact on T cell numbers and cytokine production, this study suggests that IFN-γ/IL-33-treated MSC may target Th1/Th17 and Tregs skewing capabilities.

2. Materials and methods

2.1. Mice

Six- to eight week-old C57BL/6 and Balb/c mice were obtained and maintained in the facilities of Facultad de Medicina of Universidad de los Andes. Breeding of C57B/6 ($H2^b$) and Balb/c ($H2^d$) were set up to obtain F1 hybrids ($H2^{bxd}$). All experimental procedures were approved by the Ethics Committee of Facultad de Medicina of Universidad de los Andes.

2.2. Skin transplantation

Tail skin grafts from C57BL/6 (syngeneic group) or F1 mice (allogeneic group) were transplanted onto the dorsal area of 6–8 weeks old C57BL/6 mice. As mentioned in the figures, groups of mice were administered 10^6 MSC cells via intraperitoneal (i.p.) injection one day before surgery (day −1). At day 13, skin graft-draining lymph nodes (dLNs) and skin transplants were collected and analyzed for cell number and phenotype.

2.3. MSC treatment

Bone marrow-derived MSC which express the reporter protein RFP (Cyagen, CA, USA) were used between passages 9–16. Cells were cultured in α-MEM medium (Corning, MA, USA) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco, BRL, USA). For characterization cultures, 2×10^5 MSCs were seeded into 6-well plates, next day, MSCs were stimulated with 20 ng/mL of recombinant murine IFN-γ, 20 ng/ml of recombinant murine IL-33 (all from Peprotech, NJ, USA) or a combination of both cytokines at the same concentration for 24 h. Then, the supernatants were collected for cytokine analysis, and a fraction of these cells were used for a phenotype analysis by flow cytometry or for total RNA isolation, as described below.

2.4. Flow cytometry

Anti-mouse antibodies used for MSCs surface analysis were: α-CD45 (clone I3/2.3), α-CD11b (clone M1/70), α-CD117 (clone 2B8), α-CD29 (clone HA2/5), α-MHC-II (clone M5/114.15.2), α-CD44 (clone IM7), α-CD73 (clone TY/11.8) and Sca-1 (clone D7, from eBioscience, San Diego, CA, USA). For T cells analysis, α-CD4 (clone RM4-5), α-CD8 (clone 53-6.7), α-CD25 (clone PC61), all from Biolegend (San Diego, CA, USA) and α-Foxp3 (clone FJK-16S) from eBioscience (San Diego, CA, USA), all conjugated either to FITC, PE, PerCP, PE-Cy5.5, PE-Cy7, APC or Pacific Blue. Data acquisition was performed on a FACSCanto II™ (Becton Dickinson, Mountain View, CA, USA) and data analysed using FlowJo software (Treestar Inc., Ashland, OR, USA).

Table 1
List of primers used in this study for qRT-PCR assay.

Gene	Forward primer	Reverse primer
Arg-1	TTT TAG GGT TAC GGC CGG TG	CCT CGA GGC TGT CCT TTT GA
iNOS	CCT TGG TGA AGG GAC TGA GC	CAA CGT TCT CCG TTC TCT TGC
IL-17RA	AAA TAC CAC AGT TCC CAA GCC	TGG GCG AAC TTT AGG ACC AC
IL-33	AAC AGG CCT TCT TCG TCC TT	GGA CCA GGG CTT CGC CT
GAPDH	CCA GGT TGT CTC CTG CGA CTT	CCT GTT GCT GTA GCC GTA TTC A
ST2	GTG ATA GTC TTA AAA GTG TTC TGG	TCA AAA GTG TTT CAG GTC TAA GCA

2.5. qRT-PCR

RNA of differentially stimulated MSCs was extracted using E.Z.N.A total RNA kit (Omega Bio-tek, Norcross, GA). cDNA samples were prepared with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Expression of iNOS, ARG-1, IL-33 and ST2 were performed in Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA) using 5× HOT FIREPol® Evagreen® qPCR supermix (Solis BioDyne, Tartu, Estonia) as fluorescent detector. Primers used are shown in Table 1. For analysis, expression of the mentioned genes was normalized with respect to the expression of the housekeeping gene glyceraldehyde 3-phophate dehydrogenase (GAPDH).

2.6. Lymph nodes cells stimulation ex vivo

At day 13 post-surgery, dLNs cells were obtained and concentrated at 1 x 10^6/mL of culture media. 10^6 cells were plated and polyclonally activated using 5 μg/mL of soluble α-CD3 antibody (clone 2c11, Biolegend, USA). After 3 days in complete RPMI medium at 37 °C and 5% CO_2, supernatants were harvested for cytokine quantification.

2.7. ELISA

Supernatants from MSC differentially stimulated and from ex vivo stimulated dLN cells were collected and stored at −80 °C for cytokine quantification by ELISA (sandwich) test. Pure and Biotin-conjugated antibodies for the following cytokines were purchased from Biolegend (San Diego, CA, USA): IFN-γ, IL-6, IL-10, IL-17, IL-33 and TGF-β, and the recombinant murine cytokines for standard curves (Peprotech, NJ, USA).

2.8. Statistical analysis

Data were analyzed using an unpaired Student's *t*-test or a Mann-Whitney test (two-tailed). In all cases, $p < 0.05$ was considered with statistical significance. For data analysis, Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used.

3. Results

3.1. IL-33 does not affect the expression of MSC classical markers, maintains IFN-γ-dependent MHC-II expression, and greatly increases IL-6, TGF-β and iNOS expression

The alarmin IL-33 is a cytokine with several functions, including the modulation of the immune response, impacting T helper polarization [3], Treg differentiation and Myeloid-derived Suppressor Cells (MDSC) frequencies [23,24], among others. Based on these observations, we decided to evaluate the role of IL-33 on MSC, a cell population of stromal origin, with the capacity to facilitate regeneration and healing of damaged tissues [4]. In parallel, many reports have demonstrated that MSC may regulate immune responses, mainly by producing factors