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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

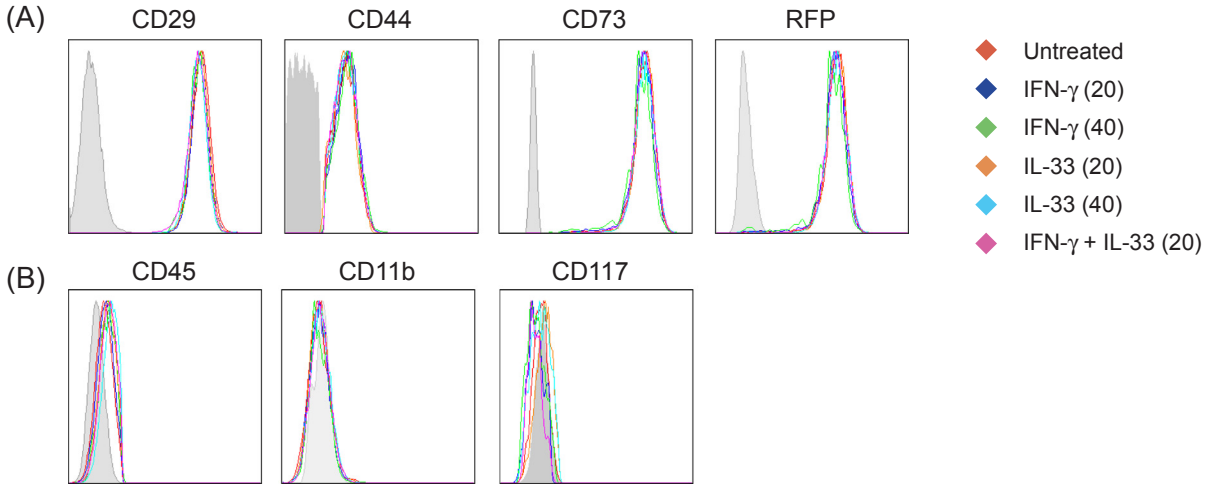

Fig. 1. IFN-γ/IL-33-treated MSC does not change their classical phenotypic identity. Bone marrow-derived MSCs were cultured in media supplemented with 20 ng/mL of IFN-γ (blue), with 20 ng/mL of IL-33 (green) or both cytokines at 20 ng/mL each (red) for 24 h. Representative histograms for the indicated classical surface markers analyzed by flow cytometry. n = 8 independent cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

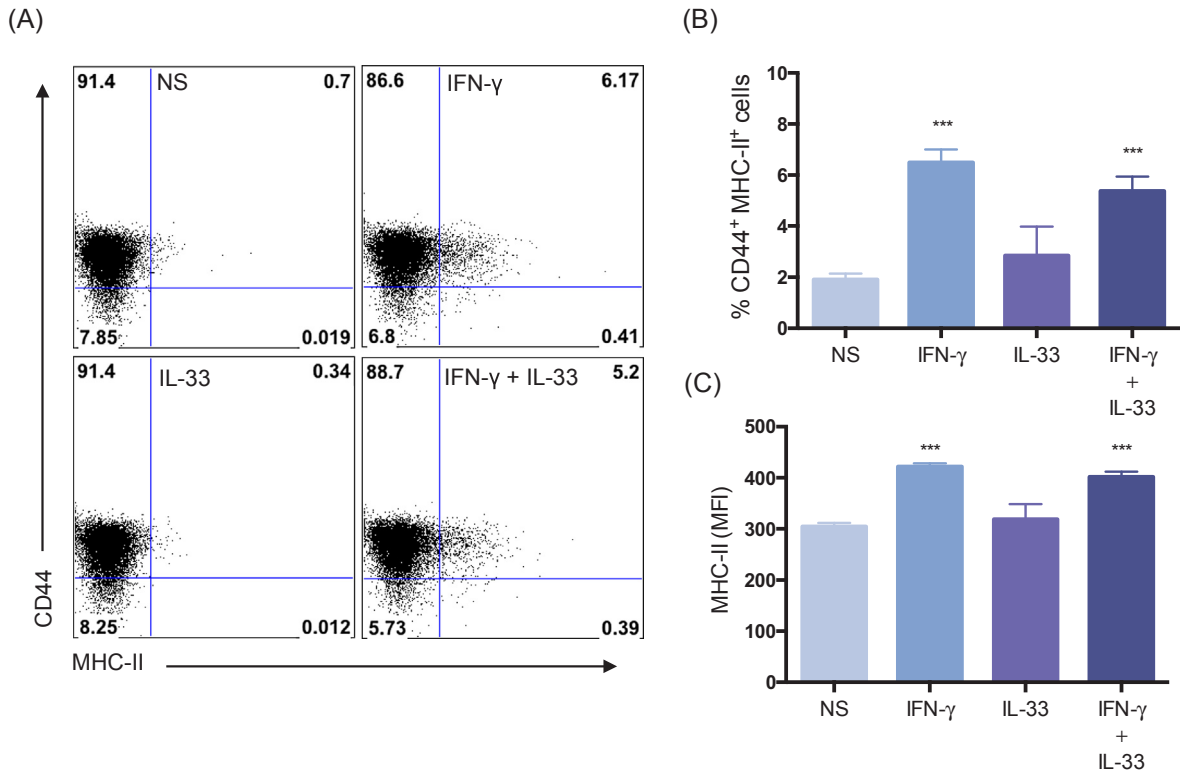

Fig. 2. IL-33 treatment maintains IFN-γ-dependent MHC-II expression on MSC. Bone marrow-derived MSCs were cultured in media supplemented with the indicated cytokines at 20 ng/mL for 24 h. Flow cytometry was used to study the expression of MHC-II. (A) Representative dot plots of MSC depicting CD44 and MHC-II expression under the different conditions tested. (B- and C) Bar graphs showing the frequency (B) of CD44 + MHC-II + MSC or the MFI (C) for MHC-II on CD44 + MSC. Pooled data from 8 independent experiments. Error bars represent SEM and the statistic significance was calculated using Mann-Whitney test, considering *$p < 0.05$ and ***$p < 0.001$. Statistical values were obtained by comparing with non-stimulated condition (NS).

such as NO, IL-6, and TGF-β, among others [8,9], which lead to the establishment of tolerance. Thus, considering the above, we tested the role of IL-33 on MSC. For the study, we used a commercially available bone marrow-derived MSC line that expresses the reporter protein RFP[1] (Cyagen). MSC at 70% confluence were stimulated with IFN-γ, IL-33 or both at 20 ng/mL, for 24 h. After this time, cells and supernatants were recovered for analysis. For all cytometric studies, the first gate strategy used for the analysis was on CD45-RFP + cells. As shown in Fig. 1A- and B, the expression of CD29, CD44, CD73, CD45, CD11b and CD117 molecules (markers used to define MSC phenotype) in this MSC gate was not modified upon cytokine stimulation, neither was RFP. Since IFN-γ induces the expression of MHC-II on MSC, suggesting a potential role as antigen presenting cell (APC), we also evaluated MHC-II expression, observing that MSC treated with IFN-γ and IL-33 maintain

[1] http://www.cyagen.com/us/en/product/c57bl6-mesenchymal-stem-cells-rfp.html.

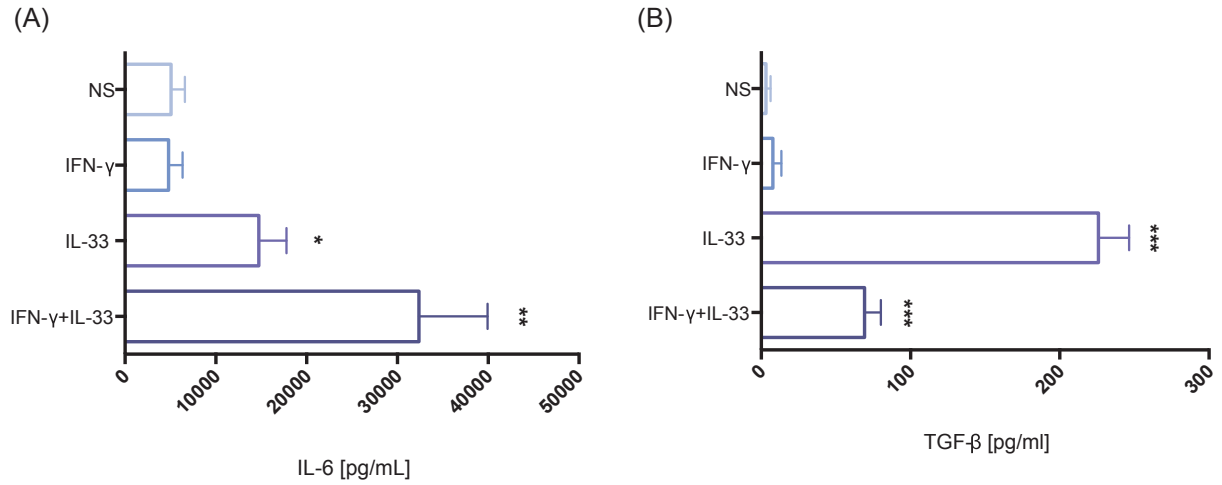

Fig. 3. IL-33 treatment triggers IL-6 and TGF-β production on MSC. Bone marrow-derived MSCs were cultured in media supplemented with the indicated cytokines at 20 ng/mL for 24 h. After this time, supernatants were harvested to assay cytokine production by ELISA test. Bar graphs displaying IL-6 (A) and TGF-β (B) secretion by MSC under the indicated conditions. Pooled data from 8 independent experiments. Error bars represent SEM and the statistic significance was calculated using Mann-Whitney test, considering *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Statistical values were obtained by comparing with non-stimulated condition (NS).

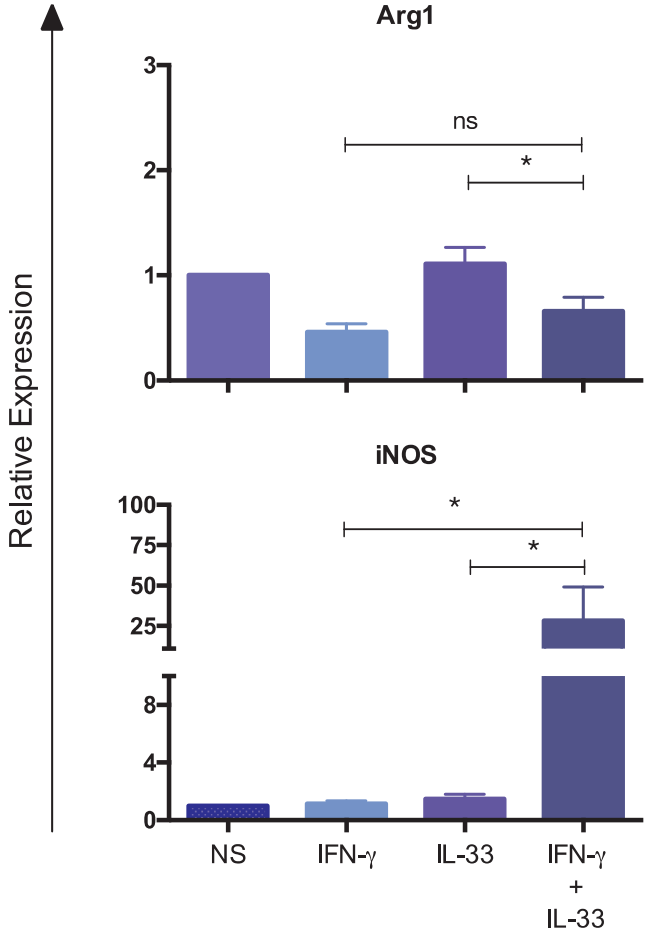

Fig. 4. IL-33 and IFN-γ treatment on MSC synergizes for up-regulating iNOS gene expression. Bone marrow-derived MSCs were cultured in media supplemented with the indicated cytokines at 20 ng/mL for 24 h. After this time, cells were harvested and RNA isolated. cDNA was further prepared and the expression of ARG1 and iNOS was evaluated by qRT-PCR. Relative expression was obtained by the delta-delta ct method of the mentioned genes using GAPDH gene as housekeeping control. Error bars represent SEM and the statistic significance was calculated using Mann-Whitney test, considering *$p < 0.05$ and "ns" as non-significant".

both the frequency of MSC MHC-II + (~7%), Fig. 2A- and B, and the MFI for MHC-II (~400) as in IFN-γ alone (~6% and 400, respectively), Fig. 2C. The treatment with IL-33 alone does not induce MHC-II expression. Since no drastic changes were promoted by the insertion of IL-33 in MSC treatment, we next analyzed the secretion of key cytokines all relevant in the polarization of the immune response. First, we assayed the production of IL-10 since this cytokine has been co-related with a regulatory function by MSC [25,26], but none of the treatments lead to IL-10 secretion. Similarly, we did not detect IL-17 or IL-33 in any of the samples (data not shown). Another important cytokine that has been described as a regulatory factor derived from MSC, with relevant effects *in vitro* and *in vivo*, corresponds to IL-6. This molecule is a cytokine with important properties, such as the ability of inhibiting monocyte differentiation and their ability to stimulate lymphocytes, in addition to participate in apoptosis of lymphocytes and neutrophils [5]. Thus, we quantified IL-6 in the different samples, detecting an important increment in its concentration when MSCs are stimulated with IL-33 alone (~20,000 pg/mL), in comparison with ~5000 for non-stimulated or IFN-γ alone conditions. Interestingly, the treatment with both IFN-γ and IL-33 augmented in 1.5 fold the concentration of IL-6 obtained with IL-33 alone (~30,000 pg/mL, Fig. 3A).

In addition, we also observed a dramatic up-regulation in the production of TGF-β when MSCs are treated with IL-33 only (~250 pg/mL, in comparison with ~20 pg/mL for non-stimulated and IFN-γ alone conditions), which was inhibited when MSCs were cultured in the presence of IL-33 and IFN-γ (~80 pg/mL), Fig. 3B. These data suggest that IL-33 affects MSC biology at least by modulating IL-6 and TGF-β secretion, key cytokines in the differentiation of IL-17-producing cells, such as Th17 and IL-17 + Foxp3 + Tregs [27–31]. To further complement whether IL-33 may target MSC modulatory activity, we next studied mRNA levels of pivotal genes widely described as factors responsible for MSC modulatory function, such as Arg1 and iNOS. Arg1 and iNOS encode for enzymes involved in arginine metabolism, and their activity control T cell proliferation/activation [6]. Based on the current study, our data indicate a slight decrease in the levels of Arg1 mRNA when IFN-γ is present in the culture, either alone or together with IL-33 (~50% reduction), but these changes were rather modest if we consider the drastic increment in iNOS mRNA levels when IFN-γ and IL-33 are used together in the treatment of MSC (~30, in comparison with ≤2 for non-stimulated, IFN-γ or IL-33 alone), Fig. 4, left column. Altogether our results suggest that IL-33 targets some of the classical molecules that render regulatory functions to MSC, such as IL-6, TGF-β and iNOS.

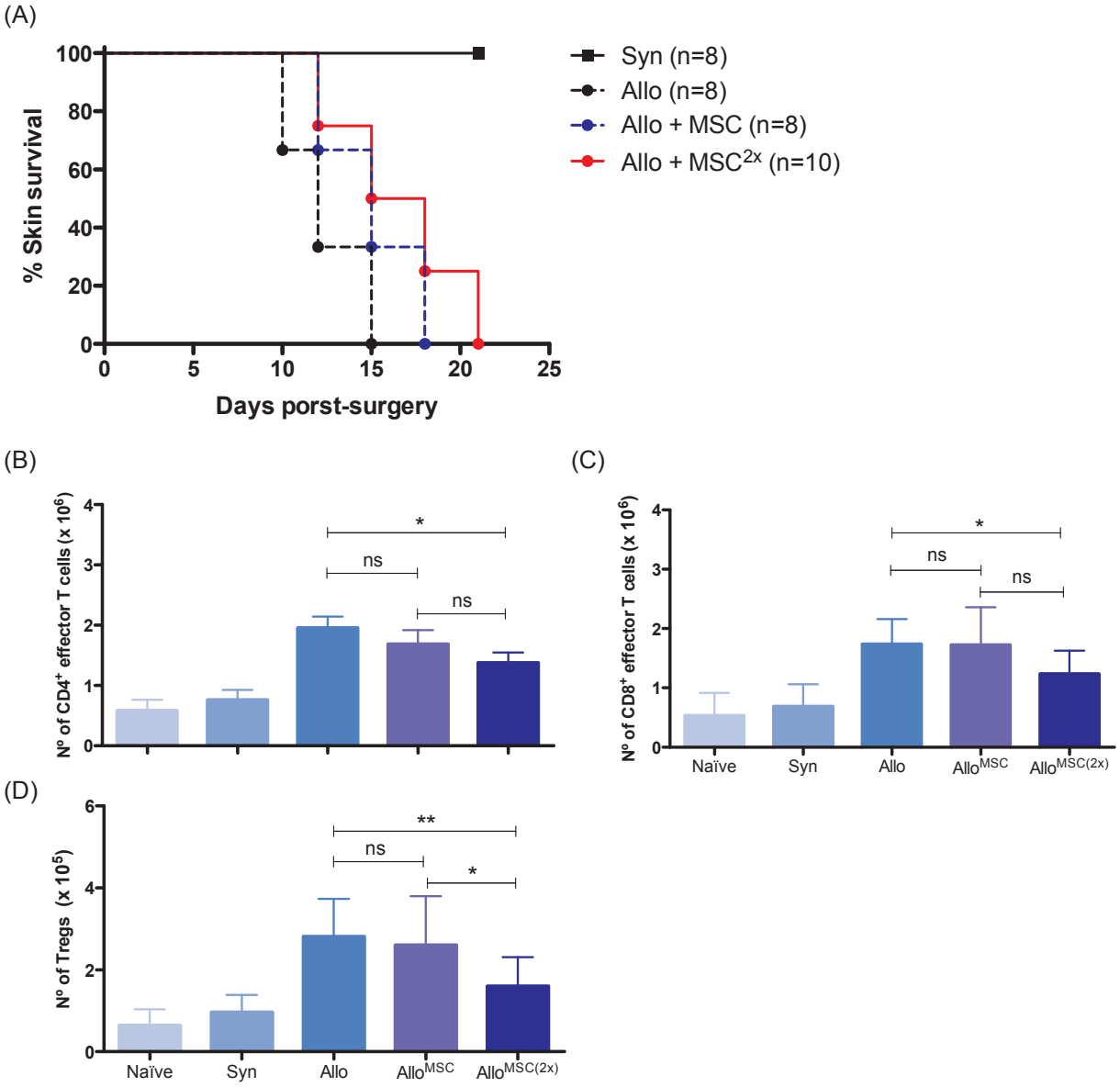

Fig. 5. Administration of IFN-γ/IL-33-treated MSC does not prevent allograft rejection but diminishes T cell number. Bone marrow-derived MSCs were cultured in media supplemented with IFN-γ and IL-33 (both at 20 ng/mL) for 24 h or alone. At day −1, mice received 10^6 of either non-treated MSC (MSC) or IFN-γ/IL-33-treated MSC (MSC^{2X}) cells via i.p. Next day (day 0), mice were transplanted with syngeneic (C57BL/6) or allogeneic (F1) skin grafts. Skin graft survival was monitored three times per week. After 14 days, dLN were removed, and cell counts and phenotype were obtained. (A) Survival graph, (B) Graph depicting the number of CD4 + T effector cells, (C) CD8 + T effector cells and (D) Foxp3 + regulatory T cells. Pooled data from 4 independent experiments, with n = 3–5 mice per group. Error bars represent SEM and the statistic significance was calculated using Mann-Whitney test, considering *p < 0.05, **p < 0.01 and "ns" as non significant. Statistical values were obtained by comparing with allogeneic group ("Allo").

3.2. IFN-γ/IL-33-treated MSC affect Th1/Th17 axis in vivo

Based on the data supporting that MSC treated with IFN-γ in conjunction with IL-33 may be inducing a potential modulatory phenotype (high production of IL-6 and TGF-β, in addition to enhanced iNOS gene expression, Figs. 2–4), we sought to test the administration of cytokine-modulated MSC in a skin transplantation model *in vivo*. In these experiments, we utilized a skin transplantation model established in our laboratory in which C57BL/6 recipient mice receive either syngeneic skin (from C57BL/6 donor) or allogeneic skin (from C57BL/6 × Balb/c, or F1, donor) at day 0. Between days 9–10 the first signs of rejection are visible (characterized by necrotic grafts), which lead to full rejection in the following days. Thus, in our study we consider only two experimental groups, beside the control ones: non-treated MSC (or MSC) and IFN-γ/IL-33-treated MSC (or MSC^{2X}) as indicated in (Figs. 5 and 6), since the modulation of MSC phenotype by IFN-γ alone has been tested before [7] and IL-33 (alone)-treated MSC showed mild effects in cytokine and gene expression analysis. Thus, mice received a single injection of 10^6 MSC or MSC^{2X} via i.p at day −1, and the following day (day 0) recipients were transplanted with syngeneic or allogeneic skin grafts. Skin transplants were monitored three times per week, during three weeks. As expected, syngeneic grafts were fully accepted, allografts were rejected by ~day 14 post-surgery, and both MSC- and MSC^{2X}-treated groups did not prevent transplant rejection, as shown in Fig. 5A. To understand at the cellular level the behavior of T cell-mediated immunity under these conditions, we studied their number and phenotype after 14 days post-transplantation. As shown in Fig. 5B-–D, only the treatment with MSC^{2X} seems to affect T cell populations in dLN, resulting in a decrease of CD4 + T effector and Treg cells (~1.5×10^6 and ~1×10^5 cells, versus ~2×10^6 and ~3×10^5 Tregs in untreated

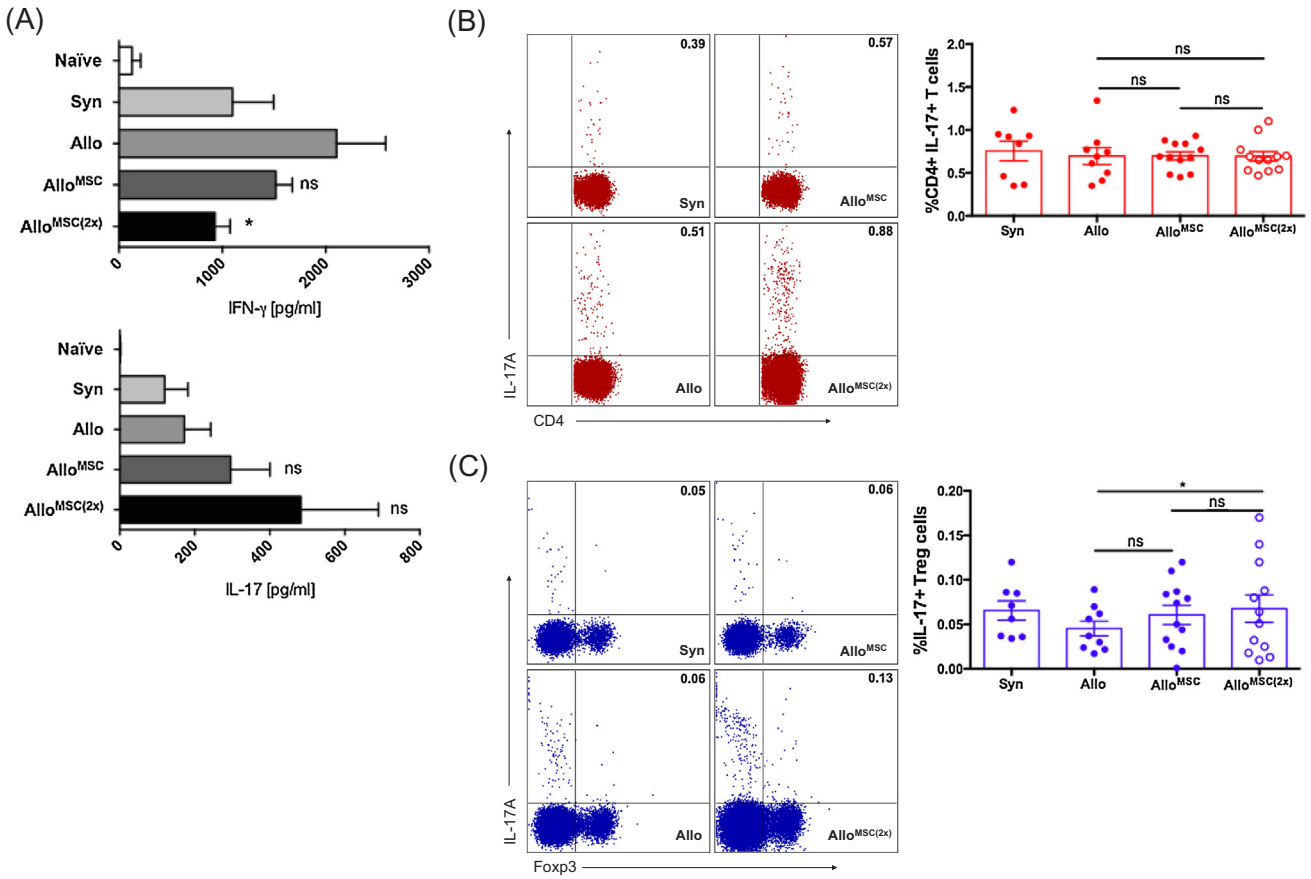

Fig. 6. IFN-γ/IL-33-treated MSC target Th1/Th17 axis in skin-transplanted mice. At day −1, mice received 10^6 of either non-treated MSC (MSC) or IFN-γ/IL-33-treated MSC cells (MSC^{2X}) via i.p. Next day (day 0), mice were transplanted with syngeneic (C57BL/6) or allogeneic (F1) skin grafts. After 14 days, dLN were removed, and cell counts were obtained. 10^6 cells were polyclonally activated with anti-CD3 (5 μg/mL) for 72 h, time after which supernatants were harvested for ELISA assays or further re-stimulated with PMA/Iono for 6 h to perform intracellular cytokine staining. (A) Bar graphs displaying IFN-γ (top) or IL-17 (bottom) production from supernatants after anti-CD3 stimulation. Representative bar plots from 3 independent experiments with n = 3–5 mice per group. Representative dot plots (left) and bar graph (right) depicting the frequencies of CD4 + IL-17A + T cells (B) or CD4 + Foxp3 + IL-17A + T cells (C) at the indicated conditions. Bar graphs showing pooled data from 2 independent experiments including n = 4–11 mice per group. Error bars represent SEM and the statistic significance was calculated using Mann-Whitney test, considering *p < 0.05 and "ns" as non significant. Statistical values were obtained by comparing with allogeneic group ("Allo").

allogeneic group; Fig. 5B and D, respectively), and CD8 + T effector cells (~1 × 10^6 cells and ~2 × 10^6 cells in untreated allogeneic group; Fig. 5C). Lastly, we quantified cytokine production from dLN cells activated polyclonally *in vitro*. In these experiments, supernatants were harvested after 72 h in culture, and IFN-γ, IL-17 and IL-10 production was tested. As shown in Fig. 6A, IFN-γ production was inhibited after MSC^{2X} treatment, but the production of IL-17 (Fig. 6B) shows a slight increment with either MSC used (in comparison with untreated allogeneic group). No changes in IL-10 were detected (Supplementary Fig. 1). The data above suggests that MSC^{2X} may modulate the immune response *in vivo* by interfering with Th1/Th17 axis, since IL-17-producing CD4 + T cells (including Foxp3 + cells) seem to enrich in dLN of animals treated with MSC^{2X}, Fig. 6C- and D, although additional work is needed to complement this observation.

4. Discussion

During these last years MSCs have become an interesting cell population due to their therapeutic benefits. To date, several clinical trials using MSCs have shown promising results in the fields of regenerative medicine, and also in immunological pathologies. All of these positive applications are based on the regulatory properties that MSCs exert physiologically. For example, cytokine-licensed MSC display modulatory functions such as T cell inhibitory outcomes, production of anti-inflammatory cytokines, tissue regeneration and wound healing. Among the most relevant molecules involved in MSC regulatory mechanisms, iNOS, Arg1, IL-6, IL-17 and TGF-β, in addition to surface molecules such as PD-L1, act as potent players in MSC mechanisms of action. On the other hand, the alarmin IL-33 has been extensively studied, and the reports indicate that IL-33 may be involved with inflammatory and anti-inflammatory functions. Since our interest is to understand the mechanisms behind immune tolerance, we decided to investigate the potential link between IL-33 and MSCs. Our results indicate that the treatment of MSC with IL-33 does not affect the expression of classical surface markers, including IFN-γ-induced MHC-II (Figs. 1 and 2). On the contrary, secretion of relevant cytokines such as IL-6 and TGF-β is greatly enhanced by those MSC previously treated with IL-33, suggesting a possible involvement in T helper differentiation, including modulation of Foxp3 + Treg subset plasticity, as stated later here and elsewhere [27,30,31]. As mentioned above, enzymes like iNOS in mouse, and IDO in human, are known for their role in MSC biology [1,3,7]. Our data shows that IL-33 has a great impact in iNOS expression when added to MSC in conjunction with IFN-γ, demonstrating a synergistic effect of these two cytokines (Fig. 4), but nevertheless the quantity of nitric oxide (NO) generated by these differentially treated-MSC did not differ among conditions (Supplementary Fig. 2), which could be explained by mechanisms regulating the translation of iNOS mRNA, iNOS activity or NO usage, which may be differentially consumed based on the nature of the response. Additionally, IFN-γ and IL-33 cooperative activity was not observed in the

case of Arg1 expression, where IL-33 could not revert the down-regulatory effect of IFN-γ, (Fig. 4).

In the *in vivo* setting, allogeneic skin grafted animals treated with MSC^{2X} showed no benefit in organ rejection, but a decrease in T cell numbers, including effector CD4 + and CD8 + T cells plus Foxp3 + Tregs in dLN, Fig. 5. The above is complemented with cytokine production data (Fig. 6A), where the amount of T cell-dependent IFN-γ secretion is reduced in MSC^{2X}-treated animals, and IL-17 production is slightly incremented. Supporting the latter, we also observed that dLN from MSC^{2X}-treated animals contains an increased frequency of IL-17 + CD4 + Foxp3 + T cells, Fig. 6B- and C. Overall, these data permits to hypothesize that at the time of analysis (14 days after surgery), CD4 + T cells may undergo into cellular plasticity (pointing overall to Th17 polarization), since IFN-γ secretion is decreasing and IL-17 secretion remains unchanged. In accordance with this, the CD4 + T cell compartment (regulatory) could be loosing their cell stability resulting in skewing the immune response toward Th17-driven, hence no transplant tolerance was achieved. This argument is supported by other studies in which T cells (naïve or Tregs) exposed to IL-6 and TGF-β (cytokines produced by stimulated-MSCs), change their phenotype toward Th17 [27–31], and that Tregs could produce IL-17 before Foxp3 down-regulation [31], giving rise to a transient IL-17 + Foxp3 + T subset. These cells retain the immunosuppressive properties of Tregs, mainly producing Granzyme B and perforin [17], but, at the same time, they can exert an effector response secreting IL-17 and other effector cytokines, such IL-2 and IFN-γ [32]. Furthermore, since tolerance was not established with the strategy described here, we believe that different doses of MSC and/or alternative administration route and timing may be considered for future experiments. Altogether, the administration of IFN-γ/IL-33-treated MSC modulates the immune response by favoring Th1/Th17 axis, instead of facilitating immune tolerance. This effect is highly relevant in models of solid tumor, for which our group is currently testing the potential applicability in additional experimental animal models.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.09.013.

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