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Mesenchymal cells recruit and regulate T regulatory cells

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Objective. **Despite much investigation into T regulatory cells (Tregs), little is known about the mechanism controlling their recruitment and function. Because multipotent mesenchymal stromal cells (MSCs) exert an immune regulatory function and suppress T-cell proliferation, this in vitro study investigated their role in Treg recruitment and function.**

Materials and Methods. **Human MSCs and different T cell populations ($CD3^+$, $CD3^+/CD45RA^+$, $CD3^+/CD45RO^+$, $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RO^+$, $CD4^+/CD25^+/CD45RA^+$) from healthy donors were cocultured for up to 15 days. Harvested lymphocytes were analyzed by flow cytometry and FoxP3 and CD127 expressions were measured by real-time polymerase chain reaction. Their regulatory activity was assessed.**

Results. **We demonstrate MSC recruit Tregs from a fraction of $CD3^+$ and from immunoselected $CD3^+/CD45RA^+$ and $CD3^+/CD45RO^+$ fractions. After culture with MSCs both immunoselected fractions registered increases in the $CD4^+/CD25^{bright}$/FoxP3 subset and CD127 expression was downregulated. When purified Treg populations ($CD4/CD25^+$, $CD4/CD25^+/CD45RA^+$, and $CD4/CD25^+/CD45RO^+$) are used in MSC cocultures, they maintain FoxP3 expression and CD127 expression is downregulated. Treg suppressive capacity was maintained in Treg populations that were layered on MSC for up to 15 days while control Tregs lost all suppressive activity after 5 days culture.**

Conclusions. **In conclusion, our study demonstrates that MSCs recruit, regulate, and maintain T-regulatory phenotype and function over time.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Natural regulatory cells $CD4^+/CD25^+$ are primarily involved in maintenance of self-tolerance [1], i.e., they suppress effector T-cell proliferation and cytokine production in a cytokine-independent manner requiring cell-to-cell contact [2]. Apart from autoreactive T-cell suppression, $CD4^+/CD25^+$ cells also dampen immune responses against infectious pathogens, cancer, and allogeneic organ and stem cell grafts [3].

Only $CD4^+/CD25^+$ cells expressing the highest levels of CD25 (called CD25 bright cells) are reported to be Tregs [4]. They are characterized by high FoxP3 expression [5], which is required for Treg development [6]. Tregs are also associated with low expression of CD127, the interleukin (IL)-7 receptor α-chain [7]. Indeed, CD127 downregulation is associated with acquisition of regulatory function [8] and inversely correlates with FoxP3 expression [9]. Although Tregs in adult peripheral blood were believed to be exclusively $CD45RO^+$ memory cells, recent findings demonstrated they contained naïve $CD45RA^+$ cells [10]. Only this subpopulation maintained robust suppressive activity after expansion in vitro [11].

Despite much investigation into human Tregs, little is known about the mechanism controlling their recruitment and function. An intriguing question is whether multipotent mesenchymal stromal cells (MSCs) [12,13] play a role in Treg recruitment and function. MSCs exert an immune regulatory function and suppress T-cell proliferation in vitro [14,], in vivo in animal models [15] and in humans [16]. They provide cell-to-cell contact and, even in the absence of the thymus, produce growth factors for cell hematopoiesis [17]. Despite substantial interest in MSC, data on interactions between MSC and Tregs are scarce and divergent. In an in vitro MSC T-cell coculture model, T cells displayed a regulatory phenotype [18–20], and Treg recruitment was

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hypothesized to be involved in MSC downregulation of T-cell responses [21]. Another investigation into T-cell coculture with MSCs reported the MSC inhibitory effect was not mediated through Tregs [22]. Consequently, better characterization of the MSC–Treg interaction and elucidation of its mechanisms are crucial.

In the present study, we investigated MSC–T-cell interactions and demonstrate MSC are involved in Treg recruitment and regulation. MSCs recruit Tregs from total $CD3^+$ cells and from separated $CD3^+/CD45RO^+$ and $CD3^+/CD45RA^+$ fractions, with the highest recruitment level being observed in $CD45RA^+$ Tregs. These results suggest the stromal environment constitutes a homeostatic niche for $CD4^+/CD25^+$ regulatory T cells.

MSCs regulate Treg from immunoselected $CD4^+/CD25^+$ and from sorted $CD4^+/CD25^+/CD45RO^+$ (memory Tregs) and $CD4^+/CD25^+/CD45RA^+$ (naïve Tregs). After coculture with MSC, suppressive activity is maintained over time, FoxP3 is upregulated and CD127 downregulated, indicating MSCs regulate the Treg phenotype and function. Consequently, coculture with human MSC might provide a feasible strategy for enhancing naïve and memory Treg phenotypes for Treg-based cellular therapy.

Materials and methods

Peripheral blood mononuclear cells (PBMCs) from leukapheresis and MSCs from bone marrow were obtained from eight healthy adults. MSCs in all the present experiments were allogeneic to the PBMCs employed for MSC/T-cell cultures and for the suppression assays. All donors provided informed consent (Helsinki Declaration).

$CD3^+$ cells

$CD3^+$ cells were isolated by positive selection (auto magnetic-activated cells sorting separator with CD3 microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany).

Naïve and memory T cells

$CD3^+/CD45RA^+$ cells (naïve) and $CD3^+/CD45RO^+$ cells (memory) were isolated by immunoselection (MidiMACS separator with CD45RO magnetic beads; Miltenyi Biotec). $CD3^+/CD45RA^+$ cells are recovered from the negative fraction and $CD3^+/CD45RO^+$ cells from the positive.

Regulatory T cells

$CD4^+/CD25^+$ regulatory cells were isolated using an isolation kit (Miltenyi Biotec). $CD4^+/CD25^+$ cells were labeled with (ECD)-conjugated anti-CD45RA (Beckman-Coulter Corporation, Hialeah, FL, USA) or ECD-conjugated anti-CD45RO (Beckman-Coulter) and sorted via EPICS ELITE (Beckman-Coulter) to provide $CD4^+/CD25^+/CD45RA^+$ (naïve Treg) and $CD4^+/CD25^+/CD45RO^+$ (memory Treg). Within $CD4^+/CD25^+$ subset, the bright cells were considered the cells with fluorescence over the first logarithm. The mouse IgG1 isotype control antibody was used to assess background fluorescence.

MSC generation

Multipotent MSCs were obtained from bone marrow aspirate from eight healthy subjects after they had given informed consent, as described elsewhere [23]. Briefly, marrow mononuclear cells were resuspended at a concentration of 2×10^6 cells/mL in complete culture medium. Medium contained α-modified Eagle's medium (GIBCO BRL Life Technologies, Grand Island, NY, USA), 10% human serum, L-glutamine (2 mmol/L), 2-mercaptophenol (10^{-4} mol/L), inositol (0.2 mmol/L), and folic acid (20 μmol/L). Cells were cultured in 175 cm^2 flasks and incubated at 37°C 5%CO_2 in a humidified thermostat for 2 to 4 weeks until the confluent layer was formed.

MSC/T-cell cocultures

MSCs were cocultured with $CD3^+$ cells and with $CD3^+/CD45RA^+$ cells, $CD3^+/CD45RO^+$ cells for 5 days (ratio 1:5) and with $CD4^+/CD25^+$, $CD4^+/CD25^+CD45RA^+$, and $CD4^+/CD25^+/CD45RO^+$ cells for 15 days (ratio 1:5) in RPMI-1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated human AB serum, 1% penicillin/streptomycin, and 2 mM L-glutamine.

Flow cytometry

T-cell populations were analyzed by monoclonal antibodies directed against CD3, CD4, CD25, CD127, CD45RA, CD45RO, CCR5, CCR7 (Coulter Corporation, Hialeah, FL, USA), and FoxP3 (BioLegend, San Diego, CA, USA). MSCs were studied by monoclonal antibodies directed against CD45, CD90, CD105 (Beckman-Coulter) CD127 and FoxP3. Cells were analyzed by CYTOMICS FC500 (Beckman-Coulter) on days 0 and +5 of culture.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qPCR) was performed using TaqMan Gene Expression Assay (Hs01085835_m1) for FoxP3 and (Hs00902338_g1) for CD127. Expression levels were calculated as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = [\Delta Ct\ (sample) - \Delta Ct\ (calibrator)]$ and $\Delta Ct = [Ct\ (sample) - Ct\ (housekeeping)]$. Relative expression was determined by normalization to 18S.

Suppression assays

The suppression assay for MSC/CD3 cocultures was as follows. Cultures of 10^5 irradiated stimulator cells, 2×10^4 CD3 responder (Tresp) cells and allogeneic suppressor MSCs were analyzed on day +5. Cultures of 10^5 irradiated stimulator cells with 2×10^4 CD3 responder (Tresp) cells act as control (mixed lymphocyte reaction). Cells were pulsed with 1 μCi/well [^{3}H] thymidine (Amersham Biosciences, Buckinghamshire, UK) for the last 16 hours, and incorporated [^{3}H] thymidine was measured with a beta-counter. Data are presented as stimulation index values calculated by the following formula: cpm of resting or activated Tresp cells with or without MSC/cpm of Tresp cells alone.

The suppression assay for the Treg/$CD4^+/CD25^-$ cocultures was as follows. Tregs used in the suppression assay has previously been cultured on a layer of MSCs for up to 15 days. To exclude possible MSC contamination, CD45 and CD90 stainings were performed before Tregs were used in the suppression assay. Cultures of 10^5 irradiated stimulator cells, 2×10^4 responder cells $CD4^+/CD25^-$ (Tresp) and autologous suppressor subpopulations, i.e., $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$ or $CD4^+/CD25^+/CD45RO^+$ (Tregs) were analyzed on days 0 (before MSC

cocultures), +5 and +15. Cultures of 10^5 irradiated stimulator cells with 2×10^4 responder cells $CD4^+/CD25^-$ (Tresp) act as controls. The final suppressor to responder ratio was 1:1. Cells were pulsed with 1 μCi/well [^{3}H] thymidine (Amersham Biosciences) for the last 16 hours, and incorporated [^{3}H] thymidine was measured with a beta-counter. Data are presented as proliferation percentages calculated by the following formula: cpm of activated Tresp cells alone/cmp Tresp + Tregs cells with or without MSC × 100.

Statistical analysis with Student's t-test

Significance was <0.05. Results are always at least a mean ± SD of three experiments.

Results

Mesenchymal cells

Flow cytometry analysis established MSCs were negative for CD45 and CD14 antigen expression and positive for CD90 (98.15% ± 0.27%) and CD105 (87.6% ± 0.94%), thus establishing purity and ruling out hematopoietic contamination in cells used in this study. MSCs differentiated into osteoblasts, adipocytes, and chondroblasts in vitro [12] (data not shown). At immunofluorescence, MSCs were negative for FoxP3 and strongly positive for surface CD127 (Fig. 1A). qRT-PCR showed FoxP3 mRNA was 42.18 ± 37.8 times lower than in $CD3^+$ control cells. CD127 mRNA was 1218 ± 300 times lower than in $CD3^+$ control cells.

MSC and $CD3^+$ cell cocultures

The immune regulatory capacity of ex vivo expanded MSCs was determined by testing MSC inhibition of the alloantigen-specific immune response in primary mixed lymphocyte reaction. MSCs significantly inhibited alloantigen-induced lymphocyte proliferation ($p < 0.05$; control responses in cpm = 27,224 ± 3752) (Fig. 1B). MSC and $CD3^+$ cells were cocultured to investigate $CD4^+/CD25^+$ recruitment. After 5 days coculture the $CD4^+/CD25^+$ count was similar to controls (CD3 alone) (56% ± 5.3% vs 51% ± 4.8%) (Fig. 1C). To distinguish $CD4^+/CD25^+$ Tregs from activated T cells, $CD4^+/CD25^{bright}$ and $CD4^+/CD25^{bright}/Foxp3^+$ cells were counted within the $CD4^+CD25^+$ T-cell subset by cytofluorimetric analysis. Percentages of $CD4^+/CD25^{bright}$ and $CD4^+/CD25^{bright}/Foxp3^+$ cells were higher in the presence of MSCs (42% ± 4.1% vs 34% ± 3.26% for the $CD4^+/CD25^{bright}$ and 20.5% ± 4.2% vs 3.5% ± 2.8% for the $CD4^+/CD25^{bright}/Foxp3^+$) (Fig. 1C and D). CD45RA and CD45RO distribution in $CD4^+/CD25^{bright}$ cells was also analyzed. CD45RA increased from 3% ± 2.3% on day 0 to 27% ± 5% in the presence of MSCs vs 12% ± 3.5% in controls. CD45RO decreased from 97% ± 2.3% on day 0 to 57% ± 4.8% in the presence of MSCs vs 80% ± 10% in controls (Fig. 1E).

MSCs and CD3/CD45RA or CD3/CD45RO cells cocultures

To investigate $CD4^+/CD25^+$ (Treg) recruitment, MSCs were cocultured with immunoselected CD3/CD45RA (92% ± 6% purity) cells. After 5 days' culture the Treg starting fraction of 0.05% ± 0.01% rose to 0.2% ± 0.14%. CD127 expression was downregulated from an initial 3% ± 1.2% to 0.29% ± 0.2%. The percentage of the $FoxP3^+$ cells, calculated by gating on the $CD4^+/CD25^{bright}$ cells, increased from 3% ± 2% (day 0) to 27% ± 4.2% (after 5 days' culture) (Fig. 2A). After 5 days culture of CD3/CD45RA cells alone (control) no change was observed in their $CD4^+/CD25^{bright}/FoxP3^+$ cell count (vs day 0).

MSCs were cocultured with immunoselected CD3/CD45RO (90.5% ± 0.43% purity). After 5 days' culture the Treg starting fraction of 0.3% ± 0.05% rose to 1.5% ± 0.9%. CD127 was downregulated from an initial 15% ± 1.2% to 1.32% ± 0.34%. The percentage of the $FoxP3^+$ cells, calculated at baseline and after 5 days' culture by gating on the $CD4^+/CD25^{bright}$ cells, increased from 2% ± 1.1% (day 0) to 36.6% ± 3.8% (after culture) (Fig. 2B). After 5 days' culture of CD3/CD45RO cells alone (control) no change was observed in their $CD4^+/CD25^{bright}/FoxP3^+$ cell count (vs day 0).

$CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$, and $CD4^+/CD25^+/CD45RO^+$ cells (Tregs)

In order to obtain pure populations of different types of Tregs, immunoselection of $CD4^+/CD25^+$ cells was followed by cell sorting for naïve and memory Tregs. An initial fraction of 200×10^6 (range, $180–250 \times 10^6$) cells contained 68.6% ±.3.9% $CD4^+/CD25^+$ and 7.25% ± 0.46% $CD4^+/CD25^{+bright}$ when gated on $CD4^+$ cells (Fig. 3A). Immunoselection yielded 2.7×10^6 ± 1.3 $CD4^+/CD25^+$ (purity 93.3% ± 5%) and 62.1% ± 23% $CD4^+/CD25^{bright}$ (Fig. 3C). CD127 expression was 10.6 ± 5.7 in $CD4^+$ cells (Fig. 3A), 55% ± 37%, in $CD4^+/CD25^-$ cells (Fig. 3B), 7% ± 5.7% in $CD4^+/CD25^+$ cells, and 0.4 ± 01 in $CD4^+/CD25^{+bright}$ cells (Fig. 3C).

Expression of CD45RA, CD45RO, CCR7, and CCR5 was analyzed in Tregs. When gated on $CD4^+$ cells a starting fraction contained 38% ± 12% CD45RA, 71% ± 25.7% CD45RO, 26.1% ± 18% CCR7, and 10% ± 7.5% CCR5 (Fig. 3A). Immunoselection yielded a $CD4^+/CD25^+$ fraction containing 10.7% ± 3.9% CD45RA, 93.7% ± 4.7% CD45RO, 16.3% ± 15.1% CCR7, and 25.2 ± 9.1 CCR5 (Fig. 3C). The $CD4^+/CD25^{bright}$ fraction contained 0.3% ± 0.4% CD45RA, 99.9% ± 0.2% CD45RO, 3.1% ± 3.4% CCR7, and 32% ± 8% CCR5 (Fig. 3C).

After cell sorting $CD4^+/CD25^+/CD45RA^+$ were 1.5×10^5 ± 0.3 (98% ± 0.3% purity) and $CD4^+/CD25^+/CD45RO^+$ cells were 1.2×10^6 ± 0.5 (97.4% ± 0.4% purity). The $CD4^+/CD25^+/CD45RA^+$ and $CD4^+/CD25^+/CD45RO^+$ populations do not express CD127. CCR7 was

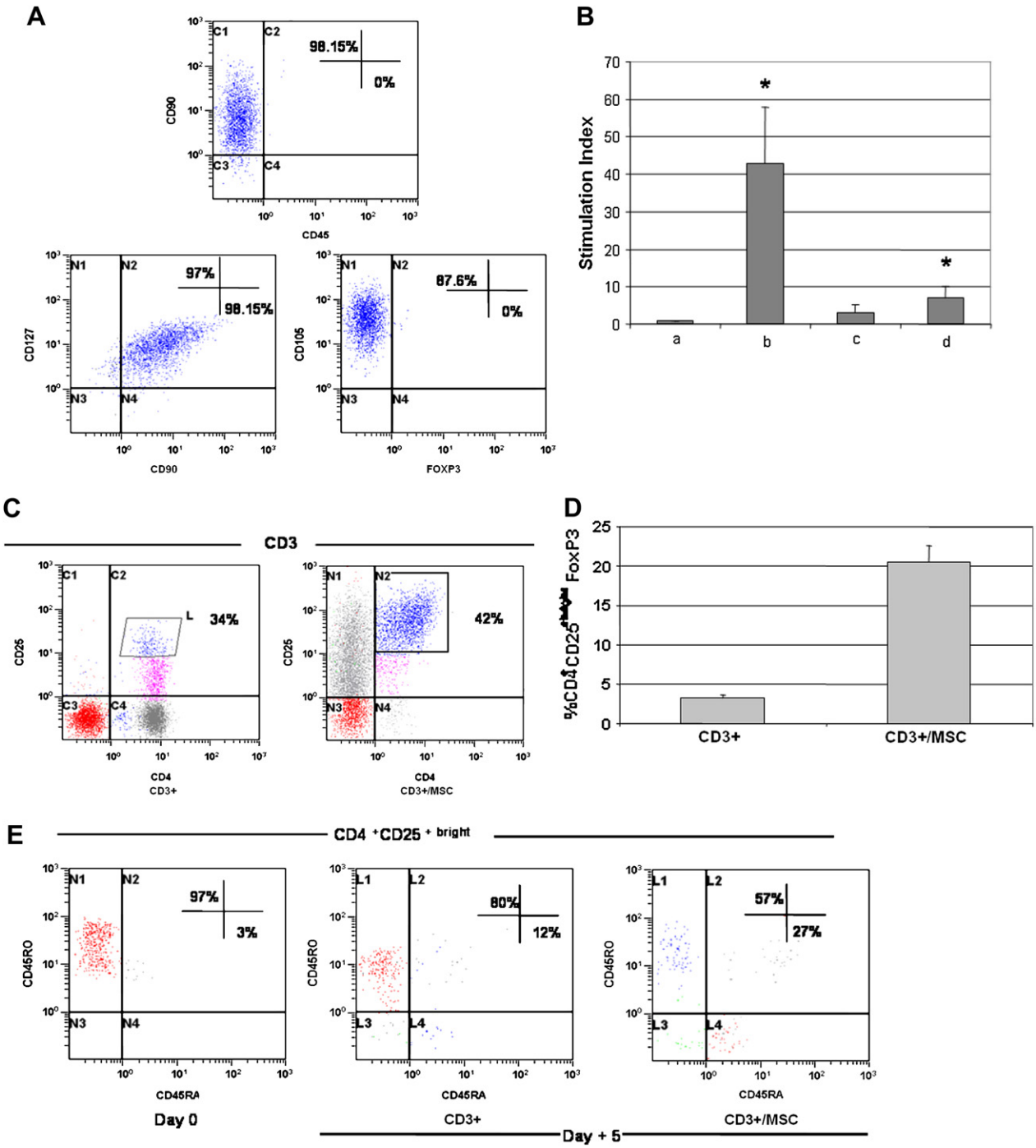

Figure 1. Mesenchymal cells (MSCs) and $CD3^+$ cell cultures. (**A**) Expression of CD127 and FoxP3 on MSCs. Plots are gated for $CD45^-CD90^+$ cells and for $CD45^-CD105^+$. $CD90^+/CD127^+$ and $CD106^+/FoxP3^-$ are boxed and the percentage of cells in the box is shown. (**B**) MSCs inhibit mixed lymphocyte reaction (MLR). The graph shows the stimulation index after 5 days of MLR. Column a: resting lymphocytes; column b: activated lymphocytes; column c: resting lymphocytes + MSCs; column d: activated lymphocytes + MSCs. *Statistically significant difference. (**C**) After coculture with or without MSCs, $CD3^+$ cells were analyzed. CD3 alone acts as control. Plots are gated for $CD4^+CD25^+$ cells and for $CD4^+CD25^{bright}$ cells. Positive cells are boxed and the percentage of the positive cells in the box is shown. (**D**) The graph shows the percentage of $CD4^+CD25^{bright}$ FoxP3 in presence of MSCs and in control cells. (**E**) Expression of CD45RA and CD45RO on T regulatory cells over 5 days, in presence or in absence of MSCs. Plots are gated for $CD4^+CD25^{bright}$ cells. The percentage of positive cells is shown.

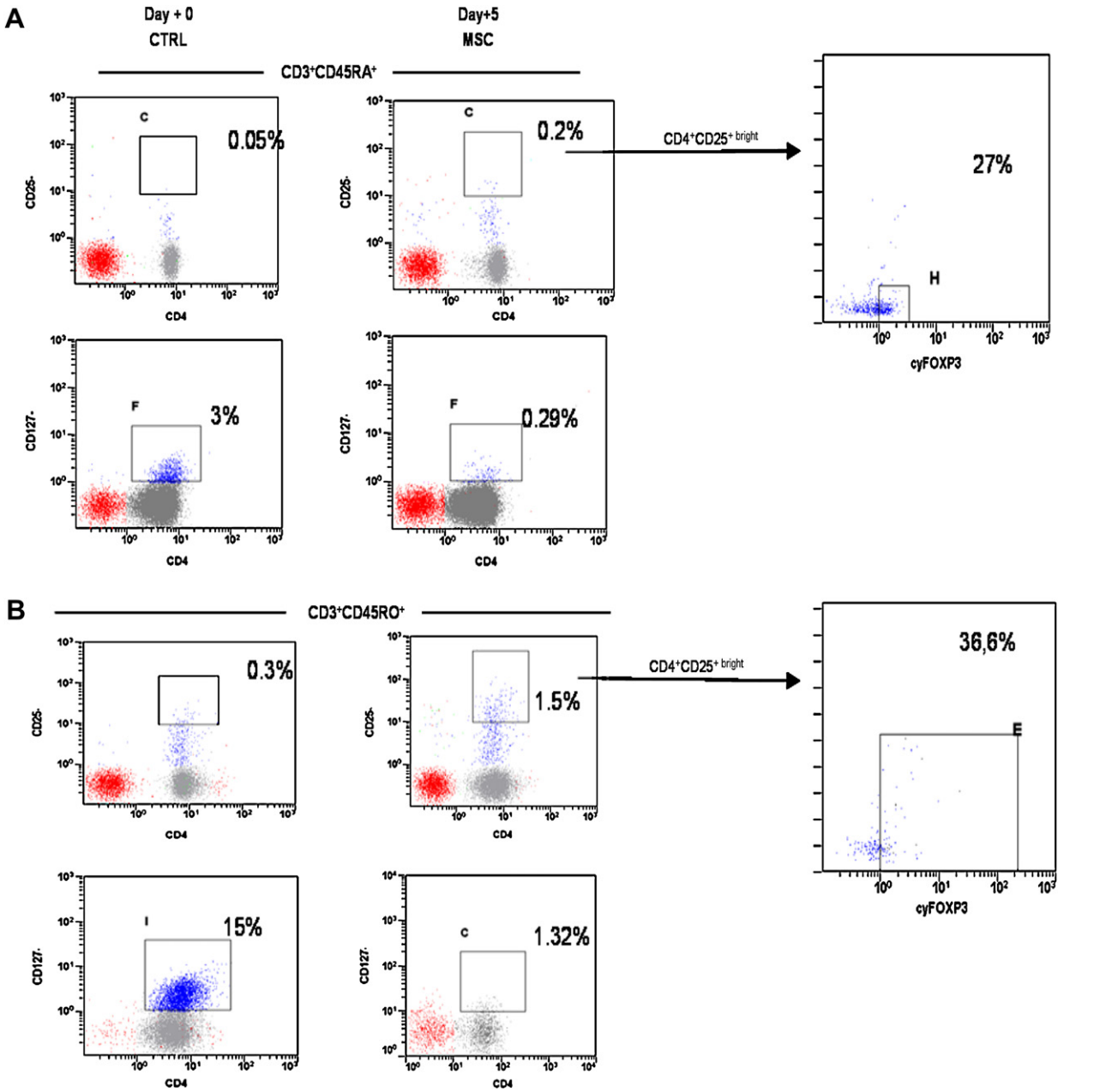

Figure 2. Mesenchymal cells (MSCs) and $CD3^+/CD45RA^+$ or $CD3^+/CD45RO^+$ cell cocultures. After coculture with or without MSCs, $CD45RA^+$ (**A**) and $CD45RO^+$ (B) cells were analyzed. $CD3^+/CD45RA^+$ or $CD3^+/CD45RO^+$ alone act as control. Plots are gated for $CD4^+CD25^+$, for $CD4^+CD25^{bright}$, $CD4^+CD25^{bright}$ $FoxP3^+$, and for $CD4^+/CD127^-$ cells. Positive cells are boxed and the percentage of the positive cells in the box is shown.

not detected in either fraction. CCR5 expression was 0.3% ± 1.7% in the $CD4^+/CD25^+/CD45RA^+$, and 11% ± 3.4% in the $CD4^+/CD25^+/CD45RO^+$ cells.

qRT-PCR on Tregs showed FoxP3 expression was higher than in the initial fraction (+7.48 ± 0.07-fold in $CD4^+/CD25^+$, +33.9 ± 13.3-fold in $CD4^+/CD25^+/CD45RA^+$, and +60.9 ± 1.8-fold in $CD4^+/CD25^+/CD45RO^+$ cells) (Fig. 3D) ($p < 0.05$ vs controls for all separated fractions). FoxP3 expression was 1.82-fold ± 0.17-fold greater in $CD4^+/CD25^+/CD45RO^+$ cells vs $CD4^+/CD25^+/CD45RA^+$. CD127 expression decreased by −3.8-fold ± 1.5-fold in $CD4^+/CD25^+$, −16.85-fold ± 9.9-fold in $CD4^+/CD25^+/CD45RA^+$, and −16.57-fold ± 11.9-fold in $CD4^+/CD25^+/CD45RO^+$ cells (Fig. 3E) in line with results of immunophenotyping.

The immune suppressive capacity of purified Treg populations was tested before culture in a proliferation assay. $CD4/CD25^+$ cells reduce $CD4^+/CD25^-$ cell proliferation to 18% ± 3%. $CD4/CD25^+/CD45RA^+$ cells reduced proliferation to 4% ± 1.1% and $CD4/CD25^+/CD45RO^+$ cells

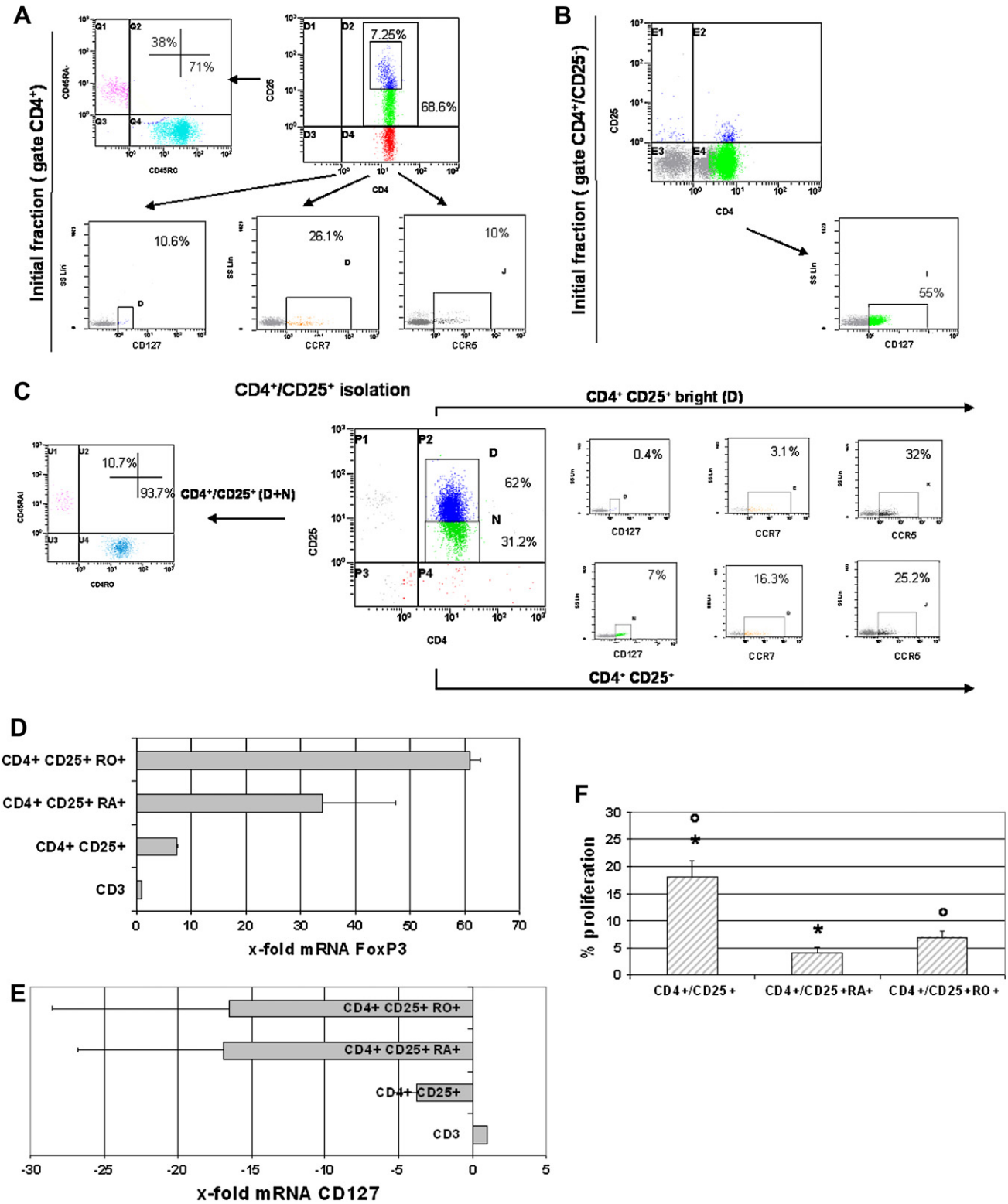

Figure 3. T regulatory cells (Treg) isolation. (**A**, **B**) T cell starting fraction. Plots are gated for $CD4^+$ cells (**A**) and for $CD4^+/CD25^-$ cells (**B**). (**C**) $CD4^+/CD25^+$ and $CD4^+/CD25^{bright}$ cell fraction. Positive cells are boxed and the percentage of cells in the box is shown. (**D**) Quantitative analysis of FoxP3 mRNA from selected $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$ and $CD4^+/CD25^+/CD45RO^+$ cells. $CD3^+$ cells are the starting fraction. (**E**) Quantitative analysis of CD127 mRNA from selected $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$, and $CD4^+/CD25^+/CD45RO^+$ cells. $CD3^+$ cells are the starting fraction. (**F**) Suppression assays: At a Treg to T responder cell (Tresp) ratio of 1:1 suppression capacity is indicated by the residual proliferation capacity of responder cells. The suppression capacity of the different Treg subpopulations is indicated. ○ and * indicate statistically significant differences.

to 7% ± 1.2% (Fig. 3F), showing all Tregs exert powerful immunosuppressive activity (control responses in cpm = 21,900 ± 4128).

These experiments provided three different highly purified populations of Tregs (CD4/CD25$^+$, CD4/CD25$^+$/CD45RA$^+$, and CD4/CD25$^+$/CD45RO$^+$).

FoxP3 expression was highest in CD4/CD25$^+$/CD45RO$^+$ cells and was greater in CD4/CD25$^+$/CD45RA$^+$ than in CD4/CD25$^+$. CD127 downregulation was similar in naïve and memory Tregs.

MSCs and CD4$^+$/CD25$^+$, CD4$^+$/CD25$^+$/CD45RA$^+$, and CD4$^+$/CD25$^+$/CD45RO$^+$ (Tregs) cocultures

To investigate the role of MSC in Treg regulation, the three Treg populations were cultured with MSCs for 15 days. On day +5 of culture FoxP3 expression did not change in CD4$^+$/CD25$^+$ cells. It increased 5.38-fold ± 2.3-fold in CD4$^+$/CD25$^+$/CD45RA$^+$ and 7.98-fold ± 1.9-fold in CD4$^+$/CD25$^+$/CD45RO$^+$ cells. CD127 expression decreased by −582-fold ± 29.2-fold in CD4$^+$/CD25$^+$ cells, by −216-fold ± 17.5-fold in CD4$^+$/CD25$^+$/CD45RA$^+$ and by −71.95-fold ± 12.6-fold in CD4$^+$/CD25$^+$/CD45RO$^+$ cells (Fig. 4A). Cytofluorimetric analysis performed only on the CD4$^+$/CD25$^+$ fraction, showed when MSCs were used CD4$^+$/CD25$^+$/FoxP3$^+$ was upregulated to 14% ± 4% and CD127 downregulated to 4.4% ± 1.5% vs respectively 0.2% ± 0.28% and 21.9% ± 3.5% in controls without MSCs (CD4$^+$/CD25$^+$ alone) (Fig. 4B). FoxP3 upregulation was even more marked in CD4$^+$/CD25^{bright} cells (51% ± 10% vs 0.1% ± 0.7% in controls). Cytofluorimetric results are in line with molecular analysis.

On days +5 and +15 suppression assays were performed. On day +5 of MSC cocultures CD4$^+$/CD25$^-$ proliferation, with CD4$^+$/CD25$^+$ cells as suppressor cells, was 2.4% ± 4% vs 9.3 ± 2 in controls; with CD4/CD25$^+$/CD45RA$^+$ as suppressor cells proliferation was 1.2% ± 6% vs 5% ± 2% in controls and with CD4$^+$/CD25$^+$/RO$^+$ as suppressor cells proliferation was 3.2% ± 2.1% vs 17.9 ± 4 in controls. In CD4$^+$/CD25$^+$ and CD4$^+$/CD25$^+$/RO$^+$ cells, the suppressive capacity was reduced but not completely abrogated when cells were maintained without MSC (Fig. 4C) (control responses in cpm = 37,871 ± 9347).

On day +15, CD4$^+$/CD25$^-$ proliferation, withCD4$^+$/CD25$^+$ cells as suppressor cells, was 1.7 ± 0.8 vs 45.2 ± 12 in controls; with CD4/CD25$^+$/CD45RA$^+$ as suppressor cells it was 5.4 ± 3.1, vs 21.3 ± 11.2 in controls, and with CD4$^+$/CD25$^+$/RO$^+$ as suppressor cells it was 2.3 ± 2 vs 33.5 ± 8.7 in controls (Fig. 4C) (control responses in cpm = 30,219 ± 12,753).

Discussion

As we intended to use MSCs as a potential "homeostatic niche" for Tregs [24] to investigate their role in Treg recruitment and regulation, we first analyzed our findings in light of reports of FoxP3 expression in the thymic microenvironment where Tregs develop. We demonstrate MSCs do not express FoxP3 protein, even though a very low level of mRNA was found. Liston et al. [25], demonstrated the thymic microenvironment does not express detectable levels of FoxP3 protein. Therefore, FoxP3 molecule production does not appear crucial in MSC-mediated Treg recruitment.

CD127 expression needed to be quantified in the MSCs before the coculture experiments. We found MSC strongly expressed the IL-7 receptor. Interestingly, bone marrow fibroblasts are a source of Flt3 ligand and interferon-β, which, in an autocrine loop, act as potent inducers of CD127 [26]. The demonstration of IL-7 receptors in nonlymphoid cells including primary marrow stromal cells challenged the view that IL-7 exerts effects mostly within lymphoid populations [27]. IL-7–mediated signaling through its receptor on MSC may be distinct from signaling mediated by IL-7 receptors on lymphoid cells. As Tregs are IL-7–independent [9], it is beyond the scope of this study to investigate the role of high CD127 expression on MSCs. However, understanding our MSCs were FoxP3 negative and strongly positive for CD127, served to prevent misleading results in the coculture experiments.

In cocultures, we confirmed MSC inhibited CD3$^+$ proliferation. Inhibition has been hypothesized to be linked to Treg recruitment [21]. Hoffmann et al. [11] recently demonstrated that only the CD45RA$^+$/CD62L$^+$/CCR7$^+$ subset maintains the Treg-cell phenotype and function throughout culture. Over time, in our MSC/CD3$^+$ cell cocultures, the CD45RO$^+$ subset (memory Tregs) decreased while the CD45RA$^+$ subset (naïve Tregs) significantly increased. After coculture with MSCs, both immunoselected fractions registered similar increases in the CD4$^+$/CD25^{bright}/FoxP3 subset, suggesting the specific advantage of CD45RA over CD45RO was lost. These distribution patterns suggest that MSC, when cocultured with total CD3$^+$ cells, recruit Tregs mainly through CD45RA$^+$ (naïve Treg) upregulation. When MSCs are cocultured with immunoselected CD45RA$^+$ or CD45RO$^+$ both naïve and memory subsets are involved in Treg recruitment.

In both naïve and memory Treg populations, we observed for the first time that coculture with MSCs downregulates CD127 expression and that CD127 inversely correlates with FoxP3 expression, providing evidence in support of another report that CD127 expression correlates inversely with FoxP3 expression within Treg cells [7]. The mechanism underlying CD127 inhibition has been investigated by others who showed IL-2 acts as negative regulator of CD127 through defective downstream phosphatidylinositol-3-kinase signaling [28].

In the present study, we also showed that, in both naïve- and memory-derived Tregs, MSCs regulate and maintain Treg function over time. After immunoselection, all Treg

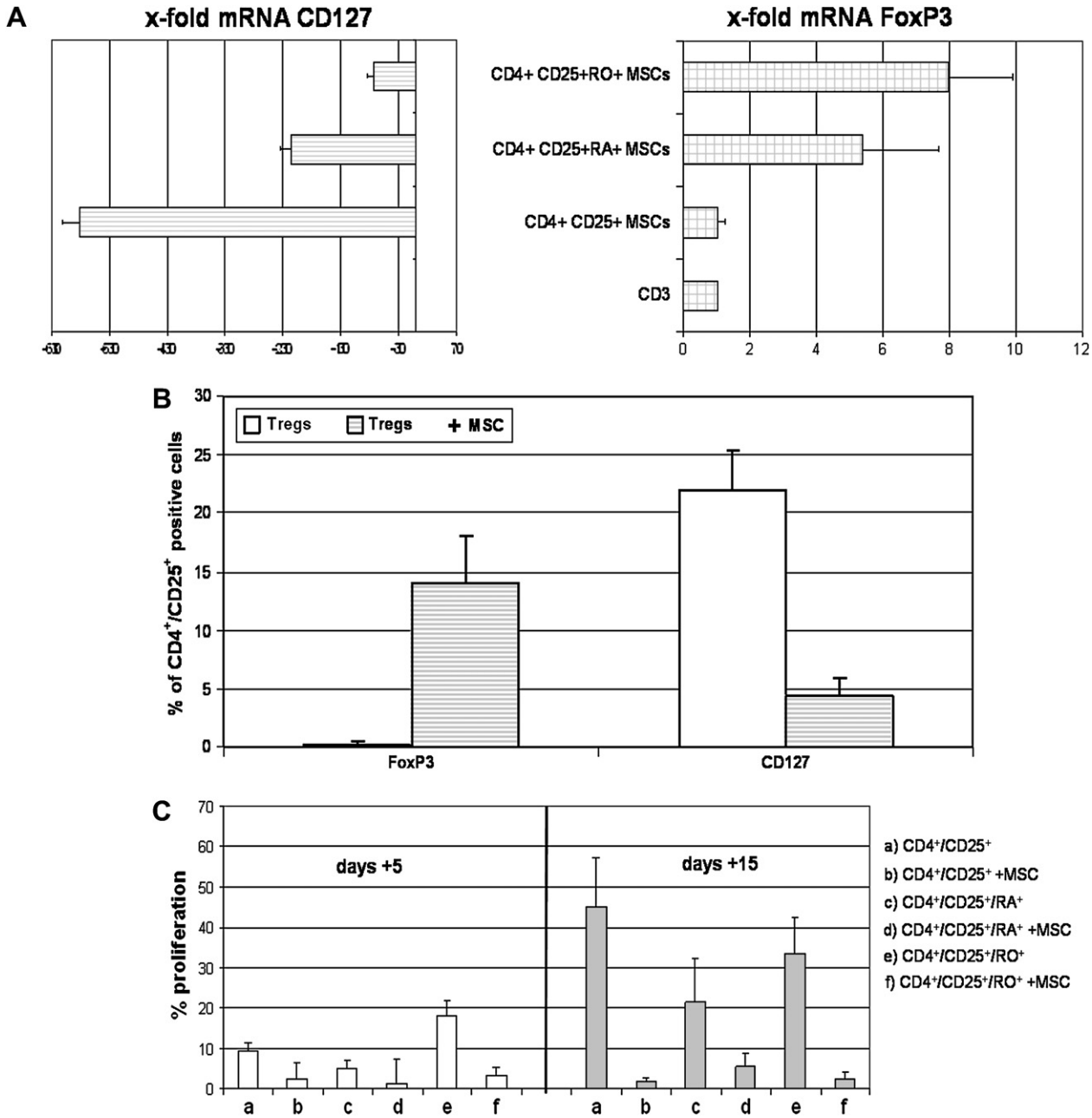

Figure 4. Mesenchymal cells (MSCs) cells and T regulatory cells (Treg) subpopulations cocultures. (**A**) Quantitative analysis of FoxP3 and CD127 mRNAs by selected $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$, and $CD4^+/CD25^+/CD45RO^+$ cells cultured in presence of MSCs. $CD3^+$ cells acts as control. (**B**) The graph shows the percentage of $CD4^+CD25^+$ expressing FoxP3 and CD127 in presence of MSCs and in control cells ($CD4^+/CD25^+$ without MSCs). (**C**) Suppression assays: At a Treg to T responder cell (Tresp) ratio of 1:1 suppression capacity is indicated by the residual proliferation capacity of responder cells. Suppression assays were performed on days +5 and +15. The suppression capacity of the different Treg subpopulations cultured with or without MSCs is indicated.

fractions exerted similar suppressive activity on human $CD4^+/CD25^-$ populations, indicating Treg function had been maintained. Remarkably Treg suppressive capacity was maintained in all Treg populations that were layered on MSC for up to 15 days while control Tregs (without MSCs) lost all suppressive activity after 5 days culture. Despite $CCR5^+$ cell enrichment by immunoselection and cell sorting within the $CD4^+/CD25^+/CD45RO^+$ fraction, we were unable to confirm the link between CCR5 and stronger suppressive activity as described by Kallikourdis et al. [29].

Our experiments also provide evidence for the first time that after culture with MSCs FoxP3 expression is maintained in naïve and memory subtypes and that MSCs downregulate the CD127 molecule on highly purified Treg populations. In MSC-free cultures, only the $CD45RA^+$ subset of $CD4^+/CD25^{bright}$ is reported to maintain FoxP3

expression after Treg expansion [11]. Continued expression of FoxP3 is needed to maintain the lineage identity and function of peripheral mature Treg cells [6], while low CD127 expression is associated with acquisition of regulatory functions [8]. Exposure to MSC therefore guarantees high FoxP3 expression, and maintenance of Treg function.

Although the mechanism underlying Treg upregulation in the presence of MSCs is unknown, T cell receptor undoubtedly has a determinant role in conferring $CD4^+/CD25^+$ Treg cell specificity and differentiation [30]. Barda-Saad et al. [31], showed TCR engagement was increased in nonadherent T cells in MSC cocultures. Moreover, TCR triggering in peripheral T cells generally downregulates CD127 expression [26]. So one may speculate that MSCs, through TCR engagement, downregulate CD127 expression on Tregs, thus maintaining the Treg phenotype. The specific action on FoxP3, which was evident when the total $CD3^+$ fraction was used, may be less important when purified Treg populations are cultured with MSC.

Tregs appear to be a good candidate for cellular therapy, as they can prevent development of autoimmune diseases [32], tumor immunity [32], graft rejection [16], and graft-vs-host disease [16]. Although several studies have described strategies for expanding Tregs in vitro, largely by using costimulatory antibodies against CD3 and CD28 in conjunction with an extremely high dose of IL-2 [33], they may be associated with the risk of triggering $CD4^+/CD25^+$ effector cell expansion and consequent T-cell–mediated alloreactions when used as adoptive therapy.

The results of the present in vitro studies show how the naïve subtype-related difficulties in obtaining a sufficient number of Tregs for clinical purposes, can be overcome because MSC cocultures maintain the Treg function over time, particularly in the abundant $CD4/CD25/CD45RA^-$ fraction. Furthermore, cultivating Tregs on a layer of MSCs reduces the risk of eliciting an unwanted alloreactive response in vivo.

In conclusion, as MSC recruit, regulate, and maintain Treg function over time, coculture with human MSCs might provide a feasible strategy to enhance naïve and memory Treg phenotypes in an attempt to develop Treg-based cellular therapy.

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