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Multipotent mesenchymal stromal cells express FoxP3: a marker for the immunosuppressive capacity?

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Abstract

Multipotent mesenchymal stromal cells (MSCs) have immunosuppressive capacity but the exact mechanism by which they suppress proliferation of T lymphocytes is not fully understood. Recently, the characteristics and function of regulatory T lymphocytes (Tregs) have become better defined. Tregs and MSCs have immunosuppressive features in common. Here, we looked for a common basis for immunosuppression in these distinct cell types. FoxP3 and CD39 expression in MSCs was measured by flow cytometry and RT-qPCR. The importance of FoxP3 in MSC-mediated immunosuppression was investigated by siRNA technology and mixed lymphocyte culture (MLC). The effect of 5-azacytidine and other immunosuppressive drugs on FoxP3 expression and immunosuppression by MSCs was explored by flow cytometry, MLC, and RT-qPCR. MSCs express FoxP3 at variable levels, but they do not express CD39. FoxP3^{high} MSCs suppress MLC to a greater extent than cells with lower FoxP3 expression. However, FoxP3-decreased MSCs were found to retain their immunosuppressive properties. 5-azacytitine had no effect on FoxP3 expression or MLC suppression by MSCs. However, immunosuppressive drugs led to increased FoxP3 levels and MLC inhibition in FoxP3^{low} MSCs. This is the first demonstration of FoxP3 expression by MSCs. Although MSCs share several features with Tregs, and FoxP3^{high} MSCs tend to be more immunosuppressive, MSCs do not require functional FoxP3

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for their immunosuppressive activity. The increased MSC-mediated suppression of immune responses by immunosuppressive drugs deserves further investigation.

Keywords

transplantation; forkhead box 3; mesenchymal stem cells

Introduction

Naturally occurring regulatory T lymphocytes (Tregs) with the $CD4^+CD25^+FoxP3^+$ phenotype have become a major focus of immunological studies. Tregs have pleiotropic suppressive effects on immune responses to alloantigens, tumor antigens, and infectious agents[1]. The suppression of both $CD4^+$ and $CD8^+$ T lymphocytes *in vitro* is mediated by a cell contact-dependent/cytokine-independent mechanism, although suppression in *in vivo* models may require Tregs to produce the interleukin (IL)-10[2–3]. In the control of certain autoimmune diseases, primarily inflammatory bowel disease, studies have identified production of tumor growth factor (TGF)-β by Tregs as a key factor[4–5]. Similarly to Tregs, cell contact, IL-10, and TGF-β all mediate immunosuppression by multipotent mesenchymal stromal cells (MSCs)[6–9]. Further studies have identified several other soluble molecules including indoleamin-2,3-dioxygenase (IDO) and prostaglandin E_2, as being important for MSC-mediated immunosuppression. However, blocking of any single molecule does not restore immunosuppression[9]. MSCs are regarded as hypoimmunogenic and have been successfully transplanted over HLA barriers without rejection[10–12]. Thus, MSCs may serve as a “universal donor” and, in combination with simple expansion procedures, may have a future in cellular therapy. MSCs have been explored *in vivo* as treatment for ischemic acute renal failure[13], toxic lung damage[14], and autoimmune encephalomyelitis[15] with encouraging results. In clinical transplantation, MSC infusions improved the outcome of severe graft-versus-host-disease (GVHD), which is a frequent and threatening complication of hematopoietic stem cell transplantation[10,16–18]. As stated above, Tregs express forkhead box P3 (FoxP3), which is a transcription factor that is an essential and a sufficient regulator of Treg development and function[19–21]. FoxP3 associates with histone acetyltransferase (HAT) and class II histone deacetylases (HDAC), among other transcription regulatory proteins, to form a functional complex inducing transcriptional repression[22–23]. Tregs may also share some wound healing features of MSCs; a subset of Tregs express CD39. This molecule degrades extracellular ATP released during tissue injury, thereby reducing inflammation[24]. Since FoxP3 expression is a critical feature of functional Tregs, and CD39 expression may confer wound-healing properties, we investigated whether FoxP3 and CD39 are also involved in MSC function.

Material and methods

Isolation and ex vivo culture of cells

Bone marrow aspirates of approximately 50 mL were taken from the iliac crest of healthy donors with a median age of 26 (range 1–32) years screened by history, physical examination, and serology for HIV and hepatitis viruses. Expansion of clinical-grade MSCs

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was performed according to the guidelines of the MSC consortium of the European Blood and Marrow Transplantation Group, as previously described in detail[25]. Characterized by flow cytometry, the MSCs uniformly fulfilled minimal criteria[26]. Peripheral blood lymphocytes (PBLs) were isolated from peripheral blood of healthy donors, as described elsewhere[27]. Donors of both MSCs and PBLs gave informed consent and the study was approved by the Regional Ethics Review Board.

Flow cytometry to investigate FoxP3 and CD39 expression

PBLs (n = 5) were co-cultured for 24, 48, and 72 h with adherent MSCs (n = 5) at a ratio of 10:1, in RPMI supplemented with 10% human AB serum. PBLs without MSCs in RPMI and a mixed lymphocyte culture (MLC) of PBLs stimulated with PBLs pooled from donors were used as negative and positive controls, respectively. MSCs without PBLs in MSC culture medium were used to control for possible effects of RPMI on MSCs. In addition, 25 μM carboxyfluorescien diacetate succinimidyl ester (CFSE) was used to label MSCs in another set of cultures, otherwise prepared as above and then cultured for 7 days. CFSE dilution as a measure of MSC proliferation was calculated as follows: mean flouresence intensity (MFI) after culture divided by MFI before culture (i.e. positive control value).

After co-culture, PBLs were removed by washing the MSCs with PBS. MSCs were collected by detaching them using trypsin. The MSCs were then stained with antibodies to CD3-PerCP (BD Biosciences, San José, CA)/CD105-FITC (Ancell, Bayport, MN) or CD39-FITC (Abcam, Cambridge, UK). In the next step, cells stained for CD3 and CD105 were fixed in 4% formaldehyde, washed in 0.5% saponin, and stained with an antibody to FoxP3-PE (eBioscience, San Diego, CA). Finally, washed cells were assayed in a flow cytometer (FACSort; BD Biosciences). Fluorescence signals from 5×10^4 cells were counted and analyzed.

Lymphocyte proliferation assays regarding FoxP3 expression

After flow cytometric analysis, two MSCs with differences in FoxP3 phenotype, $FoxP3^{low}$ and $FoxP3^{high}$, were selected for further investigation using lymphocyte proliferation assays as previously described[27–28]. PBLs (n = 5) were challenged with a pool of allogeneic PBLs in the presence of 10% MSCs, supernatant from MSC culture, or supernatant from MLCs with 10% MSCs present. Proliferation data, in CPM, were calculated as the mean of triplicate determinations and autologous counts (background control) were subtracted.

Silencing of FoxP3 expression by siRNA

For siRNA knockdown of FoxP3 expression, MSCs (n = 5) were resuspended in hypo-osmolar buffer at a concentration of 1×10^6 cells per ml. Seventy-five μl of cell suspension was mixed with 2.25 μg FoxP3 siRNA (1 ID#s27191, 2 s27192) or control siRNA (Applied Biosystems, Austin, TX) in sterile 1-mm cuvettes. Cells were electroporated using 2 pulses of 920 V at a pulse length of 100 μs using the Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA). Following electroporation, the cells were allowed to recover for 10 min at 37°C prior to seeding in complete medium.

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For protein analysis, the cells were lysed directly in Laemmli lysis buffer containing 2-β mercaptoethanol, and heated to 95°C for 10 min. Samples were fractionated on 4–12% gradient SDS gels (Invitrogen, Carlsbad, CA) and transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK). For immunoblotting, membranes were incubated overnight with antibodies raised against FoxP3 (ab10901 and ab22510; Abcam plc, Cambridge, UK) and or β-actin (AC15; Sigma Aldrich, St. Louis, MO), followed by incubation with relevant HRP-conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL). For detection, the membranes were incubated with ECL (GE Healthcare) or Pierce Super-Signal (Thermo Fisher Scientific) detection reagents and exposed to Hyperfilm-ECL (GE Healthcare). The silencing was also confirmed by real-time quantitative PCR (RT-qPCR) for FoxP3 RNA. Primer and probe sequences, RNA preparation, reverse transcription, and RT-qPCR (with the ABI 7000 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) were used as previously described[29].

Effect of demethylating agents and immunosuppressants on FoxP3 expression and immuno-suppression

MSC cultures (n = 5) were exposed to the demethylating agent 5-azacytidine (at 0.01, 0.1, 0.5, and 1 μM; Sigma, St. Louis, MO) for 72 h. Thereafter, the FoxP3 expression of MSCs was investigated by flow cytometry for FoxP3 and their immunosuppressive capacity was investigated by lymphocyte proliferation assay, as described above.

In another set of experiments, MSC cultures (n = 9) were exposed to the immunosuppressants tacrolimus (25 ng/mL; Astellas Ireland Co. Ltd., Killorglin, Ireland), sirolimus (15 ng/mL; Wyeth Medica Ireland, Little Connell, Ireland), and methylprednisolone (10 μg/ml; Pfizer Inc., New York, NY) in clinically relevant doses for 4 h[30–32]. Thereafter, MSC FoxP3 expression and immunosuppressive capacity were investigated by flow cytometry (at 4 h) and RT-qPCR for FoxP3 (at 4 and 48 h) and lymphocyte proliferation assays (at 4 h), respectively, as described above.

Statistical analysis

The results were analyzed with the Mann-Whitney U-test. Statistical significance was considered at the 5% level.

Results

MSCs express FoxP3

By intracellular flow cytometry, it was determined that MSCs (n = 5) express FoxP3, but not CD39. Lymphocyte contamination was ruled out by staining for CD3 (Fig. 1a and b). FoxP3 expression in MSCs was variable over time in culture and also upon co-culture with lymphocytes for 24, 48, and 72 h. MSC 64 was identified as $FoxP3^{low}$ and MSC 81 as $FoxP3^{high}$ (Fig. 1c).

FoxP3 expression and suppression of lymphocyte proliferation

Regarding MSC proliferation, there were no significant differences between $FoxP3^{low}$ and $FoxP3^{high}$ cells (CFSE dilution 0.03 ± 0.003 vs. 0.02 ± 0.001, n = 5). After co-culture with

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lymphocytes, the MSCs showed stable FoxP3 levels and proliferation compared to MSCs unexposed to lymphocytes (mean Fox P3 positivity 10.3 ± 7.6% vs. 11.9 ± 6.8%, and CFSE dilution 0.025 ± 0.005 vs. 0.022 ± 0.009, n = 5).

The immunosuppressive effect of MSCs was investigated by inhibition of allogeneic MLCs. The inhibitory capacity ranged from 0% to 99%. MSC 64 and MSC 81 showed 11% and 99% inhibition, respectively (Fig. 2a). Upon repeated testing with MSC 64 and MSC 81 and their respective culture and MLC supernatants, MSC 81 ($FoxP3^{high}$) was found to be superior to MSC 64 ($FoxP3^{low}$) in inhibiting the MLC (Fig. 2a). The superiority of $FoxP3^{high}$ MSCs to $FoxP3^{low}$ MSCs in suppressing MLCs were confirmed in another set of experiments below (82–100% inhibition, n = 5 versus 0–67% inhibition, n = 4; $p < 0.05$) (Fig. 3ab, **middle panel**).

To determine whether FoxP3 expression was essential for immunosuppressive activity of MSCs, FoxP3-2-siRNA (as FoxP3-1-siRNA had only a moderate effect) was used to knock down FoxP3 expression. MSC 81 silenced for FoxP3 showed a 50–70% reduction in FoxP3 expression as shown by western blot and RT-qPCR (Fig. 2b), but the inhibition of MLC was not affected (normalized inhibition of MLC 0.95 ± 0.05 vs. 0.93 ± 0.04, n = 3). Comparable results were also obtained using other MSCs FoxP3-silenced at the level 50–70% (normalized inhibition of MLC 0.61 ± 0.11 vs. 0.64 ± 0.08, n = 3). In addition, reduced cell survival was observed after FoxP3 silencing with siRNA (data not shown).

Immunosuppressive agents modulate FoxP3 expression by MSCs and augment immunosuppression by weakly suppressing MSCs

MSCs (n = 5 out of 9) with more than 5% $FoxP3^{+}$ cells by flow cytometry and strong suppression of MLC showed stable FoxP3 levels and suppression of alloactivated lymphocytes after treatment with calcineurin inhibitors and the corticosteroid (p = no significant changes). RT-qPCR showed stable FoxP3 RNA expression 4 h after exposure, decreasing for untreated cells at 48 h with a trend suggesting that the immunosuppressive drugs could maintain higher FoxP3 expression (p = no significant changes) (Fig. 3a).

MSCs (n = 4 out of 9) showing less than 5% $FoxP3^{+}$ cells and poor inhibition showed a trend of increased $FoxP3^{+}$ cell numbers and suppression of MLC after treatment with calcineurin inhibitors and the corticosteroid (p = no significant changes). RT-qPCR showed stable expression at 4 h with a trend towards higher FoxP3 RNA expression in both untreated and treated cells at 48 h (p = no significant changes) (Fig. 3b).

Significantly higher FoxP3 levels and suppression of alloactivated lymphocytes were seen in $FoxP3^{high}$ (n = 5) compared to $FoxP3^{low}$ MSCs (n = 4, $p < 0.05$), but not in FoxP3 RNA levels (p = no significant differences) (Fig. 3ab).

5-azacytidine does not modulate FoxP3 expression by MSCs and has no effect on MSC-mediated immunosuppression

By intracellular flow cytometry, MSCs showed no significant changes in FoxP3 expression after exposure to 0.01–1 μM 5-azacytidine (mean FoxP3 positivity 5.7 ± 2.2% vs. 2.7–9.8%, n = 7). The treatment with 5-azacytidine had no effect on the MSC-mediated

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immunosuppression measured by inhibition of allogeneic MLC (normalized inhibition of MLC 0.62 ± 0.08 vs. 0.60–0.71, n = 5).

Discussion

The immunosuppression mediated by MSCs has been thoroughly investigated over the last decade, and MSCs are clearly effective at controlling the GVHD alloresponse in human stem cell transplant recipients[33], but the cellular mechanisms controlling the immunosuppressive capacity of MSCs remain poorly understood. Tregs are also important in controlling alloresponses after stem cell transplantation[34–36]. Thus, MSCs and Tregs have comparable functional significance in the field of transplantation. FoxP3 expression is a functional marker for Tregs, but subsets of Tregs express ectonucleotidases CD39 and CD73[37–38], which confer anti-inflammatory properties. As both MSCs and Tregs express CD73 and have similar immunosuppressive features, we investigated whether MSCs and Tregs share fundamental similarities in the molecular pathways leading to their characteristic functions of immunosuppression and anti-inflammatory action. This is the first demonstration that MSCs expanded from healthy donors express FoxP3. The expression is highly variable between cells and over time. The expression of FoxP3 was intrinsic to cell growth and maturity and was not affected by exposure to allogeneic lymphocytes. CD73 expression is a consistent feature of human MSCs[26]. CD73 is an ectonucleotidase involved in the formation of adenosine and it may contribute to suppression of T lymphocytes[38]. While another ectonucleotidase, CD39, is involved in suppression of immune responses by degrading adenosine triphosphate (ATP) in a subset of Tregs[37], CD39 expression has not been investigated in MSCs previously. We found that MSCs express CD73 but not CD39, in common with the majority of human Tregs.

We also examined the functional relationship between FoxP3 expression and immunosuppression. Compared to $FoxP3^{low}$ MSCs, $FoxP3^{high}$ cells showed greater suppression of MLC. The effect could be demonstrated when MSCs were in contact with the lymphocytes as well as when the lymphocytes were incubated with culture supernatants from unstimulated or MLC-stimulated MSC cultures. This indicates that the immunosuppressive effect was derived from soluble factors. In Tregs, it is clear that FoxP3 has a principal role in gene regulation[23]. FoxP3 unambiguously regulates activity since mice with FoxP3 mutations show immune dysregulation[39], and dendritic cells expressing transgenic FoxP3 display regulatory features from altered cytokine expression[40]. To determine whether functional FoxP3 is essential for immunosuppression by MSCs, we tested MLC inhibition after siRNA-mediated knock down of FoxP3. Although siRNA-treated MSCs still inhibited the MLC, it should be noted that we did not achieve more than a 50% reduction in FoxP3 RNA expression and a 70% reduction in FoxP3 protein levels (determined by RT-qPCR and western blot, respectively). This raises the possibility that the residual FoxP3 may have been sufficient for the immunosuppressive effects observed. Since FoxP3 was a marker for MSCs with high immunosuppressive capacity, our results suggest that while FoxP3 may be required for *initiation* of an immunosuppressive phenotype, its persistent expression is not essential for MSC-mediated immunosuppression.

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MSCs are being used in clinical trials worldwide, and their role in treating complications related to SCT is under investigation[16–18,25,33]. SCT recipients regularly receive calcineurin inhibitors and corticosteroids, which could affect the FoxP3 expression and MSC-mediated immunosuppression. Indeed, MSCs exposed to calcineurin inhibitors may have enhanced immunosuppressive capacity[41]. Moreover, FoxP3 regulation is highly dependent on epigenetic events[23,42]. We therefore explored the effect of immunosuppressants on FoxP3 expression and immunosuppression by MSCs. On exposure to various immunosuppressants, $FoxP3^{high}$ MSCs showed stable FoxP3 levels and consistent suppression of allogeneic MLC, whereas $FoxP3^{low}$ MSCs had a trend towards increased FoxP3 expression and suppressive capacity of alloreactive lymphocytes. Demethylating agents can upregulate a wide variety of promotors. We therefore investigated the effect of 5-azacytidine on FoxP3 expression by MSCs. However, exposure to 5-azacytidine did not change either FoxP3 expression or suppressive activity. These results suggest that while immunosuppressive drugs enhance MSC-mediated immunosuppression, epigenetic modulation is not sufficient to change the FoxP3 expression and immunosuppressive activity. This once again suggests that FoxP3 is expressed at insignificantly low levels by MSCs but that it might be a surrogate marker for immunosuppressive capacity rather than a functional necessity for immunosuppression.

In conclusion, this is the first report to show FoxP3 expression and absence of CD39 expression by MSCs. MSCs have some features in common with Tregs, but they express lower levels of FoxP3 than Tregs; and while there is a correlation between FoxP3 expression and the immunosuppressive capacity of MSCs, their immunosuppressive function is not as tightly linked to FoxP3-regulated gene activity as is the case with Tregs. Nevertheless, in the context of SCT it remains to be determined whether immunosuppressive drugs act synergistically with infused MSCs to increase their therapeutic effect on GVHD.

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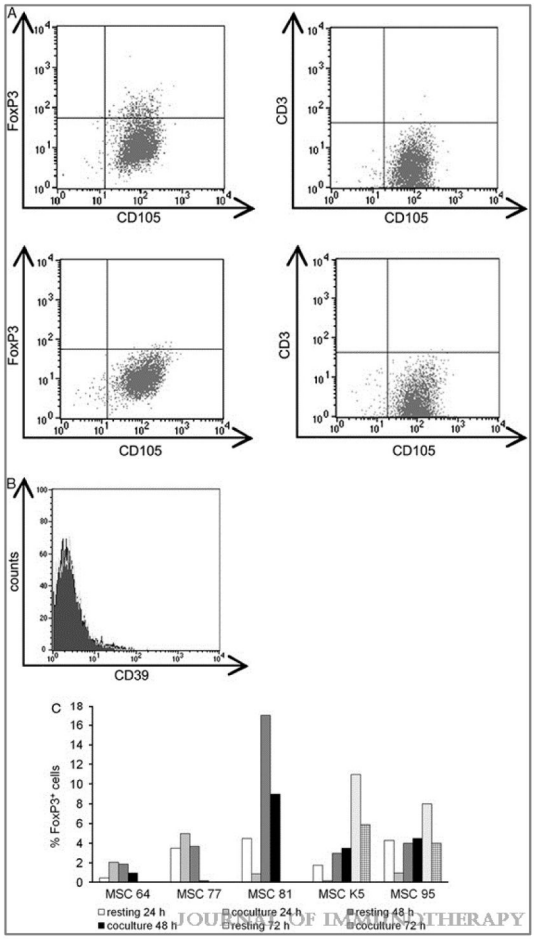

Figure 1. MSCs show variable expression of FoxP3, but are negative for CD39 expression

FoxP3^{high} MSCs (upper panel) and FoxP3^{low} MSCs (lower panel) were stained for (**a**) FoxP3 and the MSC marker CD105, and (**b**) CD39, and analyzed by flow cytometry. (**c**) FoxP3 levels were highly variable in resting MSCs and upon co-culture with allogeneic lymphocytes for up to 72 h. Contamination by lymphocytes was ruled out by staining for CD105 and CD3. Dashed line denotes isotype control. Representative graphs are shown.

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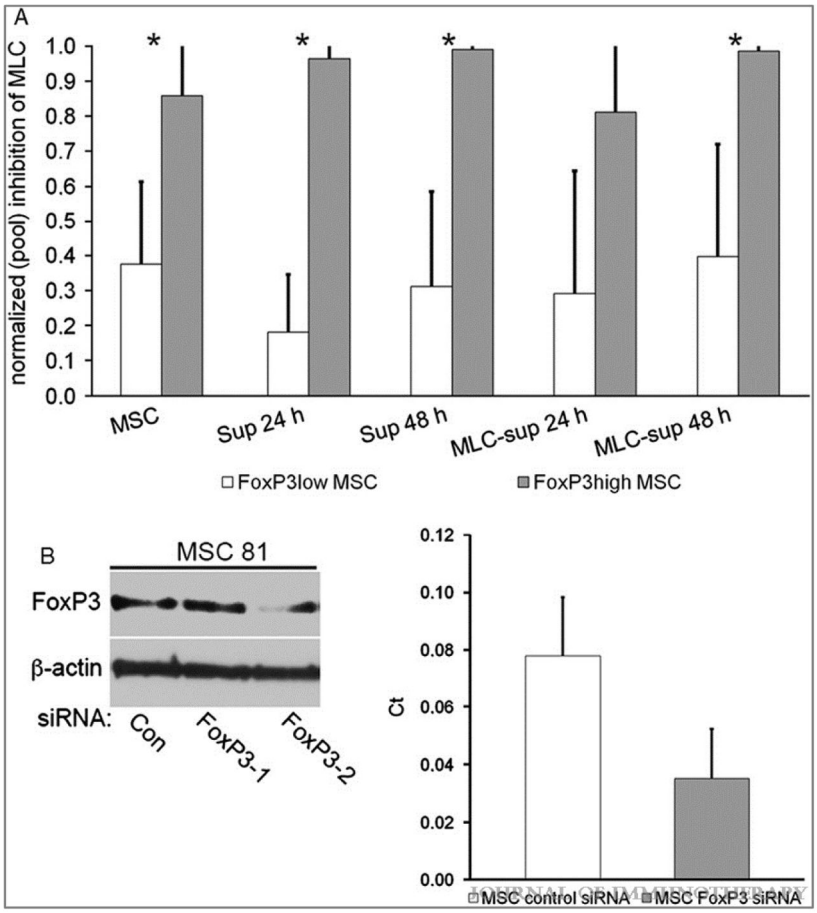

Figure 2. MSC-mediated immunosuppression in relation to FoxP3 expression and FoxP3 silencing

(**a**) FoxP3^{low} or FoxP3^{high} MSCs and supernantants from MSC culture medium (Sup) or from MLC with MSCs present for 24 and 48 h, were added to MLCs (n = 5). (**b**) Two different siRNAs targeting FoxP3, denoted FoxP3-1 and -2, and one nonsense siRNA, denoted con/control, were used for knock down of FoxP3 expression in MSCs as demonstrated by western blot (left panel). The decrease in FoxP3 RNA expression by FoxP3-2-siRNA seemed superior to FoxP3-1-siRNA, why FoxP3-2-siRNA treated MSCs were evaluated in RT-qPCR and displayed a significant decrease in RNA expression (right panel).

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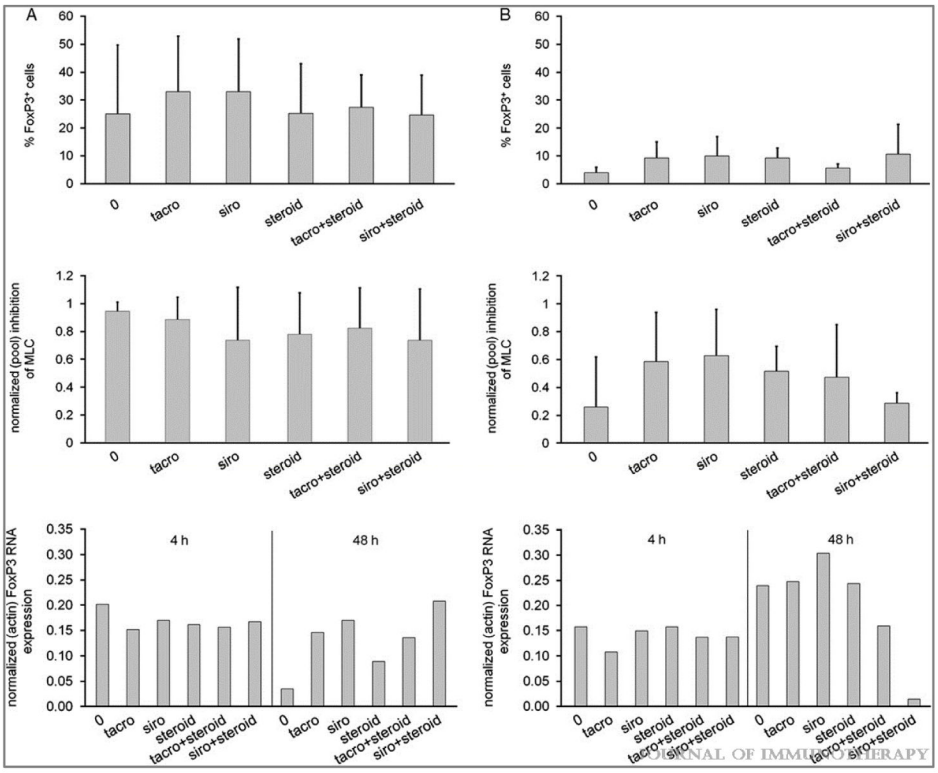

Figure 3. Immunosuppressive agents modulate FoxP3 levels in MSCs

(**a**) FoxP3^{high} MSCs (n = 5) and (**b**) FoxP3^{low} MSCs (n = 4) were treated with immunosuppressive drugs. FoxP3 expression was determined by flow cytometry (upper panel). The immunosuppressive capacity of the MSCs was tested in MLC (middle panel). FoxP3 RNA levels over time (at 4 and 48 h) were determined by RT-qPCR (lower panel). 0 = untreated, tacro = tacrolimus, siro = sirolimus, steroid = methylprednisolone, tacro+steroid = tacrolimus in combination with methylprednisolone, and siro+steroid = sirolimus in combination with methylprednisolone.