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Human Embryonic Stem Cells Suppress T Cell Responses via Arginase I-Dependent Mechanism

Nurit Yachimovich-Cohen,* Sharona Even-Ram,* Yoel Shufaro,*‡ Jacob Rachmilewitz,‡ and Benjamin Reubinoff*†

Human embryonic stem cells (hESCs) can proliferate extensively in culture and give rise to progeny of the three germ layers. Several reports suggested that mouse and hESCs may attenuate immune responses. In this study, we focused on the mechanism by which hESCs inhibit T cell responses. Using coculture experiments, we demonstrate that hESCs inhibit cytokine secretion and T cell proliferation in response to potent T cell activators. Furthermore, we show that hESCs downmodulate the TCR-associated CD3-ζ chain. These effects are maintained when hESCs are replaced by their conditioned media and can be restored by the addition of L-arginine to hESC-conditioned media or by treatment of hESCs with a specific arginase inhibitor. Moreover, we show arginase-I expression and activity in hESCs. We further demonstrate that mouse ESCs (mESCs) similarly inhibit T cell activation via arginase I, suggesting an evolutionary conserved mechanism of T cell suppression by ESCs. In addition, we demonstrate that arginase I expression is not limited to ESCs in culture, but can also be detected in the inner cell mass and the trophectoderm of preimplantation mouse embryos and hESC-derived trophectoderm cells. Finally, T cells infiltrating ESC-derived teratomas have significantly lower levels of CD3-ζ chain. Collectively, the data indicate a role for ESC-arginase I activity in the attenuation of T cell activation. The Journal of Immunology, 2010, 184: 1300–1308.

Human embryonic stem cells (hESCs) can proliferate extensively in culture and give rise to progeny of the three germ layers. These cells can be propagated indefinitely in the undifferentiated state and can be induced to differentiate in vitro into many cell types representing all three embryonic germ layers (1, 2).

Several reports suggest that hESCs have immune-privilege properties and can inhibit an immune response (3–6). In vivo experiments showed that rat ESCs were not rejected when transplanted into the liver portal vein of allogeneic recipients, but rather induced transplantation tolerance (3). Lack of rejection was also reported after injecting mESCs into the supraorbital vein of allogeneic recipients (4). In both the rat and mouse in vivo experiments, FasL expression by the ESC was suggested to mediate the immunosuppressive activity via donor-specific T lymphocyte apoptosis in the recipients’ thymus (3, 4). Graft acceptance was also observed with hESCs 2 d after xenograft transplantation to mice muscle (5), and 3 wk after transplantation under the kidney capsule of an allogeneic mouse model (6). Nevertheless, other reports demonstrated rejection after transplantation of mouse and hESCs. Following allogeneic engraftment of mESCs into the portal vein, initial spreading throughout the liver was shown, but later stage analysis could not trace the cells, suggesting their rejection (7). In addition, following xenogeneic transplantation of hESCs to the gastrocnemius muscles of mice, an adaptive donor-specific immune response developed (8). It should be noted that there are significant differences between the studies regarding issues such as the type of transplantation (allo- or xenogeneic), the number of transplanted cells, the method and location of engraftment, and the length of follow-up. These differences may possibly contribute to the variable results.

In vitro experimental systems support the notion that both hESCs and mESCs have immunosuppressive properties. Accordingly, hESCs inhibit proliferation of PBMCs (5, 9), express low levels of class I MHC molecules, do not express class II MHC, and do not induce NK cell lysis activity (5, 7, 10).

In the current study, we explored the mechanism underlying the immunoregulatory properties of hESCs, focusing on their ability to inhibit T cell activation. We uncovered a mechanism by which hESCs inhibit T cell activation. Using coculture systems of hESC with activated PBMCs, we demonstrate for the first time that hESCs inhibit cytokine secretion and T cell proliferation via their arginase I activity and L-arginine consumption, which leads to reduced expression of the TCR-ζ chain. This well-documented mechanism used by tumors to evade local T cell responses in vivo (11–13) may potentially also serve hESCs to locally inhibit T cell activity. In support of this notion, we demonstrate the downregulation of the CD3-ζ chain in T cells infiltrating teratomas generated by transplantation of mESCs to allogeneic immunocompetent mice. We further show that arginase I is expressed by the preimplantation embryo, suggesting its potential role in attenuating maternal T cell activity in the embryo’s microenvironment.

Materials and Methods

Cell maintenance and preparation of conditioned medium

HES-1 and HES-2 cell lines were derived and characterized previously (2). H7 cell line was obtained from The National Stem Cell Bank (Madison, Wisconsin). hESCs were maintained on human foreskin fibroblasts (a gift from...
M. Revel; Rehovot, Israel) treated for 2.5 h with 10 μg/ml mitomycin-C (Sigma-Aldrich, St. Louis, MO), and plated in gelatin-coated 9.5-cm2 well plates (Nunc, Glostrup, Denmark; 3 x 104 feeders/well). hESCs were routinely cultured in 85% knockout DMEM supplemented with 14% knockout serum replacement, 1 mM t-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acids (10 mM of each amino acid), 100 μM β-mercaptoethanol (Life Technologies, Carlsbad, CA) and 4-mg/ml basic fibroblast growth factor (Cytolab, Rehovot, Israel). The medium was changed every day. The cells were passaged weekly as small clusters following digestion with collagenase type IV (1 mg/ml; 200 U/ml) for 1 h. For FACS analysis, urea quantification assay, and immunofluorescence staining, single cells were obtained by washing hESC colonies twice with PBS without calcium and magnesium, followed by 10 min incubation in 0.05% EDTA in PBS (Biological Industries, Beit-Haemek, Israel). The cells were then triturated to single cells and separated from feeder cells. The cells were used to propagate the foreskin feeder cells and adult fibroblasts from skin biopsies of healthy donors (a gift from H. Ben-Bassat, Jerusalem, Israel). It was composed of high-glucose DMEM (Life Technologies) supplemented with 10% FCS (Biological Industries), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies).

E14 mESCs (a gift from H. Cedar, Jerusalem, Israel) and C57BL/6 mESCs (a gift from D. Eilat, Jerusalem, Israel) were routinely cultured in 85% knockout DMEM (Life Technologies) supplemented with 15% ES approved FCS (Biological Industries), 2 mM t-glutamine, 100 U/ml penicillin, 150 μg/ml streptomycin, 1% nonessential amino acids (10 mM of each amino acid), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol (all supplements were purchased from Life Technologies), and 10 U/ml LIF (Chemicon, Huissen, The Netherlands). The cells grew in gelatin-coated 6-cm plates, and the medium was changed every 3–4 d as single cells, using 0.04% trypsin (Life Technologies) digestion for 2 min. All cells were grown at 37°C in 5% CO2, 4% O2 atmosphere.

Three milliliters of hESCs and mESC-conditioned medium (CM) were collected at days 7 and 3 after ESC plating, respectively. At this stage, the 9.5-cm2 wells contained ∼5 x 103 hESCs and mESCs per well. In some experiments, several concentrations of Nω-hydroxy-nor-l-arginine (Noha, Calbiochem, San Diego, CA), were added to hESC or mESC culture 24 h prior to collection of CM. For preparation of CM, cells were depleted by centrifugation, and media were filtered through a 0.22-μM filter to remove any remaining cell debris.

hESCs were immunostained using either mouse anti-human SSEA-4 (1:100), TRA-1-60 (1:100) or TRA-1-81 (1:100) primary Abs and the appropriate isotype control Abs (all from Chemicon), and FITC-conjugated polyclonal goat anti-mouse Igs (1:100; Dako) for detection; 5 μg/ml propidium iodide (Sigma-Aldrich) served for dead cell exclusion.

IFN-γ quantification and determination of T cell proliferation
Supernatants were collected from cocultures of activated and nonactivated PBMCs with hESCs, control cells, or their CM. IFN-γ levels in triplicate samples was quantified using commercial ELISA (R&D Systems, Minneapolis, MN).

To track PBMC’s proliferation, CFSE-labeled (Molecular Probes, Leiden, The Netherlands) cells were activated with 5 ng/ml OKT3. After 72 h, the cells and their fluorescence intensity was determined by flow cytometry.

Flow cytometry
PBMCs were collected from cocultures of activated and nonactivated PBMCs with hESCs, control cells, or their CM. The cells were washed by flow cytometry.

Immunofluorescent staining of hESCs and mESCs
To characterize hESC-specific markers by immunofluorescence, the cells were plated on glass coverslips pretreated with poly-o-lysine (30–70 KDAs, 10μg/ml; Sigma-Aldrich) and laminin (4μg/ml; Sigma-Aldrich) and were incubated in hESC or mESC culture 24 h prior to collection of CM. For preparation of CM, cells were depleted by centrifugation, and media were filtered through a 0.22-μM filter to remove any remaining cell debris. For fixation of hESC-CM, media were collected as above and placed into 1350, 3500, and 12,000–14,000 Da pore size dialysis bags (Medicell International, London, U.K.). CM was then dialyzed for 24 h at 4°C, against fresh hESC medium at a 1:10 ratio of fresh medium to CM volume. Two rounds of 24-h dialysis were performed.

Coculture of PBMCs with hESCs, mESCs, or their CM
PBMCs were purified from the venous blood of healthy donors by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich), according to manufacturer’s instructions. Blood was collected under approval of the Haartal Medical Center Helsinki Ethics Committee. Cocultures containing 105 PBMCs/well with either fresh medium, 5 × 105 hESCs growing on mitomycin-C-treated feeders, 3 × 105 foreskin feeders, and 3 × 105 adult fibroblasts, or the conditioned media of these cells prepared as described above, were incubated for 72 h with or without 1 ng/ml supernatant staphylococcal enterotoxin B (SEB, Sigma-Aldrich) or 5 ng/ml anti-CD3 Ab (OKT3, eBioscience, San Diego, CA). In some experiments, PBMCs were added to 0.2 μm transwell tissue culture inserts (Nunc), placed into 1.9-cm2 wells containing hESCs, and incubated for 72 h. In some experiments, the amino acids l-arginine, l-cysteine, l-histidine, l-lysine, and l-tryptophan (all purchased from Sigma-Aldrich) were added to conditioned media.

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For CD3-ζ chain expression, anti-CD3 stimulated human PBMCs, which were cultured with fresh medium, hESC-CM or foreskin-CM were collected and stained with APC-labeled anti-human CD3 (1:20; IQP, Minneapolis, MN). The cells were subsequently fixed with 4% paraformaldehyde for 20 min on ice, permeabilized with 0.1% saponin (Sigma-Aldrich) at room temperature for 10 min. The cells were then stained with mouse anti-human CD3-ζ chain PE (1:10; Santa Cruz Biotechnology). For the determination of CD3-ζ chain levels in teratoma-derived T cells, lymphocytes were stained with APC-labeled anti-mouse CD3 Ab, then washed, fixed in 1% paraformaldehyde for 20 min on ice, and permeabilized with 0.1% saponin at room temperature for 10 min. The cells were then stained with anti–PEC-conjugated CD3-ζ chain (1:10; Santa Cruz Biotechnology). For the detection of hESC-derived trophoblast cells were immunostained with an anti-HLA-G Ab (clone MEM-G/099) followed by FITC-conjugated polyclonal goat anti-mouse Igs (1:100; Dako) for detection.

For each sample, 105 cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences) using CELLQuest (BD Biosciences) or FCS Express V3 (De Novo Software, Thornhill, Ontario, Canada) softwares.

RT-PCR
Total RNA was isolated from hESCs (7 d after passage), human foreskin feeder cells, human adult fibroblasts, mESCs, and mouse embryonic feeder (MEF) cells. RNA was isolated using TriReagent (Sigma-Aldrich) according to the manufacturer’s instructions. cDNA was prepared using M-MLV reverse transcriptase using oligo-dT primers (both from Promega, Madison, WI). β-Actin PCR served to validate the quality of cDNA and the PCR re-action. PCR was performed using standard protocols with Taq DNA Polymerase (Promega). Amplification conditions were as follows: denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The number of cycles varied between 30 and 36, depending on the particular mRNA abundance. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

The primer pairs that were used for PCR were as follows: human arginase I forward 5’-TGGAACATTCTGACATGACA-3’ and human arginase I reverse 5’-AAGTCCGAAAACAGGCAA-3’ yielded a 253-bp band. Human arginase II forward 5’-GACATGGCCAGACCTTGTG-3’ and human arginase II reverse 5’-CCTGTGATCCATCTTCTGTG-3’ yielded a 304-bp band. Cat-2B forward 5’-GCTAAGGCTGATACTGCT-3’ and Cat-2B reverse 5’-TGCACTGAGCCGGAAGC-3’ yielded a 121-bp band. Human β-actin forward 5’-TTCACCCACGCGCCGACG-3’ and human β-actin reverse 5’-TCTCCCTTCTGCAATCTTGGC-3’ yielded a 291-bp band. Mouse arginase I forward 5’-AAGAAAAAGCGGATTACCTAC-3’ and mouse arginase I reverse 5’-CACCCTCTCTGCTGTC-3’ yielded a 449-bp band. Mouse β-actin forward 5’-AAGTGTGAGACACTGCTT-3’ and mouse β-actin reverse 5’-GGCGATCTCCACACCTTCA-3’ yielded a 189-bp band.

Measurement of arginase enzymatic activity
Urea is a product of L-arginine hydrolysis by arginase; therefore, arginase activity was measured in fresh cell lysates by colorimetric assay for the detection of urea. Accordingly, 5 × 105 cells were lysed in 50 μl 0.1% Triton (Sigma-Aldrich) containing 9 μl protease inhibitor mixture set IV
produced per hour per 10^6 cells. Where indicated, 200 or 400 at 540 nm was measured. Results were expressed as micrograms of urea and 25 μL α-isonitrosoprosopophenone (Sigma-Aldrich) was added and incubated for 45 min at 100°C. A standard curve was obtained by adding 100 μL (1, 2.5, 10, 20 μg) urea (J. T. Baker, Phillipsburg, NJ) to 400 μL acidic mixture and 25 μL α-isonitrosoprosopophenone. After 10 min in the dark, absorbance at 540 nm was measured. Results were expressed as micrograms of urea produced per hour per 10^6 cells. Where indicated, 200 or 400 μM of the arginase I inhibitor 2(S)-amino-6-borono-hexanoic acid NH4 (ABH; Axxora, San-Diego, CA) was added to the assay.

Immunostaining of mouse blastocysts

Blastocysts were generated from (C56BL/6xFVB)F1 mice using standard methods (14), and were immobilized by fibrin gel-embedding (15), followed by fixation with 4% PFA/PBS 5% sucrose, and stained with anti-arginase I Ab or isotype control as described above. The blastocysts were then visualized using an Olympus Fluoview laser-assisted confocal microscope (Tokyo, Japan).

Derivation of trophoderm cells from hESCs

hESC-derived trophoblast cells were prepared using an established protocol (16). hESCs were detached from foreskin plates using EDTA as described above and plated on permanox slides (Nunc) coated with 75% matrigel (BD Biosciences). The cells were grown for 14 d with 100 ng/mL bone morphogenetic protein 4 (PeproTech, Rocky Hill, NJ) in hESC medium and then either fixed with 4% PFA/PBS or arginase I immunofluorescent staining or detached from matrigel for HLA-G staining and flow cytometric analysis using 0.04% Trypsin (Life Technologies).

Teratoma formation and the isolation of spleen and teratoma-infiltrating lymphocytes

Teratomas were generated in immuno-compotent allogeneic mice by injection of 15 × 10^6 C57BL/6 hESCs to 5-wk-old BALB/c mice. The cells were transplanted s.c. into the lateral upper thigh of the right hind limb of each mouse. Teratomas appeared in 7 of 18 mice after 2–4 wk and removed when teratoma size reached 0.8–1.5 cm^3. Teratomas were then washed twice in 10 mL PBS to remove the remaining blood. Tissue was cut into 1-mm pieces and incubated in 2 mL DMEM (Life Technologies) for 20 min with 300 U/ml collagenase type IV and 50 U/ml DNaseI (Worthington Biochemical, Lakewood, NJ) at 37°C. Following incubation, the cells were purified by density centrifugation using 50 mM Tris/HCl, pH 9.7. The reaction was stopped by the addition of 400 μL acidic mixture H2SO4/H3PO4/H2O (1:3:7, v/v/v). For quantification of urea produced, 25 μL 9% α-isonitrosoprosopophenone (Sigma-Aldrich) was added and incubated for 45 min at 100°C. A standard curve was obtained by adding 100 μL (1, 2.5, 10, 20 μg) urea (J. T. Baker, Phillipsburg, NJ) to 400 μL acidic mixture and 25 μL α-isonitrosoprosopophenone. After 10 min in the dark, absorbance at 540 nm was measured. Results were expressed as micrograms of urea produced per hour per 10^6 cells. Where indicated, 200 or 400 μM of the arginase I inhibitor 2(S)-amino-6-borono-hexanoic acid NH4 (ABH; Axxora, San-Diego, CA) was added to the assay.

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Statistical methods

All data are presented as mean ± SD. The statistical analysis was performed using the Student t test.

Results

hESCs inhibit PBMC responses by a contact-independent mechanism using small, soluble molecules

To study the effect of hESCs on T cell response to Ag, hESCs were cocultured with human PBMCs from healthy donors. The hESCs (HES-1, 2, and H7) (1, 2) were cultured on human foreskin feeders (17). The hESCs exhibited the morphology and expressed markers of undifferentiated cells (Supplemental Fig. 1A–F). FACS analysis showed that, under these culture conditions, the level of background differentiation was low and the cultures comprised mainly undifferentiated stem cells (85–99% of total cells expressed various markers of pluripotent stem cells; Supplemental Fig. 1G–J). To test the ability of hESCs to inhibit T cell activation, as measured by cytokine secretion and T cell proliferation, PBMCs were either untreated or activated with SEB. In the absence of SEB, IFN-γ secretion was not detected in the culture medium of hESCs, foreskin feeders without hESCs, PBMCs, or PBMCs cocultured with hESCs or feeders (Fig. 1A). In the presence of SEB, PBMCs, but not hESCs or feeder cells, secreted IFN-γ. The secretion of IFN-γ was significantly reduced (93.9 ± 3.6%) when the SEB-activated PBMCs were cocultured with hESCs (Fig. 1A, 1B). The secretion of IFN-γ by PBMCs was inversely proportional to the number of seeded hESCs (Fig. 1A). Minimal reduction in IFN-γ secretion was observed in control coculture experiments of PBMCs with foreskin feeder cells or with primary adult fibroblasts obtained from skin biopsies (Fig. 1B).

To further analyze the inhibitory effect of hESCs on activated PBMCs, we inquired whether the inhibition was dependent on cell–cell contact or on soluble factors. In transwell experiments in which direct contact between the hESCs and PBMCs was prevented, significant inhibition of IFN-γ secretion was observed, similar to the inhibition observed when cell–cell contact was permitted. We therefore concluded that soluble factors play a role in the inhibitory effect of hESCs on PBMC activation (Fig. 1B). In line with this conclusion, hESC-CM could replicate the inhibitory effect of cocultured hESCs, in contrast to foreskin CM used as control (Fig. 1B).

To identify the soluble factors responsible for the inhibitory activity of hESCs, we tested the inhibitory activity of hESC-CM after dialysis against fresh hESC medium using several cut-off pore sizes (12–14, 3.5, and 1 kDa). Surprisingly, IFN-γ secretion was restored following dialysis of hESC-CM, even when we used the smallest pore size membrane (1 kDa), which enables the transfer of only small peptides and metabolites (Fig. 1B). This finding suggested a role for small molecules in the inhibition of PBMCs activation by hESCs.
Supplementation of hESC-CM with L-arginine restores T cell activation

The finding that small soluble molecules have a role in the inhibitory activity of hESCs led us to consider a mechanism that involves degradation and consumption of L-arginine by the enzymatic activity of arginase I. This enzyme degrades L-arginine, resulting in its depletion from the cell’s microenvironment. It has been shown that the low concentration of L-arginine in the microenvironment leads to local T cell unresponsiveness (18). Therefore, we first tested whether addition of L-arginine to hESC-CM can restore T cell responses. Supplementation of hESC-CM with L-arginine abolished the inhibition of IFN-γ secretion in a dose-dependent manner (Fig. 2A). This finding was observed when the PBMCs were activated by either SEB or the anti-CD3 Ab, OKT3 (Fig. 2B). Despite variability in the absolute level of IFN-γ secretion, and hence the level of inhibition between PBMC donors, the reversal of the inhibition of IFN-γ secretion by L-arginine was significant (Fig. 2B). Supplementation with several other amino acids (L-tryptophan, L-lysine, L-histidine and L-glycine) at the same concentrations failed to restore IFN-γ levels (data not shown), indicating that the beneficial effect of L-arginine supplementation was specific and could not be attributed to a general consumption of metabolites by hESCs.

We further analyzed T cell proliferation as another parameter of T cell activation. Proliferation was also significantly reduced when PBMCs were activated in the presence of hESC-CM, in contrast to control fresh medium and foreskin CM. Addition of L-arginine significantly restored T cell proliferation, as demonstrated by CFSE dilution analysis of CD3+ T cells (Fig. 2C). These data suggested that L-arginine depletion plays a role in the inhibitory effect of hESC-CM.

Arginase I expression and activity in hESCs

Given the depletion of L-arginine in hESC-CM, we confirmed that arginase I is expressed and active in hESCs. RT-PCR analysis showed the expression of arginase I by hESCs, but not by foreskin feeder cells or adult fibroblasts (Fig. 3A). Importantly, hESCs also express high levels of CAT2B, which is an alternatively spliced variant of CAT2 (Fig. 3B). This membrane cationic transporter mediates L-arginine uptake from the cells’ microenvironment into the cytoplasm. The combined activity of arginase I and CAT2B leads to L-arginine depletion from the cells’ microenvironment (13). In contrast with arginase I, RT-PCR analysis showed no difference in the expression of arginase II by hESCs, foreskin feeder cells, and adult fibroblasts (Supplemental Fig. 2).

We further demonstrated the expression of the arginase I protein in the cytoplasm of hESCs using immunofluorescent staining (Fig. 3C). This finding was consistent in three hESC lines: HES-1, HES-2, and H7 (Fig. 3C). Typically, 70–80% of the cells expressed the arginase I protein in each of the three lines (Fig. 3D), indicating that arginase I expression is not an incidental finding in a single hESC line.

Arginase I metabolizes L-arginine to urea and L-ornithine (19). To verify the activity of arginase I in hESCs, we determined the level of urea production in fresh hESC lysates from the HES-1 (Fig. 3E), HES-2, and H7 lines (Supplemental Fig. 3). Substantial urea production was detected in all three lines. It was significantly inhibited by ABH, a specific arginase inhibitor. In contrast, only background levels of urea were produced in foreskin feeder cells or adult fibroblasts lysates (Fig. 3E). Our data demonstrate arginase I expression and activity in several hESC lines, which in combination with CAT2B expression may lead to L-arginine consumption from the hESC microenvironment.

Arginase I activity in hESCs results in downregulation of the surface CD3 ζ-chain on activated T cells that can be recovered by L-arginine supplementation

It has been shown that Jurkat T cells cultured in L-arginine–free medium express low levels of the CD3 ζ-chain, a key molecule in the signal transduction cascade of the TCR, resulting in an attenuated T cell response. This reduction in ζ-chain surface density could be reversed by L-arginine replenishment (12). Further studies suggested that arginase I-dependent L-arginine depletion from tumors’ microenvironment downregulates ζ-chain expression, leading to T
cell unresponsiveness, which is possibly used as a mechanism for tumor evasion (11, 13). Therefore, we explored the possibility that hESC-mediated inhibition of T cell activation was dependent on CD3-ζ-chain downregulation. For that purpose, expression levels of surface CD3-ζ-chain in CD3+$^+$CD4+$^+$ (helper) T cells were analyzed by FACS after activation of PBMCs with OKT3 in the presence of either hESC-CM, foreskin CM, or fresh medium (Fig 4A). OKT3 activated helper T cells exposed to hESC-CM expressed lower levels of the CD3-ζ-chain, compared with PBMCs incubated with fresh medium or foreskin CM controls (Fig. 4B). Similar results were obtained when CD3+$^+$CD4$^-$ (cytotoxic) T cells were analyzed for CD3-ζ-chain downregulation (Supplemental Fig. 4). This reduction of CD3-ζ-chain levels was reversed by the addition of L-arginine (Fig 4B, 4D; Supplemental Fig. 4B, 4D) and was specific, because the overall levels of surface CD3 complex were not significantly changed (Fig. 4C, Supplemental Fig. 4C).

Moreover, pretreatment of hESCs with the arginase I inhibitor Noha during the preparation of CM significantly elevated CD3-ζ-chain levels, compared with conditioned media of untreated hESCs (Fig. 4E, Supplemental Fig. 4D), but not as high as with L-arginine supplementation. The usage of Noha concentrations exceeding 100 μM did not result in elevated CD3-ζ-chain levels (data not shown).

Whereas some studies have suggested that CD3-ζ-chain downregulation results in apoptosis (20), others have suggested that these two events are not linked (21). In our study, there was no significant induction of T cells apoptosis, suggesting that apoptosis has no major role in hESC-mediated T cell inhibition (data not shown).

**mESCs inhibit T cell response and downmodulate CD3-ζ-chain levels via arginase I**

Having shown that hESCs use a unique immunoregulatory mechanism involving arginase I activity, we next tested whether this mechanism also operates in mESCs. Using RT-PCR we demonstrated arginase I expression by mESCs, but not by MEF cells (Fig. 5A). Expression at the protein level was demonstrated by immunofluorescent staining, using anti-arginase I Ab, showing cytoplasmic localization of the enzyme (Fig. 5B). Similar to hESC-CM, mESC-CM inhibited PBMC activation as measured by IFN-γ secretion (Fig. 5C). This inhibition was reversed by supplementation of the mESC-CM with L-arginine (Fig. 5C). In addition, activation of PBMCs in the presence of mESC-CM reduced CD3-ζ-chain density, which could be reversed by L-arginine supplementation (Fig. 5D). Furthermore, CD3-ζ-chain expression was significantly restored by pretreatment of mESCs with the arginase I inhibitor Noha (Fig. 5D). In conclusion, these results suggest that mESCs, like hESCs, suppress T cell activation via arginase I-dependent downregulation of the CD3-ζ chain, which can be reversed by L-arginine supplementation.

**CD3-ζ chain is downmodulated in teratoma-infiltrating T cells**

It has been shown that teratomas generated by high numbers of mESCs were not rejected after allogeneic transplantation (22). This could possibly be related, at least in part, to local arginase I activity in undifferentiated mESCs, leading to inhibition of T cell activity because of the downmodulation of CD3-ζ chain in teratoma-infiltrating T cells as described for tumor-associated myeloid cells (11). To test this possibility, we performed an allogeneic transplantation of $15 \times 10^6$ C57BL/6 mESCs, s.c., into BALB/c recipients. Teratoma formation was observed in 7 of 18 (39%) of the recipient mice after 2–4 wk. We then isolated lymphocytes from the teratomas and studied the expression of the CD3-ζ chain in CD3+$^+$ gated T cells (Fig. 6). CD3-ζ levels were significantly lower ($p < 10^{-5}$) in teratoma-infiltrating T cells, compared with
splenic T cells from the same mice (Fig. 6A, 6B) or from non-transplanted control mice (Fig. 6C). This reduction in ζ-chain levels is likely to suppress TCR signal transduction of T cells infiltrating ESC-derived teratoma and therefore, at least locally, inhibit T cell activation in vivo.

Arginase I expression in preimplantation embryos

It can be speculated that arginase I expression by pluripotent stem cells is used by the preimplantation embryo to attenuate maternal T cell activity in its microenvironment and prevent rejection. We therefore inquired whether arginase I is expressed in vivo by the ICM of the blastocyst, from which ESCs are derived. Immunostaining of mouse blastocysts revealed that arginase I is expressed by the ICM and trophodermal cells (Fig. 7A–D), which form the outermost layer of cells of the blastocyst that attach and invade into the uterine wall. In line with this finding, we further demonstrated arginase I

FIGURE 4. The CD3-ζ chain is downregulated in activated T cells in the presence of hESC-CM and is restored by l-arginine supplementation. PBMCs were activated with OKT3 for 72 h in hESC-CM with or without l-arginine supplementation as well as in fresh medium and foreskin-CM as controls. The cells were immunostained with APC-conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-conjugated anti-CD3-ζ chain and analyzed by flow cytometry. PBMCs were gated for CD3⁺CD4⁺ T cells (A), and the levels of CD3-ζ chain (B) and CD3 (C) are shown. Numbers represent mean fluorescence intensity (MFI) of each histogram. CD3-ζ chain expression levels were reduced in CD4⁺ T cells that were incubated in hESC-CM (red line) and restored in the presence of l-arginine supplementation (blue line, B), whereas the expression levels of CD3-complex were similar in the various culture media (C). A summary of data from several separate experiments, as shown in B, is represented in D. The MFI of CD3-ζ chain was normalized in each experiment to the level of expression in CD4⁺ T cells activated in fresh medium. E, PBMCs were activated in CM from untreated hESCs or hESCs that were treated with various concentrations of the arginase inhibitor Noha. Noha treatment resulted in partial restoration of CD3-ζ chain expression in activated CD4⁺ T cells. The MFI of CD3-ζ chain in CD4⁺ T cells was measured and is presented in D.

FIGURE 5. Arginine-I dependent inhibition of T cell activation and CD3-ζ chain downregulation by mouse ES cells. A, RT-PCR analysis of arginase I expression in mESCs and MEF cells. B, Indirect immunofluorescence staining of mESCs showing the expression of arginase I protein (green cytoplasmatic staining). Nuclei were counterstained by DAPI (blue; scale bar = 50 μm). C, mESC-CM inhibited IFN-γ secretion by OKT3-activated PBMCs, as determined by ELISA. The percent inhibition was relative to the secretion of IFN-γ by OKT3-activated PBMCs cultured in fresh medium. Secretion of IFN-γ was restored by 2 mg/ml L-arginine supplementation. D, CD3-ζ chain expression by OKT3-activated PBMCs cultured in mESC-CM was reduced in comparison with OKT3-activated PBMCs cultured in fresh medium (left bar). The MFI of the CD3-ζ chain in CD4⁺ T cells was measured and is presented as described in Fig. 4. CD3-ζ chain expression levels were restored in the presence of 2 mg/ml l-arginine supplementation (middle bar). Treatment with the arginase inhibitor Noha during the preparation of mESC-CM mostly prevented downmodulation of CD3-ζ chain expression (right bar).
The hESC immunosuppressive effect does not require cell–cell contact, as was apparent when the PBMCs were physically separated from the hESC by transwell membranes, or when the hESC were replaced by their CM. These soluble factors are small in size, because their inhibitory effect was abrogated by dialysis of CM through 1-kDa dialysis bags. This phenomenon could be caused either by generation of small inhibitory molecules by hESCs or by depletion of essential metabolites from the hESC culture medium. The inhibition of IFN-γ secretion and T cell proliferation could be reversed by adding L-arginine to the CM, supporting the latter possibility.

We further show that three lines of hESCs express arginase I, which metabolizes L-arginine to L-ornithine and urea. Furthermore, urea production is prominent in fresh hESC lysates and considerably decreased when the arginase I inhibitor ABH is used, indicating the activity of Arginase I in hESCs. It was previously demonstrated that the combined expression of the cationic transporter CAT2B and arginase I, as shown in the present study for hESCs, enables L-arginine uptake and degradation, resulting in its depletion from the cells’ microenvironment (11). Consequently, re-emergence of the CD3-ζ chain in the TCR complex of activated T cells is inhibited, leading to suppression of their further responses (13). The reduction of CD3-ζ occurs by an unknown mechanism, and it was first demonstrated in Jurkat T cells cultured in L-arginine–free medium (12). It was later shown that murine lung carcinoma cells induce arginase I expression in tumor-associated myeloid cells as a mechanism for local immunosuppression and tumor evasion (11). Arginase I activity, as demonstrated in this study, may be used by hESCs to regulate T cell responses.

Although arginase I was originally termed liver type arginase, it is now evident that it is expressed also by other types of cells, such as RBCs (23), macrophages (11, 13), and placental cells (24, 25).

Previous reports mainly studied the immunogenicity of hESCs (10) and their capability to trigger an immune response (5, 6, 9). In the present study, we focused on the potential of hESCs to suppress the activation of T cells in response to potent stimulators. We therefore used an in vitro experimental system in which PBMCs were activated by SEB or OKT3 in the presence of hESCs. We show that hESCs have the ability to inhibit T cell immune responses as also shown by Li et al. (5). We further elucidate, for the first time, the activity of arginase I as the underlying mechanism for this hESC-mediated inhibition of T cell activation.

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Arginase I was found to be involved in several disease states, such as cancer, trauma, sepsis, and liver necrosis and following liver transplantation (26). CD3-ζ downregulation has also been implicated in a wide range of pathologies, such as tumors, autoimmune diseases, trauma, and infectious diseases (21). We demonstrate, for the first time, a role for this mechanism in T cell attenuation by hESCs and mESCs.

In addition to arginase consumption, evidence for other soluble factors that mediate inhibition of immune responses by ESCs were recently published. TGF-β was suggested to play a role in immunosuppression mediated by mESCs and their CM (22). The small products biliverdin and carbon monoxide, of hemooxygenase I enzymatic activity in hESCs, were also shown to regulate T cell proliferation by their anti-inflammatory and antiapoptotic properties (9). In addition, arginase I activity in ESCs could potentially also inhibit immune responses by metabolites of L-arginine, such as spermidine, spermine, and putrescine, which were shown to have a role in cell cycle control and in regulating the secretion of proinflammatory cytokines by monocytes (18). Nevertheless, the fact that adding L-arginine to the conditioned media could reverse most of the inhibitory effects of ESC, suggesting that the consumption of arginine is the major factor responsible for the inhibitory activity of ESCs and not the production of metabolites.

A previous study suggested that contact with the hESC membrane was required to inhibit T cell proliferation in an MLC assay and that hESC-CM was ineffective (5). However, in that study, the hESC-CM was diluted with fresh medium, which could replenish the presumably depleted L-arginine.

In agreement with a contact mediated mechanism, FasL expression by rat (3) and mouse (4) ESCs was suggested to mediate T cell modulation (3, 4). In vivo, FasL induced apoptosis of thymocytes, enabled clonal deletion of donor-specific T cells, and allowed the establishment of donor-specific transplantation tolerance (3). However, other studies failed to demonstrate the expression of FasL in ESCs (6, 27).

Several other observations may explain how ESCs avoid allore cognition by the immune system. ESCs do not express MHC class II molecules and express very low levels of MHC class I molecules (7, 10). In addition, poor killing activity of NK cells toward hESCs has been shown, presumably because of low expression of ligands for NK cell receptors by hESCs (10). An alternative explanation for this finding may be based on the ζ chain being a main signal transduction molecule for T and NK cells (21). L-arginine consumption by hESCs, as demonstrated by our results, may potentially reduce the ζ chain on NK cells, resulting in their suppression. Thus, hESC-arginase I-dependent immunomodulation may potentially serve as a central mechanism for silencing of both specific immunity represented by T cells and innate immunity components such as NK cells.

None of the aforementioned immunosuppression mechanisms are ruled out by our study. In fact, several mechanisms may potentially play a role in the induction of the immunosuppressive effects of ESCs. The observation that L-arginine supplementation could not completely reverse the immunosuppressive effects of ESCs is in line with the potential contribution of other mechanisms. Further studies are required to clarify the relative significance and contribution of each of these mechanisms.

In addition to hESCs, we also showed arginase I-mediated immunomodulation by mESCs, suggesting the conservation of this mechanism along evolution. We have therefore used mESCs for in vivo studies. It has been shown that teratomas were not rejected after allogeneic transplantation of mESCs (22), as well as after engraftment of hESCs into an allogeneic mouse model (6). This could possibly be related, at least in part, to local arginase I activity in undifferentiated ESCs within the teratomas, similar to the effect described for tumor-associated myeloid cells (11). To test this notion, we have reproduced the results by Koch et al. (22), and confirmed that mESC teratomas can develop in allogeneic recipients. We further showed that T cells within these teratomas have low levels of CD3-ζ chain, suggesting arginase I-mediated T cell suppression within the teratomas. In the context of future hESC-based cell therapy and in the unfortunate case of complicating teratomas, arginase-I activity may allow their evasion from immune rejection.

We further speculated that arginase I activity is used as a universal mechanism for T cell attenuation by embryonic cells to augment survival of the preimplantation embryo, by attenuating maternal T cell activity in the embryo’s microenvironment. We have demonstrated in this study that this enzyme is expressed by both the ICM and trophectodermal cells of mouse preimplantation embryos. In addition, we have shown that arginase I is expressed by hESC-derived trophectodermal cells. The trophectodermal cells consist of the outermost layer of cells of the blastocyst that attaches and invades into the uterine wall. Therefore, local arginine consumption may possibly contribute to the suppression of maternal T cell responses in the embryo’s microenvironment. These results are in line with the reported expression of arginase I in human term placenta and its postulated role in inducing maternal T cell hyporesponsiveness during pregnancy (25). Further studies are required to elucidate the role and significance of arginase I during the period of implantation.

In conclusion, this study demonstrates that hESCs inhibit T cell response via arginase I activity and l-arginine consumption, resulting in downregulation of the TCR CD3-ζ chain and T cell unresponsiveness. Our findings shed light on the mechanism underlying the immunomodulatory properties of ESCs.

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Disclosures
The authors have no financial conflicts of interest.

References


