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Highlighted Article

Frontline Science: Placenta-derived decidual stromal cells alter IL-2R expression and signaling in alloantigen-activated T cells

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ABSTRACT

This study investigated how stromal cells affect the IL-2 pathway in alloantigen-activated T cells. We found that decidual stromal cells (DSCs) from term placentas promoted a high production of IL-2 in cultures with alloantigen-activated T cells. The intensity of expression of cluster of differentiation 25 (CD25; IL-2Rα) on T cells was increased by DSCs, whereas the frequency and intensity of expression of the signaling subunits CD122 (IL-2Rβ) and CD132 (IL-2Rγ_c) were reduced. Consequently, uptake of IL-2 and STAT5 phosphorylation (pSTAT5) was abrogated. DSCs also decreased the proportion of pSTAT5$^+$ T cells in response to IL-15, which also use CD122 for signaling. Addition of DSCs to the allogeneic cultures did not increase the expression of programmed death 1 (PD-1) or CD95, indicating that they did not promote T cell exhaustion. However, exogenous recombinant (r)IL-2 in similar concentrations in the same setting increased the expression of CD95 and downregulated CD122 in T cells. The antiproliferative effect of sirolimus (SRL) and cyclosporine A (CsA), which target the IL-2 signaling pathway, was diminished by DSCs in vitro. To conclude, DSCs affect IL-2 production and IL-2R expression and signaling, which may contribute to the stromal cell-mediated immune modulation and phenotype shift seen in activated T cells. Altered proliferation in cultures when combining DSCs and SRL or CsA may be of clinical importance, as stromal cells are used in trials for acute inflammation and are often used in combination with conventional immunosuppressive therapies.

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Introduction

Stromal cells are important for maintaining the structural integrity of connective tissue, but recent data indicate that these nonhematopoietic cells may also have an important role in immune responses. For example, fibroblastic reticular cells found in lymphoid tissue are instrumental in the development of adaptive immune responses [1], and intestinal stromal cells express innate immune receptors, produce various cytokines, and are important for mucosal homeostasis [2]. Furthermore, stromal cells from BM, adipose tissue, and placenta have been shown to have immunosuppressive effects. Various different immune cell types, such as T cells, macrophages, and dendritic cells are affected by stromal cell-mediated suppression. Stromal cells can also inhibit differentiation of monocytes to dendritic cells and priming of naïve T cells.

Following antigen recognition, T cells are highly dependent on cytokines for further activation, expansion, and differentiation. First described as a pivotal component of T cell growth and proliferation [3], IL-2 has been shown to regulate broadly conventional T cell differentiation and expansion [4–8]. In addition, IL-2 is important in the development and proliferation of T_{regs} [9] and in activation-induced apoptosis [10]. It is mainly produced by Th cells after antigen stimulation [11, 12], but it can also be produced by cytotoxic T cells, NK T cells [13], and dendritic cells [14].

The IL-2R is a heterotrimer, consisting of the 3 subunits IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ_c (CD132). The trimeric IL-2R with its high affinity for IL-2 is not preformed but assembled upon binding of IL-2 to IL-2Rα [15, 16]. This is followed by interaction with the β and γ_c chains [17]. This leads to a heterodimerization of the cytoplasmic domains on the β and γ_c chains, which is required for activation and downstream signaling through the Janus family tyrosine kinases, JAK1 and JAK3 [18–20]. Following activation of IL-2R, the receptor complex undergoes endocytosis, whereby the β and γ_c chains

Abbreviations: BM = bone marrow, CD = cluster of differentiation, CsA = cyclosporine A, DSC = decidual stromal cell, FOXP3 = forkhead box P3, GVHD = graft-versus-host disease, HSCT = allogeneic hematopoietic stem cell transplantation, MSC = mesenchymal stromal cell, mTOR = mechanistic target of rapamycin, PD-1 = programmed death 1, PD-L1 = programmed death ligand 1, pSTAT5 = phosphorylated STAT 5, r = recombinant, s = soluble, SRL = sirolimus, T_{reg} = regulatory T cell

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and IL-2 are degraded, and the IL-2Rα is recycled to the cell surface [21].

The placenta has emerged as a promising source of stromal cells for clinical use, as a result of their great expansion capacity and immunosuppressive effects. As the placenta is normally discarded after delivery, large quantities of cells can be isolated with limited ethical consideration. As a result of the immunoregulatory effects of stromal cells, MSCs isolated from BM and DSCs from placentas have been used to treat acute inflammatory disorders, such as GVHD, when standard treatment was unable to reverse the condition [22, 23]. Several pathways may contribute to MSC-mediated immune regulation. MSCs have shown to reduce immune activation as well as act as APCs and prime adaptive immune responses [24]. Upon priming with cytokines (e.g., IFN-γ, IL-1α/β, and/or TNF-α [25]), MSCs can up-regulate expression of IDO [26], PGE_2[27], PD-L1 [28], as well as HLA class II. Primed MSCs have been shown to promote T_{regs} [29], induce a shift in macrophages toward the M2 phenotype [30], and facilitate suppression through secretion of a variety of factors, including galectins [31], exosomes [32], and NO [33].

The immunosuppressive drugs SRL (also termed rapamycin) and CsA target the IL-2 signaling pathway by inhibition of mTOR and calcineurin, respectively. CsA inhibits *IL-2* gene transcription, whereas SRL inhibits IL-2-induced proliferation of effector T cells. Both drugs are used in transplant settings to suppress alloreactive T cells, although CsA is more commonly used to prevent acute GVHD. Currently, >500 registered trials worldwide are using stromal cells as cellular therapy for a wide range of inflammatory conditions (clinicaltrials.gov). However, details regarding synergistic effects of stromal cells and these standard immunosuppressive treatments are lacking.

We have previously shown that DSCs isolated from term fetal membranes exert their suppressive effect through cell-to-cell-mediated mechanisms and have the ability to induce T_{regs} in an allogeneic setting [34]. We have also found that activated T cells cocultured with stromal cells have higher expression of IL-2Rα than alloantigen-activated T cells in the absence of stromal cells. As IL-2 is the main signal for up-regulation of IL-2Rα [35], this indicates that DSCs may affect IL-2 production. Very little is known about the role of IL-2 in the interaction between activated T cells and stromal cells. In the present study, we investigated the ability of stromal cells to alter the production of IL-2, IL-2R expression, and reactivity to IL-2 in T cells in vitro.

MATERIALS AND METHODS

Isolation of DSCs

The placenta and fetal membranes enclose the fetus during pregnancy. The fetal membranes consist of amnion and chorion and are of fetal origin. During pregnancy, the maternal endometrium develops into the decidua and is closely associated with the fetal membranes. Therefore, DSCs can be isolated from the fetal membranes from term placentas without the need for any invasive intervention to obtain the decidual tissue.

DSCs were isolated from the term placentas of healthy donors following planned caesarian section. Stromal cells from 8 donors were isolated and used separately in the experiments. Ethical approval was obtained from the Regional Ethical Review Board (2009/418-31/4, 2010/2061-32). The method of isolation and expansion of DSCs by our group has been described in detail previously [23, 34]. In brief, the fetal membrane was dissected from the placenta and washed several times with PBS (Thermo Fisher Scientific, Waltham, MA, USA) before being incubated with trypsin/EDTA (Thermo Fisher Scientific). After trypsination, the fetal membrane was washed in complete DMEM (Thermo Fisher Scientific), containing 10% FCS (Thermo Fisher Scientific), penicillin (100 μg/ml; Thermo Fisher Scientific), and streptomycin (100 μg/ml; Thermo Fisher Scientific), and cut into 2–3 cm^2 pieces. The membrane pieces and the trypsin digests were spread out and incubated at 37°C in T175 flasks (Nunc A/S, Roskilde, Denmark). The tissue explants were removed from the flasks when colonies of fibroblast-like cells appeared. The cells were harvested with trypsin/EDTA when the cells were ~90% confluent. The DSCs were expanded further to passage 3 or 4 and thereafter, used in the experiments.

Characterization of stromal cells

Flow cytometry was used to determine cell-surface expression. The DSCs were positive for the MSC markers CD29, CD73, CD90, and CD105. The cells were also positive for HLA class I, PD-L1, PD-L2, ICAM-1, and the integrin-α subunit but negative for HLA class II. The cells were negative for the markers CD14, CD31, CD34, CD45, CD86, and CD326 [23]. Unlike BM-derived MSCs, DSCs had poor differentiation capability and did not differentiate into osteocytes or chondrocytes. The cells showed a slight differentiation to adipocytes, whereas MSCs derived from BM showed complete differentiation [34]. The maternal origin of the DSCs was determined by microsatellite polymorphism analysis [23]. The DSCs also had a normal karyotype and were able to suppress proliferation in a MLR [23].

MLRs

PBMCs were isolated with Lymphoprep (Axis-Shield, Oslo, Norway) from at least 18 buffy coat donors and used separately as responder cells in the experiments. The responder cells were stimulated with an irradiated (30 Gy) allogeneic pool of PBMCs containing cells from at least 6 donors. The responder cell and stimulator cell ratio was 1:1. In cultures in which DSCs were added, the ratio between responder cells and DSCs was 10:1. The culture was incubated for 6 d at 37°C in RPMI 1640 (HyClone; GE Healthcare Life Sciences, South Logan, UT, USA), supplemented with 5% heat-inactivated AB serum (in-house), 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2. To investigate proliferation, 20 μCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to the culture on d 5. The MLR was harvested (Harverster 96; Tomtec, Hamden, CT, USA), and radioactivity was measured on d 6 (1450 TriLux MicroBeta, PerkinElmer).

ELISA

An IL-2 ELISA was performed on culture supernatants. A kit from R&D Systems (Minneapolis, MN, USA) was used according to the manufacturer's instructions.

Multiplex assay

To detect IL-2 and sIL-2Rα in supernatants from MLR and DSC cultures, a 27-plex Luminex (Bio-Rad, Hercules, CA, USA) or 39-plex Luminex (Merck Millipore, Darmstadt, Germany) was used, according to the manufacturers' instructions.

IL-2 stimulation, [^{125}I]rIL-2 uptake, IL-2 neutralization, and coculture with CsA or SRL

To mimic a condition in the MLRs where there is an abundance of IL-2, rIL-2 (2 ng/ml; Novartis, Basel, Switzerland) was added on d 3. The concentration added was based on the median IL-2 concentrations detected in supernatants by Luminex (see RESULTS). To investigate IL-2 uptake, 3 ng/ml [^{125}I]rIL-2 (42 μCi/μg; PerkinElmer) was added on d 5. [^{125}I]rIL-2, internalized by the cells, was measured with a 1450 TriLux MicroBeta (PerkinElmer) after 30 min.

For IL-2 blocking experiments, an anti-IL-2 antibody or an isotype control antibody was added on d 0 at a concentration of 2 μg/ml (R&D Systems).

To inhibit calcineurin or mTOR, CsA (400, 200, or 50 ng/ml; Novartis) or SRL (16, 8, or 2 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), respectively, was added on d 0. The concentrations of CsA and SRL used were based on desired titrations in vivo in a stem cell transplantation setting at our center [36, 37]. Pharmacokinetics showed stable concentration of the drugs during the entire incubation period.

Flow cytometry

The following antibodies were used: V450-conjugated anti-CD3 (UCHT1), FITC-conjugated anti-CD3 (UCHT1), V500-conjugated anti-CD4 (clone RPA-T4), Alexa Fluor 700-conjugated anti-CD8 (RPA-T8), PE-cyanine 7-conjugated (RPA-T8) anti-CD8, Alexa Fluor 647-conjugated anti-STAT5 (pY694), and BV421-conjugated anti-CD25 (M-A251) and anti-CD279 (EH12.1; all from BD Biosciences, San Jose, CA, USA); PE-conjugated anti-CD122 (TU27) and anti-IL-2 (MQ1-17H12) and allophycocyanin-conjugated anti-CD132 (TUGh4; BioLegend, San Diego, CA, USA); and anti-FOXP3 FITC (236A/E7; eBioscience, San Diego, CA, USA). To detect pSTAT5, the cells were stimulated with 0.01–100 ng/ml rIL-2 (Novartis) or rIL-15 (PeproTech, Rocky Hill, NJ, USA) or 0.01–10 ng/ml rIL-7 (R&D Systems) for 5–20 min before fixation. For fixation and permeabilization of the cells for pSTAT5 staining, the Perm Buffer III from BD Biosciences was used. For IL-2 staining, the BD Cytofix/Cytoperm kit (BD Biosciences) was used, according to the manufacturer's instructions. For IL-2 stimulation, optimal stimulation conditions were set at 15 min incubation with 100 ng/ml rIL-2. Data were collected on a FACSCanto research use only system (BD Biosciences) and were analyzed using FlowJo Vx software (Treestar, Ashland, OR, USA). The cells (see Fig. 3F) were starved overnight in serum-free medium before stimulation with IL-2, -7, or -15.

IL-2 isoform distribution

The *IL-2* gene has regulation by alternative splicing at the pre-mRNA level, which generates 3 possible transcript variants: the IL-2 canonical mRNA sequence and transcripts with either exon 2 (IL-2δ2) or exon 3 (IL-2δ3) missing from the canonical sequence [38, 39]. In the MLR situation and the MLR + DSC situation ($n = 3$), the relative distribution of the *IL-2* gene-specific PCR product was investigated by capillary electrophoresis fragment analysis on an Agilent 2100 bioanalyzer instrument (Agilent, Kista, Sweden) using the DNA 1000 Kit, according to the instructions of the supplier. IL-2 transcript-specific primers covering all 3 alternatively spliced *IL-2* gene products (IL-7c, IL-2δ2, and IL-2δ3) were purchased from Thermo Fisher Scientific. The sequences of the primers have been given previously [38]. RNA and cDNA were obtained using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) and the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), respectively. PCR was performed on a PTC-200 thermal cycler using Taq polymerase (Thermo Fisher Scientific). The IL-2 isoform amplification data were obtained for each sample, as described earlier [40]. Time-corrected area under the peak of a particular isoform in the sample was used to obtain the relative distribution of IL-2 isoforms for different conditions.

Statistical analysis

Statistical tests were performed using Prism software (GraphPad Software, La Jolla, CA, USA) to identify trends and statistically significant outcomes. We used the Wilcoxon matched-pair signed-rank test for matched samples and the Mann-Whitney test for unmatched samples. Friedman's test followed by Dunn's post-test was used for multiple comparisons. All outcomes with $P < 0.05$ were considered statistically significant. The notations used for significance were described in figure legends.

RESULTS

Stromal cell-augmented production of IL-2 in activated T cells

Initial kinetic experiments showed that IL-2 was produced in MLRs but that addition of DSCs markedly increased the IL-2 levels during the entire activation phase (**Fig. 1A**). We further found that IL-2 was not produced from DSCs cultured alone for 48 h (Fig. 1B), indicating that IL-2 was derived from responding PBMCs in MLRs. When the DSCs were added in a Transwell, preventing cell-to-cell contact between the DSCs and the MLRs, the IL-2 production was significantly reduced compared with the condition that allowed cell contact between MLRs and DSCs (Fig. 1C). A representative flow cytometry analysis of T cells on d 3 indicated that the production of IL-2, at least in part, originated from T cells (Fig. 1D). Measurement of sIL-2Rα revealed that there was no difference between MLRs cultured with or without DSCs after 6 d of culture ($n = 5$; Fig. 1E). Purified T cells stimulated with anti-CD3- and anti-CD28-covered beads produced high IL-2 concentrations in both the absence of DSCs [median 2.7 ng/ml (range 2.1–4.7 ng/ml)] and in the presence of DSCs [3.1 ng/ml (range 2.0–3.3 ng/ml; d 3, $n = 4$, data not shown].

IL-2 isoform distribution

IL-2 can be alternatively spliced at the pre-mRNA level, which can generate either the canonical IL-2 protein sequence from all 4 exons or the 2 alternative isoforms, IL-2δ2 and IL-2δ3. The latter 2 forms of the protein have been described to be competitive inhibitors of the canonical IL-2 protein and can inhibit proliferative responses in humans [38]. Therefore, we examined the expression of different IL-2 isoforms in MLR cultures in the absence or presence of DSCs. The full-length canonical IL-2 mRNA constituted the most predominant IL-2 transcript in all of the samples under investigation, representing >96% of the total *IL-2* gene product in the samples (Supplemental Fig. 1A). The remainder of the IL-2 transcripts included IL-2δ2 isoform-specific transcripts, and we did not find detectable amounts of IL-2δ3 transcripts.

IL-2R subunits on T cells are affected by the presence of stromal cells

As T cell activation and IL-2 regulate the expression of IL-2R subunits, the expression of the receptor subunits present on the surface of T cells was determined in the allogeneic setting. Allogeneic activation increased the expression of CD25 and CD122 on both $CD3^+CD4^+$ T cells (**Fig. 2A–C**) and $CD3^+CD8^+$ T cells. CD132 was constitutively expressed on T cells (Fig. 2D). The presence of stromal cells did not alter the frequency of cells that expressed CD25 (Fig. 2B). However, the $CD4^+$ T cells that were $CD25^+$ had an increased intensity of expression of CD25 (Fig. 2E). This was also consistent for the $CD8^+$ T cell compartment. As opposed to CD25, the signaling CD122 subunit was significantly reduced in both frequency and intensity of expression in activated $CD4^+$ and $CD8^+$ T cells when in contact with stromal cells (Fig. 2C and E). This finding also applied to CD132, even though the frequency of expression remained high when the activated T cells were in contact with stromal cells (Fig. 2D). Expression of the entire IL-2R complex (CD25, CD122, and CD132) was significantly reduced for both $CD4^+$ and $CD8^+$ T cells when DSCs were added to the MLR (Fig. 2F and G).

pSTAT5

STAT5 is a major downstream regulator of IL-2 activity in T cells. As a result of the altered expression of CD25, CD132, and CD122,

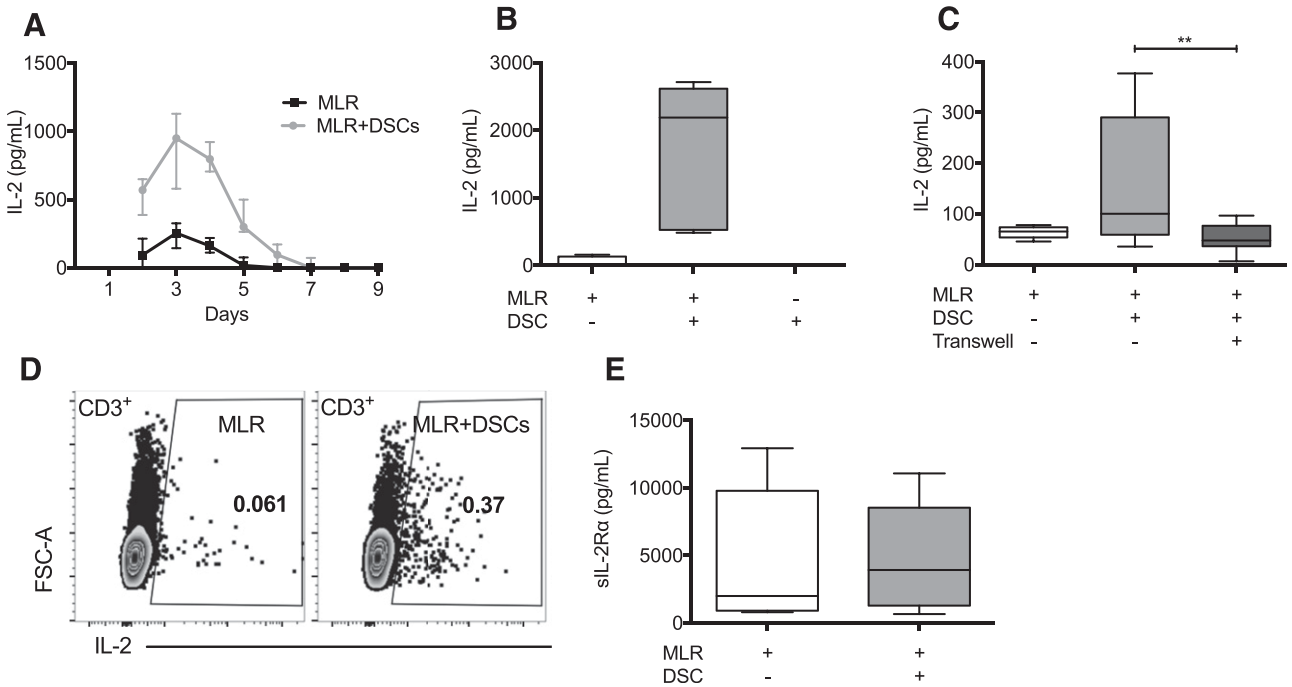

Figure 1. DSCs promote IL-2 production. (A) Kinetic experiments showed that the concentration of IL-2 peaked on d 3 of incubation ($n = 4$). (B) Concentration of IL-2 measured on d 5 by Luminex in culture supernatants from MLRs in the presence or absence of DSCs or DSCs alone ($n = 5$). (C) DSCs were added to MLRs, either directly or in a Transwell that prevented cell-to-cell contact between the alloantigen-stimulated PBMCs and the DSCs ($n = 8$). The concentration of IL-2 in supernatants was then determined with ELISA. (D) A representative flow cytometry experiment showing that $CD3^+$ T cells produce IL-2 when DSCs are added to MLR cultures ($n = 3$). FSC-A, Forward-scatter-area. (E) The concentration of sIL-2Rα was determined in culture supernatants from MLRs in the presence or absence of DSCs ($n = 5$). $^{**}P < 0.01$–0.001.

we determined the ability of DSC-exposed T cells to respond to IL-2 by examining pSTAT5. To measure maximum responsiveness to IL-2, the MLR cultures were stimulated with rIL-2 (100 ng/ml) on d 6. The proportion of $CD8^+$ T cells that were $pSTAT5^+$ was lower when stromal cells were added to the culture (**Fig. 3A** and **C**). Additionally, the intensity of pSTAT5 expression in the $pSTAT5^+$ population was significantly lower when activated $CD4^+$ T cells, $CD8^+$ T cells, and T_{regs} were in contact with stromal cells (Fig. 3D). T_{regs} are highly dependent on IL-2, and we found that the majority of T_{regs} was $pSTAT5^+$ (Fig. 3B and C). However, the intensity of $pSTAT5^+$ T_{regs} was lower in MLRs cultured with DSCs (Fig. 3D).

Despite there being a higher concentration of IL-2 in the stromal cell setting, the reduced expression of the high-affinity IL-2R (Fig. 2G) and pSTAT5 indicates an impaired ability to bind and internalize IL-2. To test this hypothesis, [^{125}I]rIL-2 was added at the end of the incubation (d 6) and was used to measure IL-2 uptake in the different conditions. The [^{125}I]rIL-2 uptake was significantly reduced under conditions of MLR + DSCs compared with control MLR (Fig. 3E).

Other cytokine receptors also use the subunits CD122 and CD132 and phosphorylate STAT5, including IL-7 (CD132) and IL-15 (CD122 and CD132). To examine how T cells that had been cultured with DSCs react to IL-7 and IL-15, pSTAT5 activation was analyzed in response to various concentrations of these cytokines after overnight starvation of the cells. MLRs with DSCs responded with less pSTAT5 expression when stimulated with high doses of IL-2 (Fig. 3F). Interestingly, T cells in MLRs that had been cultured with DSCs expressed less pSTAT5 when stimulated with IL-15 (1 ng/ml) but not when stimulated with IL-7 (Fig. 3F).

To determine further whether soluble factors in the medium from MLR cultures (with or without DSCs) could activate STAT5, we stimulated freshly isolated T cells with conditioned medium from MLR cultures and known concentrations of rIL-2. Conditioned medium from both MLRs and MLR + DSCs was able to phosphorylate STAT5 in both $CD4^+$ and $CD8^+$ T cells (Supplemental Fig. 1B), further indicating that no competitive mediator was produced by MLR + DSC cultures that could prevent activation of STAT5.

Effect of rIL-2 and DSC on IL-2R expression

To investigate whether the high level of IL-2 in allo-stimulated T cells cocultured with DSCs was the cause of the depletion of the high-affinity IL-2R, rIL-2 (2 ng/ml) was added on d 3 of culture. The concentration was based on the median levels of IL-2 detected in the supernatants, where DSCs were added to MLRs (Fig. 1B). The frequency and intensity of expression of CD122 were reduced when IL-2 was added, which was similar to the condition where stromal cells were added to the culture (**Fig. 4A** and **B**). In contrast to DSCs, rIL-2 did not alter the proportion or intensity of CD132 expression in T cells. Thus, addition of rIL-2 could partly mimic the effect of DSCs on IL-2R expression, but DSCs appeared to have a more extensive effect.

Abundance of IL-2 and reduction of receptor expression may indicate exhaustion. Therefore, T cells were stained with anti-PD-1

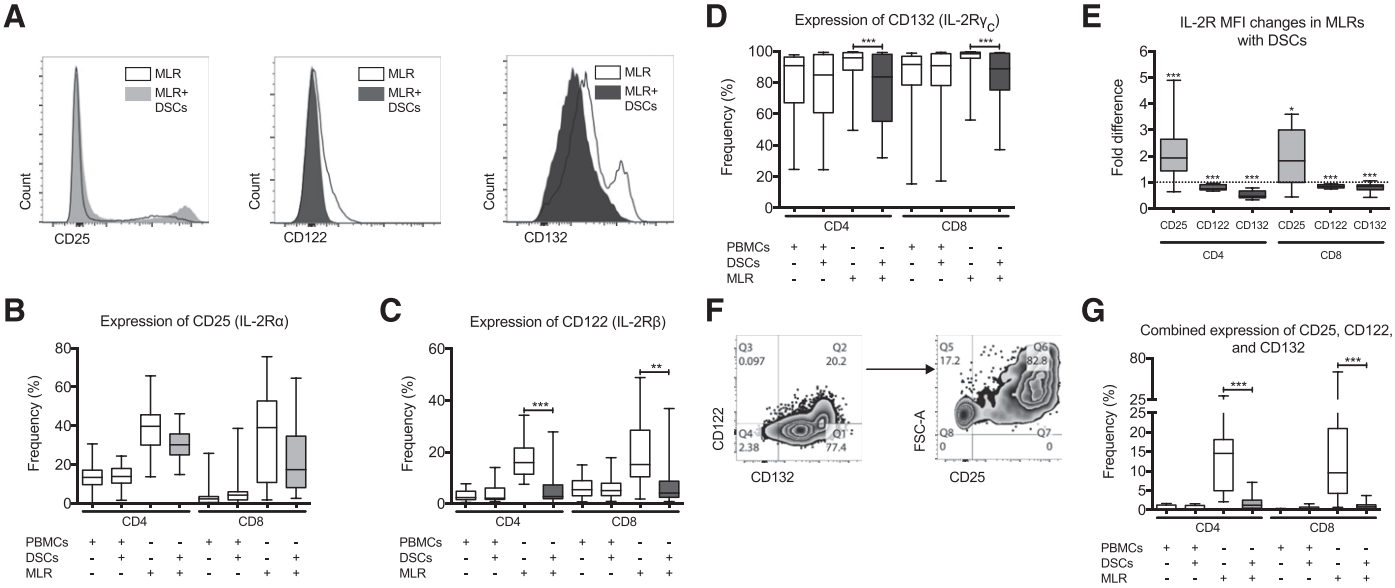

Figure 2. DSCs down-regulate the expression of the IL-2R complex on T cells. Flow cytometric staining of the 3 IL-2R subunits on T cells on d 6 of incubation in MLRs in the presence of absence of DSCs ($n = 17$). (A) Representative experiments showing the expression of CD25, CD122, and CD132 in the MLR (white) and MLR + DSC (gray, left; black, middle and right) condition in $CD4^+$ T cells. (B–D) The frequency of $CD4^+$ and $CD8^+$ T cells that were positive for CD25, CD122, and CD132, respectively, in the presence or absence of DSCs. (E) The fold difference in intensity of expression of the IL-2R subunits (MLR + DSCs/MLR). The frequency of CD25 expression was not altered when DSCs were added to MLR cultures (B), but the intensity of expression in $CD25^+$ cells was highly increased (E). CD122 and CD132 were significantly reduced in both frequency (C and D) and intensity (E) of expression. MFI, Median fluorescence intensity. (F) The gating strategy for investigation of cells with combined expression of all 3 subunits. (G) The frequency of $CD3^+CD4^+$ lymphocytes that expressed all 3 receptor subunits was almost completely abolished when DSCs were added to the culture ($n = 15$). $^*P \leq 0.05$–0.01, $^{**}P < 0.01$–0.001, and $^{***}P < 0.001$.

and anti-CD95 antibodies (Fig. 4C and D). No difference between the MLR and MLR cocultured with DSCs was detected, but CD95 was expressed in a larger proportion of the T cells when IL-2 was added to the MLR.

Stromal cells in combination with substances targeting the IL-2 pathway

Stromal cells are increasingly being used as cellular therapy to treat severe inflammatory conditions, such as GVHD. The intervention is often in addition to standard treatment, which includes a number of immunosuppressive drugs. SRL and CsA both target the IL-2 pathway and are used separately in this setting. Investigation of the combined effect on alloreactive PBMCs when both stromal cells and these substances are present might therefore be of clinical importance. DSCs and physiologically relevant concentrations of, SRL (2 ng/ml) and CsA (50 ng/ml) were independently capable of effectively suppressing proliferation of an alloreaction in vitro (**Fig. 5A**). However, the combination of DSCs and SRL (2 and 16 ng/ml) reduced the antiproliferative effect in the MLR cultures compared with SRL alone (Fig. 5B). The presence of DSCs also significantly reduced the suppressive effect of CsA at high doses (400 ng/ml), and there was a trend at 200 ng/ml ($P = 0.07$; Fig. 5C). As expected, the proliferation in MLRs was significantly reduced when a neutralizing antibody to IL-2 was added, but the blocking of IL-2 in the MLRs with DSCs did not affect the proliferative response any further (Fig. 5D). Altogether, this indicates that DSCs interfere with the immunosuppressive effect of drugs that target the IL-2 pathway.

DISCUSSION

IL-2 was originally thought to play an important role in activation and proliferation of effector T cells, but it has become apparent that IL-2 is also a negative regulator of immune responses. For example, it can promote activation-induced cell death in effector T cells [41], and it is important for T_{reg} survival [42]. In the present study, we investigated immune modulation of activated lymphocytes by stromal cells. Interestingly, we found that stromal cells induced high levels of IL-2 from alloantigen-stimulated T cells (Fig. 1A–C). As a consequence, the capacity to form the high-affinity IL-2R on T cells was reduced significantly (Fig. 2). The low expression of IL-2R suggests an impaired ability to respond to IL-2. As hypothesized, the T cells were found to have both reduced uptake of IL-2 and reduced pSTAT5 (Fig. 3). The depletion of the IL-2Rβ on T cells observed in cultures with stromal cells could, in part, be reproduced by the addition of rIL-2 (Fig. 4A). Lastly, we found that stromal cells could affect the suppressive capacity of drugs that inhibit the IL-2 pathway in cultures with alloantigen-activated PBMCs (Fig. 5).

There have been a number of publications presenting results regarding altered IL-2 production by stromal cells isolated from various sources, including BM and amniotic membranes [43–46]. However, to our knowledge, there have been no studies that have further elucidated the role of IL-2 in stromal cell-mediated immune suppression, despite elevated levels both in vitro and in vivo, indicating its importance in the clinical trials where stromal cells are used. Both stromal cells and rIL-2 have separately been widely used clinically in efforts to attenuate acute or chronic

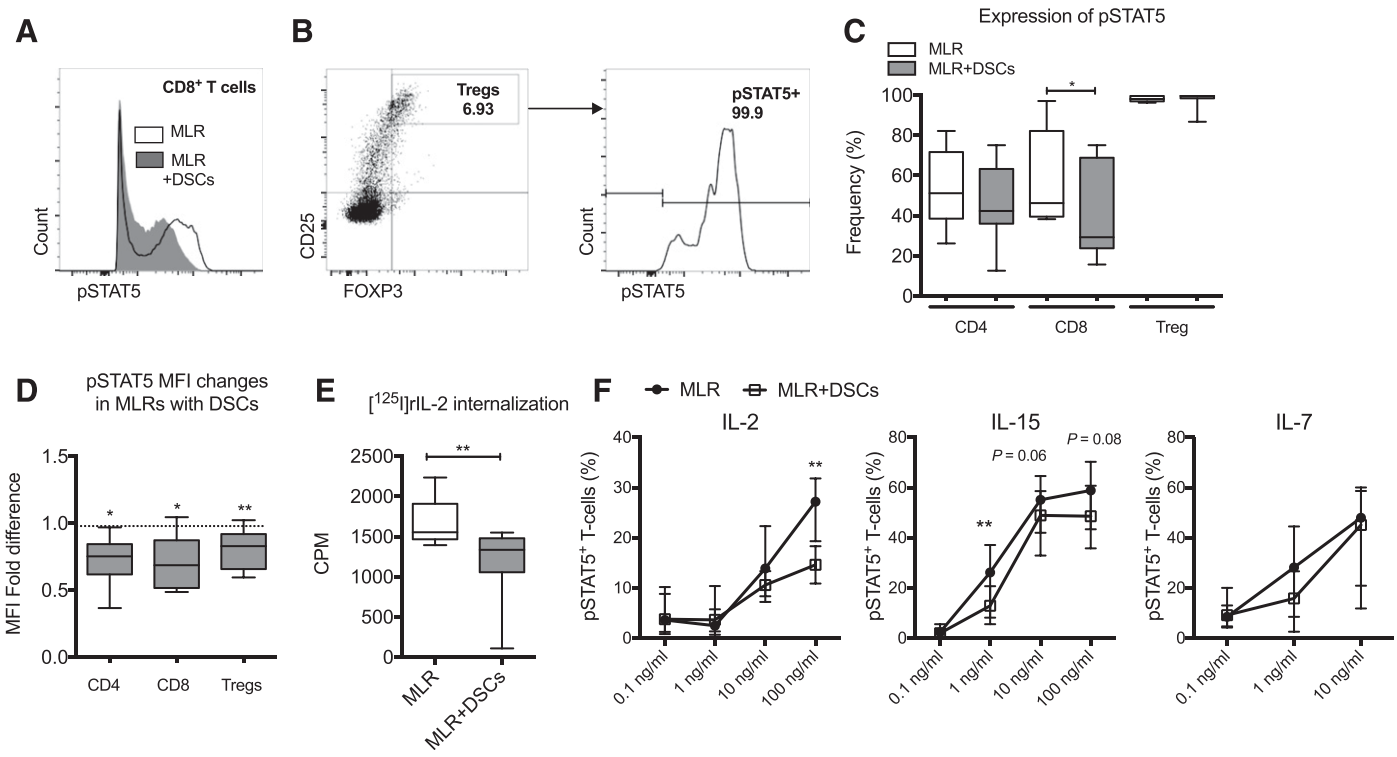

Figure 3. DSCs decrease pSTAT5 and IL-2 internalization in T cells. (A–C) The frequency of pSTAT5 expression in $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD25^{high}FOXP3^+$ T_{regs} in MLRs cultured in the presence or absence of DSCs ($n = 7$). pSTAT5 expression was analyzed after addition of 100 ng/ml rIL-2 on d 6. (D) DSCs significantly reduced pSTAT MFI in T cells. (E) The ability to internalize IL-2 in MLRs and MLRs cocultured with DSCs was determined by addition of [^{125}I]rIL-2 to the cultures ($n = 11$). DSCs significantly reduced the internalization of IL-2. (F) MLR cultures were starved overnight and stimulated with IL-2, IL-15, or IL-7 to study expression of pSTAT5 in $CD3^+$ T cells ($n = 12$). The presence of DSCs decreased the expression of pSTAT5 after IL-2 and IL-15 stimulation. *$P \leq 0.05$–0.01 and **$P < 0.01$–0.001.

inflammation. We have previously shown that the intensity of CD25 expression is altered significantly when activated T cells are in contact with stromal cells, and contact between DSCs and responding cells was found to be a necessity for suppression [34]. Consistent with these observations, we found in this study that IL-2 accumulation in the supernatant only occurs when contact is allowed (Fig. 1C). This is also in line with the increased frequency of T_{regs} that is observed when stromal cells are close to alloantigen-activated PBMCs [34, 47]. The DSCs used in this study came from the decidua parietalis. Interestingly, expression of CD25 has been reported to be higher in T cells in decidua parietalis compared with decidua basalis and in peripheral blood of pregnant women [48].

BM-derived MSCs have been reported to reduce the frequency of $CD25^+$ T cells in PHA-stimulated PBMCs [49]. We found no significant alterations in the frequency of T cells that were positive for CD25 in MLRs, but the intensity of expression of CD25 was increased in the presence of DSCs (Fig. 2A and B). CD25 does not contain any signaling domains but rather, facilitates sensitivity to IL-2, whereas CD122 and CD132 are necessary for effective signal transduction [50]. To our knowledge, CD132 and CD122 has not been investigated in a setting with stromal cells. Therefore, it is interesting that the expression of CD122 was almost completely abolished and that CD132 expression was reduced following coculture with stromal cells (Fig. 2A, C, and D). The results indicate that CD122 may be the limiting IL-2R subunit in this setting. In line with this hypothesis, MLR + DSCs with the addition of IL-15, a cytokine that uses CD122 for downstream signaling, led to a lowered STAT5 activation (Fig. 3F). In contrast, the response to IL-7 was not significantly affected by stromal cells. The signaling of this cytokine is not dependent on CD122, as the IL-7R comprises the subunits CD127 and CD132. CD122 may be down-regulated as a result of the high production of IL-2, which is supported by the results of others [51], as well as our own experiments, as addition of rIL-2 to the MLRs resulted in reduced expression of CD122 (Fig. 4A). A study by Hémar et al. [21] suggested that CD122 and CD132 are internalized and degraded, whereas CD25 may be recycled. Domains that regulate degradation of the IL-2R have been found on CD132 [52] but not on CD122 [53]. This may have implications for the feedback for CD122 surface expression. As CD132 is expressed more abundantly, simultaneous degradation of CD132 and CD122, regulated by CD132, may cause depletion of CD122, as seen in this study. The results in this study indicate that CD122 is the limiting factor in the expression of the full high-affinity IL-2R and perhaps the IL-15R in this setting.

We did not detect a change in the frequencies of cells expressing the exhaustion markers PD-1 or CD95 in MLRs with DSCs (Fig. 4C and D). However, CD95 expression was increased when rIL-2 was added to the MLRs (Fig. 4D). This

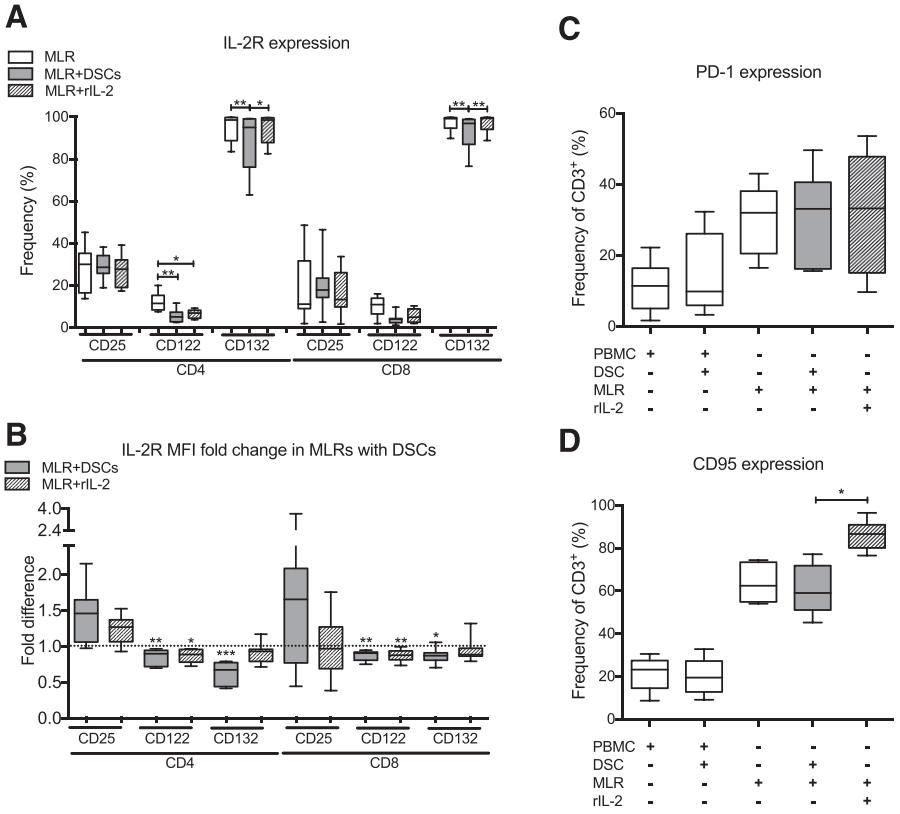

Figure 4. The effect of rIL-2 on CD25, CD122, and CD132 expression. (A and B) Addition of rIL-2 (2 ng/ml) to MLR cultures on d 3 to simulate the increased IL-2 production under conditions where DSCs are present. The frequency and intensity of expression of CD122 were reduced in $CD4^+$ T cells and $CD8^+$ T cells when rIL-2 was added to the MLRs ($n = 8$). The expression of CD25 and CD132 was not significantly affected by the addition of rIL-2 (B). (C) Expression of PD-1 on T cells showed no difference between the conditions with stimulated PBMCs ($n = 11$). (D) Expression of CD95 was increased when rIL-2 at 2 ng/ml was added to the MLR culture compared with MLRs with DSCs ($n = 9$). $*P \leq 0.05$–0.01, $**P < 0.01$–0.001, and $***P < 0.001$.

finding is in line with findings by Schmitz et al. [54]. Therefore, the DSC-induced IL-2 was unable to increase the level of exhaustion in the cultures, whereas rIL-2 increased expression of CD95. Conclusively, the IL-2R depletion and reduced pSTAT5 in the MLR + DSC setting were not a consequence of increased exhaustion based on PD-1 and CD95 expression.

To correlate the reduced pSTAT5, despite an excess of IL-2 in the culture supernatants, [^{125}I]rIL-2 was added to measure IL-2 internalization. We observed that the ability of alloantigen-stimulated PBMCs to take up IL-2 was reduced significantly when in contact with stromal cells (Fig. 3E). We interpret these findings as follows: the increased IL-2 production is followed by depletion of the high-affinity IL-2R, which in turn, reduces the responsiveness to IL-2 and expansion capability of T cells.

To examine whether stromal cells promote production of any factors that could mediate competitive binding to the IL-2R, we first measured alternative isoforms of IL-2 that have been implicated in inhibiting T cell proliferation [38]. There were no findings to suggest that stromal cells affect alternative splicing of IL-2. Furthermore, supernatants from MLRs cultured with DSCs were able to induce pSTAT5 efficiently in T cells (Supplemental Fig. 1B). Additionally, there was no observable difference between the MLRs and MLR + DSCs regarding sIL-2Rα in the culture supernatant (Fig. 1E), and overnight starvation of MLRs in serum-free medium still affected responsiveness to IL-2 (Fig. 3F). Combined, these data suggest that soluble factors do not influence the induced IL-2 insensitivity in T cells.

We have not been able to find the underlying reason for the stromal cell-induced increase in IL-2 production. One possible explanation is that IL-2 accumulates in the supernatant as a result of the fact that DSCs inhibit proliferation of T cells and therefore, prevent consumption of IL-2. However, flow cytometric intracellular staining of IL-2 indicated that a higher proportion of T cells produced IL-2 when DSCs were present (Fig. 1D). Allogeneic DSCs have a low immunostimulatory capacity (Fig. 5A) and low expression of HLA-DR [28], and it is unlikely that the HLA disparity of the DSCs would increase IL-2 production, as the number of stimulator PBMCs in our experiments is in a 10:1 ratio compared with the DSCs. The stimulator PBMC pool also contains alloantigens from at least 6 different donors. The identification of the antigen(s) on DSCs that cause the high IL-2 concentrations can provide insight regarding feto-maternal tolerance and induction of T_{regs}.

It has been shown previously that stromal cells are able to function as APCs and can prime adaptive immune responses [24], despite a lack of costimulatory molecules, such as CD80 and CD86. The primary objective in the present study was to investigate the allogeneic setting, which means that the cultures also contain APCs. Consequently, there is a possibility of indirect allorecognition of DSC-derived peptides that may increase IL-2 production. In our hands, IL-2 did not increase in supernatants when DSCs were added to cultures with purified T cells stimulated with anti-CD3- and anti-CD28-covered beads. The IL-2 concentration in the condition without DSCs was high compared with the allogeneic setting, which may mask a potential effect on IL-2 production by DSC. The titration of the bead:responder cell ratio may show if DSCs are able to increase IL-2 production independently of APCs. It also has to be taken

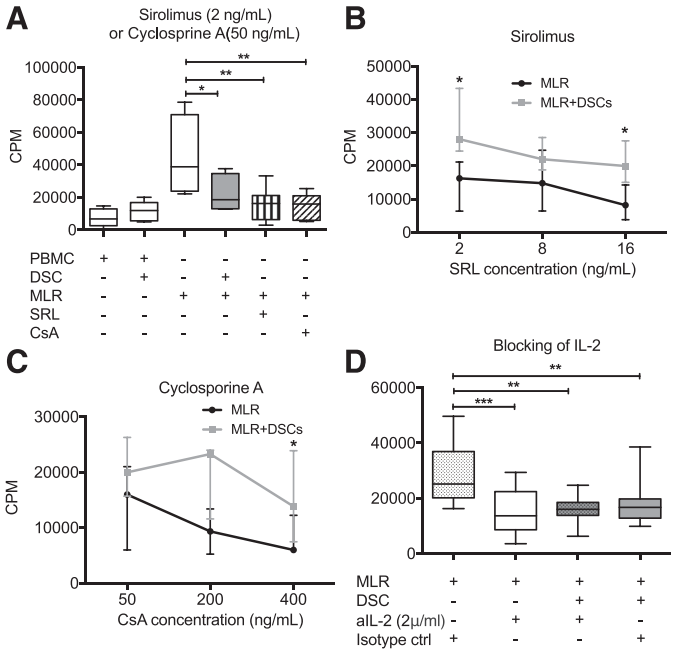

Figure 5. DSCs may interfere with drugs affecting the IL-2 pathway in vitro. (A–C) DSCs, SRL, or CsA was added to MLR cultures, and proliferation was measured on d 6 ($n = 7$). (A) DSCs, SRL, and CsA separately reduced the proliferation of the MLR, but addition of both DSCs and SRL to MLRs diminished the antiproliferative effect (B). DSCs also affected suppression by CsA (C). (D) The blocking of IL-2 with a neutralizing anti-IL-2 (aIL-2) antibody decreased the suppressive effect in MLRs but did not affect the proliferation in the MLR + DSC setting ($n = 16$). ctrl, Control. *$P \leq 0.05$–0.01, **$P < 0.01$–0.001, and ***$P < 0.001$.

into consideration that this is a short stimulation compared with the MLR, which may change the IL-2R kinetics and exhaustion profile of the T cells. This requires further investigation.

In the past decade, stromal cells have emerged as an alternative cellular therapy for a wide range of diseases, including GVHD [55], Crohn's disease [56], multiple sclerosis [57], and liver cirrhosis [58]. Stromal cells are used in HSCT therapy, as a result of presumed immune-modulatory effects [59]. In the HSCT setting, CsA and SRL are used as conventional therapy for prevention and treatment of GVHD. In vitro, the addition of rIL-2 can reverse the effect of CsA, although in a dose-dependent manner. High doses of CsA inhibit MLR proliferation, even with addition of rIL-2 [60]. In our setting, addition of stromal cells affected the inhibitory effect of CsA and SRL (Fig. 5). This suggests that functional IL-2 signal transduction may be needed for optimal stromal cell-induced suppression in vitro. This may be of clinical importance when stromal cells are used in combination with SRL and/or CsA.

Others have investigated the combined effect of stromal cells and immunosuppressive drugs. For instance, stromal cells pretreated with SRL for a short time showed an additive immunosuppressive effect in vitro and inhibited onset of GVHD in a murine model [61]. This correlation was not observed with cytostatic drugs. Other studies have suggested that there may be an additive immunoregulatory effect when stromal cells are combined with immunosuppressive drugs [49, 62, 63]. However, there are some conflicting results in line with our findings, suggesting that MSCs combined with SRL or CsA increase proliferation in MLRs [64, 65]. This could not be seen when MSCs were combined with agents that do not target the IL-2 pathway, such as mycophenolic acid. Even though there may be disparities, our observations appear to be comparable with data using stromal cells from other sources.

One aspect to consider in this setting is that DSCs may require priming with cytokines present in allogeneic cultures to be suppressive (e.g., IFN-γ, IL-1α/β, and/or TNF-α). Addition of CsA or SRL not only limits IL-2 production but also, the proinflammatory milieu in this setting. DSC expression of PD-L1 can be affected by IFN-γ [28]. PD-L1 is one of several factors that may contribute to DSC-mediated suppression [34]. In the same report, our group also found that neutralization of IFN-γ inhibits the antiproliferative potential of DSCs. The concept that immunosuppressive drugs also affect DSC immunomodulatory properties is intriguing. If the DSCs are not exposed to inflammation as a result of CsA or SRL, then the balance between inhibitory and activating markers may be skewed, and the stromal cells may augment T cell activation instead of promoting inhibition.

DSCs may have a supportive effect of lymphocytes in a quiescent state, and Blanco et al. [66] suggest that DSCs may rescue lymphocytes from apoptosis. Hence, the decreased antiproliferative effect in Fig. 5B and C could be a result of the DSCs increased survival of lymphocytes in the presence of CsA or SRL. The DSCs could also be absorbing the immunosuppressive drugs, as indicated by Girdlestone et al. [61], thus limiting the bioavailability of the drugs. Another potential interaction is that the IL-2 inhibiting/inducing effects of CsA and DSCs could balance each other.

Another study has shown that IL-2 levels increased in plasma following treatment with BM-derived MSCs [45]. IL-2 concentrations in vivo may be subject to bias as a result of tapering of immunosuppressive drugs and increased immune reconstitution following HSCT, which makes it difficult to know the significance of our in vitro findings in a patient setting. However, it is important to study the interaction between experimental cellular treatments and conventional treatments.

Both SRL and stromal cells may have positive effects on the generation of T_{regs} [34, 67], but T_{regs} are not required for stromal cell-induced immune suppression [68]. T_{regs} are highly dependent on IL-2 for expansion, and low-dose IL-2 treatment is beneficial for homeostatic T_{reg} expansion in patients [69]. Other studies have suggested an increase of T_{regs} up to 90 d after stromal cell treatment [45].

DSCs and bone marrow-derived MSCs are phenotypically and also functionally similar in terms of their immunosuppressive capacities, but there are several differences. MSCs can differentiate into adipocytes, osteoblasts, and chondrocytes, whereas DSCs lack this capacity [34], which possibly is because they are more differentiated stromal cells. DSCs also express higher levels of integrins compared with bone marrow MSCs [28], which may be an advantage for their abilities to reach damaged tissues. Only a randomized study with DSCs or BM-derived MSCs would elucidate which cell type is better suited to treat inflammatory conditions, such as GVHD.

To conclude, DSC-induced down-regulation of the IL-2R can reduce further activation and proliferation of T cells. Along with other already described suppressive mediators, such as IDO and PGE_2, the present study identified a new potential mechanism by which stromal cells exert their inhibitory effect on T cells. It remains to be determined if this is of importance in an in vivo setting, for instance, at the feto-maternal interface and in the tumor microenvironment, where stromal cells are abundant and modulate T cell responses. Our data also suggest that the interaction between stromal cells and immunosuppressive drugs should be investigated further.

AUTHORSHIP

T.E. and H.K. conceived of and designed the study. M.S. and O.R. contributed to the design. T.E., M.S., L.V., C.B., A.S., L.R., S.N., and H.K. acquired, analyzed, and interpreted data. T.E. and H.K. wrote the manuscript with input from M.S. and L.R. All authors reviewed and approved the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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