

Cord Blood Mesenchymal Stem Cells Suppress DC-T Cell Proliferation via Prostaglandin B2

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Immune suppression is a very stable property of multipotent stromal cells also known as mesenchymal stem cells (MSCs). All cell lines tested showed robust immune suppression not affected by a long culture history. Several mechanisms were described to account for this capability. Since several of the described mechanisms were not causing the immune suppression, the expression pattern of cord-blood-derived MSCs by microarray experiments was determined. Dendritic cells cocultured with cord blood MSCs were compared with cord blood MSCs. Putative immune suppressive candidates were tested to explain this inhibition. We find that cord blood MSCs themselves are hardly immunogenic as tested with allogeneic T-cells. Dendritic cells cocultured with second-party T-cells evoked abundant proliferation that was inhibited by third-party cord blood MSCs. Optimal inhibition was seen with one cord blood MSC for every dendritic cell. Blocking human leukocyte antigen G only saw partial recovery of proliferation. Several cytokines, gangliosides, enzymes like arginase, NO synthase, and indole amine 2,3-dioxygenase as well as the induction of Treg were not involved in the inhibition. The inhibiting moiety was identified as prostaglandin B2 by lipid metabolite analysis of the culture supernatant and confirmed with purified prostaglandin B2.

Introduction

HUMAN UMBILICAL CORD BLOOD (UCB) not only contains hematopoietic but also multipotent stromal cells also known as mesenchymal stem cells (MSCs) [1–3]. An adherent nonhematopoietic CD45⁻ cell population was isolated from cord blood and termed unrestricted somatic stem cell (USSC) [4]. USSCs are a separate pluripotent class of stem cells that have the ability to differentiate into many cell types, including osteoblasts, chondrocytes, adipocytes, hepatocytes, neural progenitors, and improved left-ventricular function [4–6]. In culture, USSCs can be expanded up to 10¹⁵ cells without losing their pluripotency [4,7]. Distinctive of bone marrow MSCs, expression of ICAM-3 (CD50), L-selectin (CD62L), and VCAM (CD106) is not expressed by USSCs [8]. USSCs were classified of a distinct MSC population based on microarray expression data [9]. Therefore, it is suggested that USSCs represent an immature mesodermal progenitor for MSCs.

MSCs are of inherently low immunogenicity and, more importantly, are capable of inhibiting allogeneic T-lymphocyte responses [10–14]. The molecular mechanisms reported for the immunosuppressive effects of MSCs are multiple. The reports

that describe a potential role of transforming growth factor- β (TGF- β) and hepatocyte growth factor (HGF) as mediators of T-lymphocyte inhibition are still controversial, but in general most studies agree that soluble factors are involved [11–13,15]. MSCs express only a few toll-like receptors [16] and their triggering only produces a limited cytokine response due to promoter silencing [17], illustrating their low immunogenicity. On the other hand USSCs induce an increased interleukin (IL)-12 response in mature dendritic cells [18], leading to a higher immune response. Of particular interest is the observation that in vivo administration of MSCs in baboons significantly prolongs the survival of major histocompatibility complex (MHC)-mismatched skin grafts [10]. In humans, treatment of patients with MSCs to repair tissues remains elusive [19–22]; however, MSCs showed effective reduction of graft-versus-host disease [23–25]. Since USSCs are obtained from umbilical cord, an organ connecting two only haplo identical individuals, additional immune suppressive mechanisms were expected. We find that contact is essential in this process, but that the suppressive effect is mediated by soluble factors. Here, we demonstrate that the classical immune suppressors reported for adult MSCs, like gangliosides,

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NO synthase, arginase, indole amine 2,3-dioxygenase (IDO), IL-10, human leukocyte antigen G (HLA-G), TGF- β , and induction of Treg, are marginal suppressive factors in USSC-mediated suppression. The suppressive entity is small, prompting us to analyze lipid metabolites. Analysis showed that PGB2 is present in the supernatant of those cultures and purified PGB2 showed inhibition of T-cell proliferation.

Materials and Methods

Generation and expansion of USSCs

USSCs were successfully generated according to Kögler et al. [4]. In short, cord blood was collected from the umbilical cord vein with informed consent of the mother [26]. The mononuclear cell fraction was obtained by a Ficoll (Biochrom) density barrier separation followed by ammonium chloride lysis of red blood cells. Cells were washed twice with PBS (pH 7.4) and plated out at $5\text{--}7 \times 10^6$ cells/mL in T25 culture flasks (Costar). Growth of the adherent USSC colonies was initiated using low-glucose DMEM (Invitrogen) supplemented with 30% fetal calf serum (FCS) [Perbio (Hyclone)], low dexamethasone (10^{-7} M; Sigma), and antibiotic-antimycotic (Invitrogen).

Dexamethasone of 10^{-7} M is used to get the colonies adherent. As soon as the colonies arise no dexamethasone is added anymore to the medium [27]. Expansion of the cells and further experiments were performed in the same medium as described previously, but in the absence of dexamethasone. All cells were incubated at 37°C in 5% CO₂ in a fully humidified atmosphere. When the cells reached 80% confluency, they were removed from the flask with 1× trypsin (Invitrogen) and replated 1:3 under the same medium conditions as described previously [26].

Generation of monocyte-derived DCs

Peripheral blood mononuclear cells (PBMCs) were obtained from buffycoats of healthy individuals according to institutional guidelines, including informed consent by Ficoll density barrier centrifugation. Monocytes were isolated from PBMCs by adherence to plastic culture flasks and were differentiated into immature DCs by culturing for 6–7 days in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF; 300 and 450 U/mL, respectively; Schering-Plough International) in RPMI 1640 medium containing 10% FCS. Nonadherent cells peripheral blood lymphocytes (PBLs) were collected for later use.

Mixed lymphocyte reaction

Monocyte-depleted PBLs, isolated from buffy coats of healthy donors, were used in mixed lymphocyte reactions (MLRs). PBLs (10^5) were cocultured with 6700 allogeneic DCs (ratio of 15:1) for 4–6 days and proliferation was assessed by [³H]-thymidine (0.037 MBq [1 μ Ci/well]; MP Biomedicals) incorporation for 16 h. In USSC/MLR coculture experiments, MLRs were performed in the presence of diminishing concentrations of USSCs (6700, 2220, and 740 per well). As a positive control, PBLs were stimulated with 5 μ g/mL of PHA. All cultures were performed (at least) in quadruplicate in normal USSC medium (ie, without dexamethasone). Where indicated a neutralizing antibody against

TGF- β (AB-101-NA, R&D systems; 5 μ g/mL) was added at coculture onset. PGB2, and the thromboxane antagonists L655,240, ICI 192, L670,596, and AA 2414 were obtained from Cayman Chemical Company.

Transwell analysis of USSC-associated suppressive activity

In Transwell experiments, responder PBLs were labeled with CFSE before use in MLR. To this purpose, PBLs (5×10^6 cells/mL) were resuspended in PBS containing 1% of FCS. CFSE (Molecular Probes, Inc.) was added to a final concentration of 5 μ M, and the cells were gently mixed and incubated for 10 min at 37°C in a CO₂ incubator protected from light. Adding equal amounts of FCS stopped labeling of cells, and the cells were subsequently washed twice and resuspended in normal USSC medium. CFSE-labeled responder PBLs were then cocultured with DCs in the lower chamber of a 24-well plate containing a Transwell insert (Costar, Corning, Inc.). In the upper chamber, USSCs were added at a ratio of 15PBLs:1DC:1USSC. The USSCs were separated from the other two cell types by a semipermeable membrane with a pore size of 0.4 μ m. After 7 days of culture, CFSE content of cells was determined using flow cytometry.

Conditioned medium analysis of USSC-associated suppressive activity

To produce conditioned media, USSCs were cultured at 70%–90% confluence for 4 days. The conditioned media from the cultures were then recovered and filtered through 0.45- μ m filters and used immediately. In some experiments, conditioned medium was used that was heat activated by a 10-min incubation at 85°C [28]. Responder PBLs were cocultured with DCs in the USSC-conditioned media. On day 7 of culture in the conditioned media, proliferation of the responder PBLs was measured by ³H-thymidine incorporation.

Cytometric bead array measurements

Th1 (IL-2, IFN- γ , and TNF- β) and Th2 cytokines (IL-4, IL-5, and IL-10) were quantified simultaneously using a human Th1/Th2 cytokine Cytometric bead array kit and cytometric bead array software (BD Biosciences). This array kit provides a mixture of six microbeads. Beads with distinct fluorescent intensities (FL-3) are precoated with antibodies specific for each cytokine. Fifty microliters of supernatant of MLRs with and without USSCs, along with provided standard cytokines, was added to the premixed microbeads. After the addition of 50 μ L of a mixture of PE-conjugated antibodies against the cytokines, the mixture was incubated for 3 h in the dark at room temperature. This mixture was washed and centrifuged at 200g for 5 min and the pellet was resuspended in 300 μ L of wash buffer. The BD FACSCalibur (BD Biosciences) was calibrated with setup beads and 3000 events were acquired for each sample. Individual cytokine concentrations were indicated by their fluorescent intensities.

TGF- β 1 measurements

Active TGF- β 1 levels were determined using the TGF- β -sensitive mink lung cell line MLCCL-64. To this purpose, MLCCL-64 cells were plated a day prior to transfection at a

density of 20,000 cells/well in a 48-well flat-bottomed plate (Costar) in RPMI medium supplemented with 10% FCS. Following cotransfection of 200 ng of pGL3 Basic vector containing the firefly luciferase gene and 1 ng of pRL-null (renilla luciferase) using PEI (Polysciences), cells were serum starved for 24 h. Cells were then stimulated using different concentrations of TGF- β (0, 0.1, 1, 2.5, and 5 ng/mL) (AB-101-NA; R&D Systems) in serum-free RPMI medium for 16 h. Cells were subsequently lysed and luciferase activity was measured on a Victor³ 1420 multilabel counter (Perkin Elmer) according to the Dual-Luciferase Reporter Assay System (Promega). Using this calibration curve, the level of active TGF- β 1 in supernatants of different cultures was determined.

RNA isolation and microarray hybridization

Total RNA was isolated from dendritic cells and dendritic cells cocultured with USSCs using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The quality of RNA fulfilled the criteria (28S:18S ratio > 1.5) as measured by the Agilent 2100. Two micrograms of total RNA was labeled according to the GeneChip Whole Transcript (WT) Sense Target Labeling Assay as provided by the manufacturer (Affymetrix), and hybridized to Human U133 2.0 arrays overnight before scanning in an Affymetrix GCS 3000 7G scanner. All hybridizations were carried out at the Microarray Facility of the Department of Human Genetics, Nijmegen Centre of Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands. Microarray data were deposited to GEO at the NCBI site (GSE23366). URL: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23366

Size determination of the factor

Supernatant of DC-T cell-USSC cultures was centrifuged over filters with a defined pore size (Vivaproducts), which yields a flow through of a given molecular weight cutoff. The flow through was tested in an MLR.

Lipid analysis of culture supernatants

Supernatant of DCs cultured together with allogeneic T cells, and DCs cultured together with allogeneic T cells and USSCs were collected after 6 days. After a centrifugation step to remove cells and debris, the supernatant was collected and frozen. Frozen samples were packed in dry ice and shipped to Stockholm. Analysis of 86 lipid metabolites was according to Lundström et al. [29]. Controls for extraction and yield were run in parallel.

Statistical analysis

A one-way ANOVA test was used to test the probability of significant differences between samples. Statistical significance ($P < 0.05$) is indicated in the figures as follows: *** $P < 0.0001$, ** $P < 0.0001-0.01$, * $P < 0.01-0.05$.

Results

USSCs fail to stimulate allogeneic T-lymphocytes

The level of the immune response provoked by USSCs as stimulators of allogeneic T-lymphocytes was tested. PHA or

monocyte-derived immature DCs (iDCs) were used as stimulators in control cultures. Only a slight increase in T-lymphocyte proliferation was observed when USSCs were used as stimulator cells as compared with the negative control (Fig. 1A). This lack of stimulator activity is consistent with their phenotype; like BM-MSCs, USSCs do not express MHC class II molecules, CD80 and CD86 [8].

USSCs inhibit DC-induced T-lymphocyte proliferation in a dose-dependent manner

The effect of USSCs on T-lymphocyte proliferation was evaluated by mixing different amounts of USSCs and PBLs in the presence of allogeneic DCs as stimulators. PBLs were always stimulated with DCs at a ratio of 15:1, because this ratio induces optimal T-cell stimulation. As shown in Fig. 1B, a huge reduction in T-lymphocyte proliferation is observed when DC-stimulated PBLs were cultured in the presence of USSCs. Titration studies show that this inhibitory effect is dose dependent (Fig. 1B). Optimal inhibition was observed for one USSC for every DC.

To unravel the kinetics of USSC inhibition on T-lymphocyte proliferation, USSCs were added at various time points: 0, 48, 72, 96, or 120 h, after MLR initiation (Fig. 1C). Optimal suppression of T-lymphocyte proliferation was observed when USSCs were added at the beginning of the culture. Interestingly, when USSCs were added 120 h after MLR initiation (implying only 48 h of coculture of USSCs with lymphocytes engaged for the MLR) still a marked suppression of T-lymphocyte proliferation could be observed ($P = 0.058$), indicating that USSCs are very efficient in the suppression of T-lymphocyte proliferation and not in preventing T-cell activation.

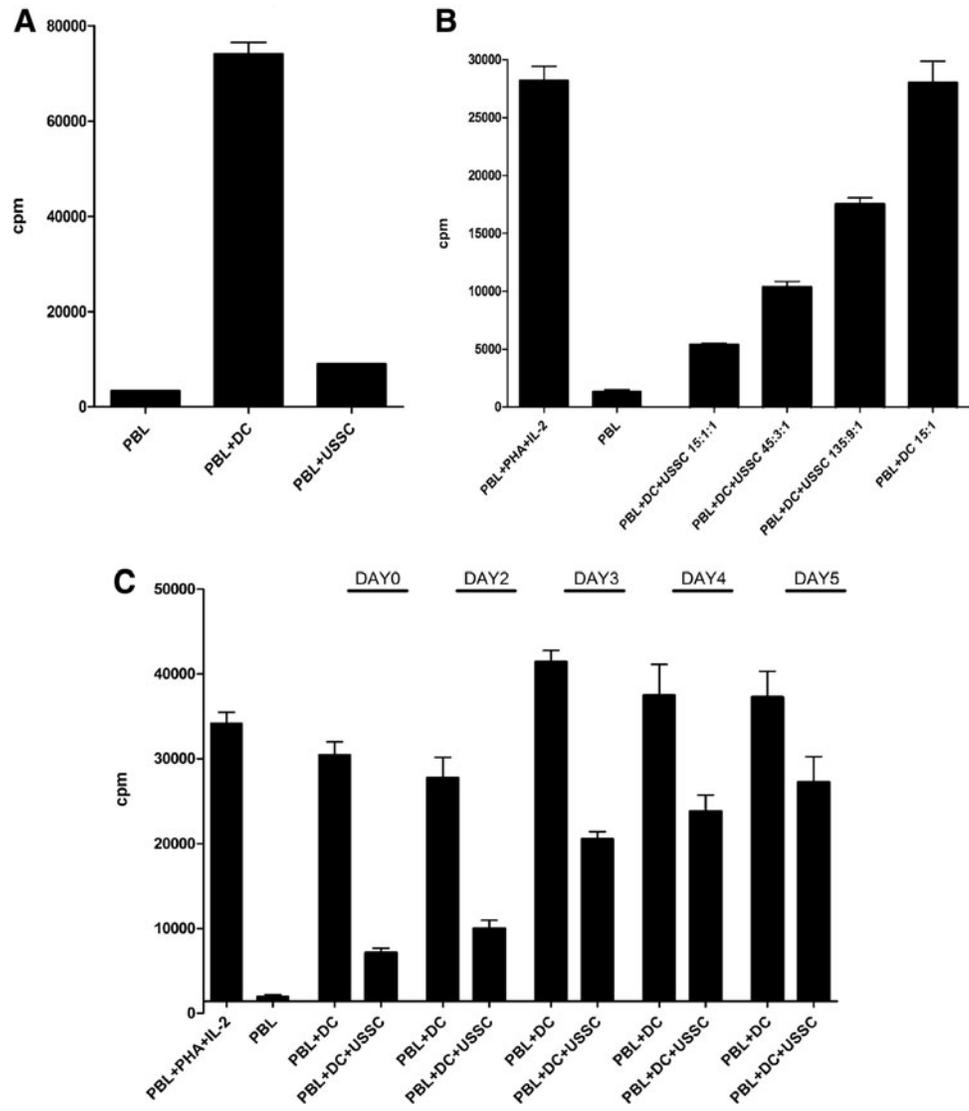
Regulatory T-cell population is not increased in MLR+ USSC cultures

Treg cells are identified by their high level of CD25 and CD4 expression, while also expressing low levels of CD127 [30]. Treg cells are further defined by expression of the forkhead family transcription factor FOXP3 (forkhead box p3) [31,32]. MSCs exert an immune regulatory function and suppress T-cell proliferation in vitro [11], in vivo in animal models [10] and in humans [33]. Previously it was shown that MSCs are able to recruit, regulate, and maintain Treg phenotype and function [34–36]. Others, however, showed that the MSC inhibitory effect on T-cell proliferation was not mediated through Tregs [12]. Our microarray data showed that CCL22, the chemokine that attracts Treg, was five times upregulated. Here, we show that USSC-mediated inhibition of T-cell proliferation is not mediated via the induction of Tregs (Fig. 2). On the contrary, the Treg population even drops from 5.5% in PBL + DC cultures to 2.8% in PBL + DC + USSC cultures.

USSC inhibitory effect requires cell contact and is mediated by soluble factors

To determine whether cell-to-cell contact is required for T-lymphocyte suppression, we compared when USSCs were cultured in contact with or physically separated by a transwell (TW) membrane from T-lymphocytes and DCs. USSCs were added to the upper chamber of a 24-well TW plate and the MLR was cultured separately in the lower chamber. When USSCs were cultured separately from the

FIG. 1. (A) Lack of proliferation of peripheral blood lymphocytes (PBLs) when cultured with allogeneic unrestricted somatic stem cells (USSCs) in mixed cell cultures. After 6 days of culturing PBLs alone, they exhibited only baseline proliferation as measured by ^3H -thymidine incorporation (counts per minute) (*first bar*). Responder PBLs cocultured with allogeneic immature DCs (iDCs) exhibited marked increase in proliferation (*middle bar*). Responder PBLs cocultured with USSCs exhibited only baseline proliferation (*third bar*). (B) USSCs inhibit iDC-induced T-lymphocyte proliferation in a concentration-dependent manner. Proliferation of human PBLs (10^5 cells/well) induced by allogeneic stimulation in the absence or presence of different numbers of USSCs was evaluated on day 6 by ^3H -thymidine uptake. Results are expressed as mean values (\pm mean standard error [SEM]) of cpm obtained in eight experiments. (C) Block of T-lymphocyte proliferation is time dependent. Proliferation of T-lymphocytes induced by allogeneic stimulation in the absence or presence of USSCs was evaluated at different time points (indicated above). Bars represent mean values (\pm SEM) obtained in four experiments. PBLs, peripheral blood lymphocytes.

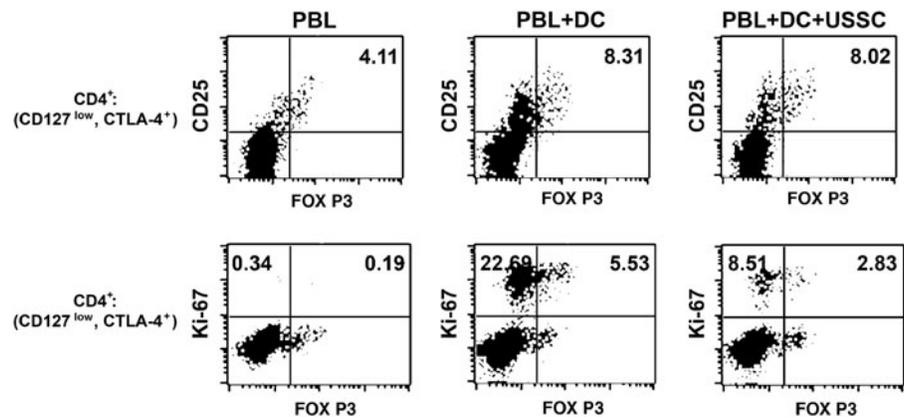


MLR, still a decrease in T-lymphocyte proliferation was observed, although not to the same extent as when USSCs were cultured in direct contact with the MLR (Fig. 3A). This indicates that both soluble factors as well as cell-to-cell contact are required for optimal suppression of T-lymphocyte proliferation.

Soluble factors involved in inhibition of T-lymphocyte proliferation

Since the Transwell system did not show complete restoration of T-lymphocyte proliferation, we investigated the relative importance of soluble factor(s) in the inhibitory

FIG. 2. Control and USSC cocultures were analyzed for Treg cells. At the end of the mixed lymphocyte reaction (MLR), cells were collected and stained with the indicated antibodies. The percentage of Treg is indicated.



effect of USSCs on T-lymphocyte proliferation. To this purpose, we tested the effect of different conditioned media on MLR cultures. Conditioned medium of USSCs contains large quantities of TGF- β . Addition of conditioned medium of USSC cultures or MLR cultures did not suppress DC-induced T-lymphocyte proliferation (Fig. 3B). Activated conditioned medium showed significant inhibition, indicating that the TGF- β in the medium is not active. Interestingly, when we tested the medium of MLR cultures in which USSCs were included, a strong inhibition in T-lymphocyte proliferation was observed (Fig. 3B). This indicates that after direct contact between the three cell types the inhibition of T-lymphocyte proliferation is mediated by soluble factors.

For MSCs, contribution of several inducible soluble factors, such as TGF- β [11], HGF [11], IL-10 [37], prostaglandin E2 (PGE2) [38,39], inducible NO synthase [40], IL-6 [41], IDO [42], and HLA-G [43,44], was described to be responsible for the observed inhibition of T-lymphocyte proliferation. Here, we investigated involvement of several of these soluble factors in USSC-mediated inhibition.

Cytokine production measurements show no upregulation of IL-10

To identify the soluble factor responsible for the observed effect, we decided to measure the levels of cytokines in su-

pernatants of MLR cultured in the presence or absence of USSCs. No differences in expression levels of IL-10 were observed when comparing supernatants of MLR cultures with that of MLR/USSC cocultures (Fig. 3C). Four other cytokines (including IL-4, IL-5, IFN- γ , and TNF- β) also showed no significant up- or downregulation. We did, however, observe a significant increase in IL-2 expression levels when the MLR was cultured in the USSCs (Fig. 3D). Most likely, this merely reflects IL-2 consumption in the MLR rather than increased IL-2 production in the USSC MLR.

TGF- β 1 is not responsible for the inhibition of T-lymphocyte proliferation induced by USSCs

Addition of neutralizing anti-TGF- β antibodies to MLR/USSC cocultures did not result in the restoration of T-lymphocyte proliferation, indicating that TGF- β is not involved in this process (Fig. 4A). To further prove this, we measured the levels of active TGF- β in the supernatant of different cultures using a TGF- β -sensitive cell line transfected with a luciferase reporter gene. Conditioned medium of USSCs contains very low amounts of active TGF- β (Fig. 4B). Heat treatment (10 min at 85°C) of this conditioned medium confirms that this mechanism activates the latent TGF- β . This active TGF- β is neutralized upon addition of anti-TGF- β antibodies during stimulation, indicating that the

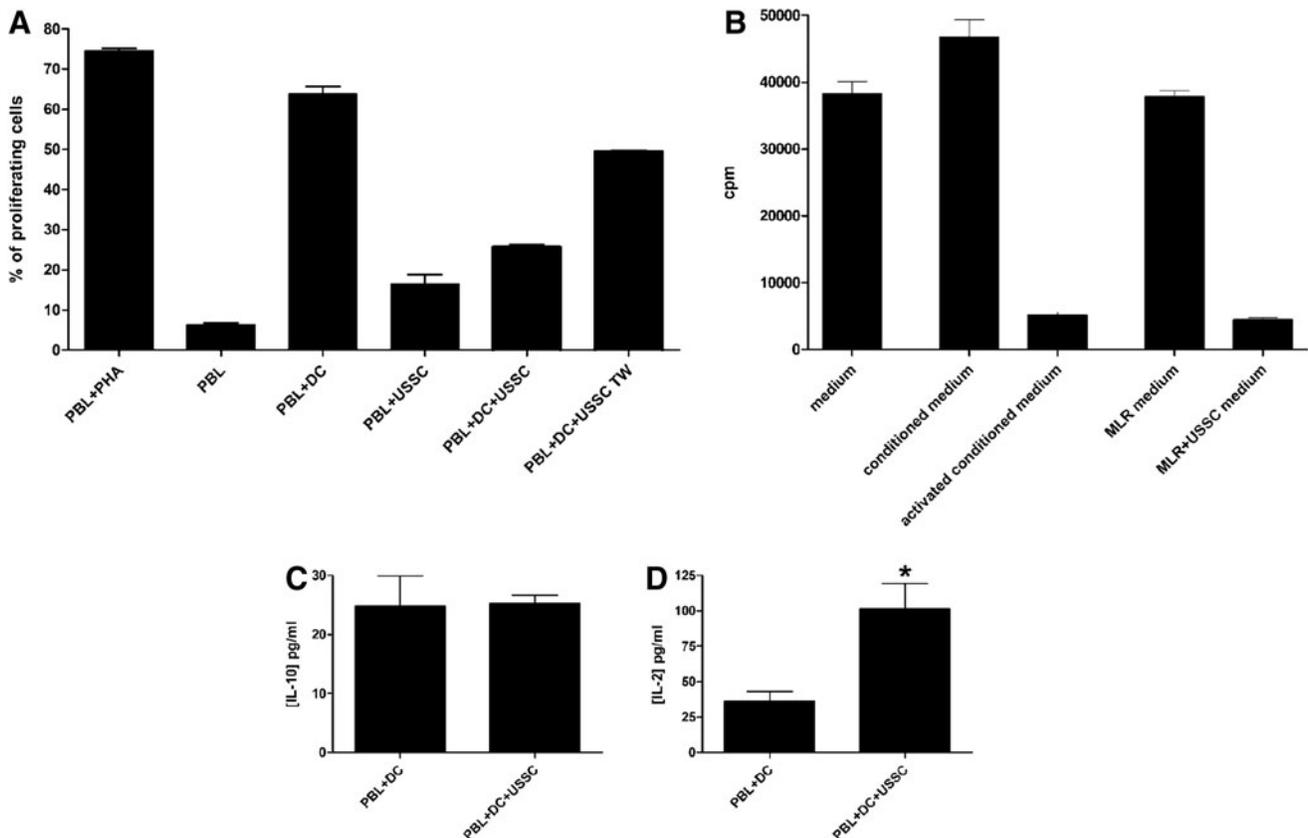


FIG. 3. (A) DCs and PBLs were cultured together in the lower compartment of a transwell plate. Where indicated USSCs were cultured in the upper compartment of the transwell plate. (B) Conditioned medium was collected and tested in a subsequent MLR experiment. Activated medium from a USSC culture showed significant inhibition (*third bar*) as was also found for supernatant collected from a DC/PBL/USSC coculture. (C) Concentration of IL-10 (C) as well as IL-2 (D) in DC/PBL and DC/PBL/USSC cultures. Results are expressed as mean values \pm SEM.

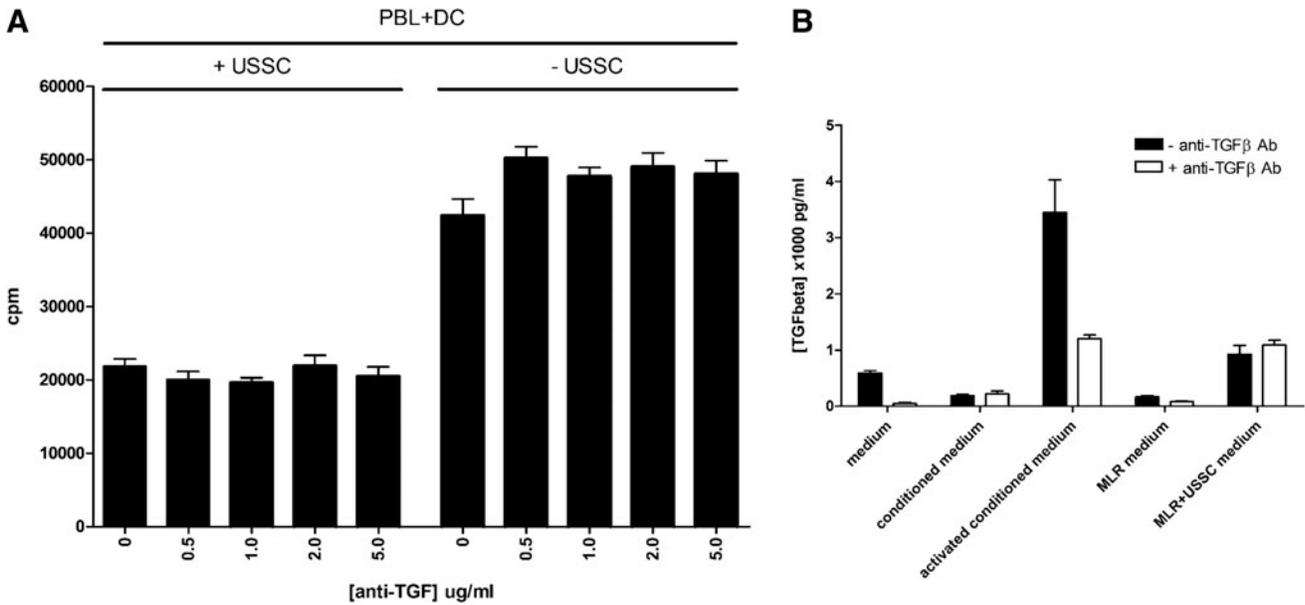


FIG. 4. (A) Titration of TGF- β neutralizing antibodies. At all concentrations tested the antibodies do not restore T-lymphocyte proliferation. (B) TGF- β 1 measurements by using a luciferase reporter gene in different supernatants of mixed cultures. Results are expressed as mean values \pm SEM.

antibody indeed can neutralize the activity of TGF- β . The levels of active TGF- β are again low in supernatants of PBL/DC cocultures, but this level increases significantly when USSCs are added to the coculture. In contrast to the activated conditioned medium of USSCs, addition of anti-TGF- β to the supernatant of PBL/DC/USSC cocultures has no effect on active TGF- β levels. This indicates that in this case the increase in luciferase activity is not caused by an elevated level of active TGF- β , but is caused by the upregulation of another factor that has a receptor on the reporter cell.

Human leukocyte antigen G

Based on the microarray data HLA-G1 and HLA-G3 were more than two times upregulated. The nonclassical HLA class I antigen HLA-G, physiologically expressed on extravillous cytotrophoblast at the fetomaternal interface during pregnancy, plays an important role in protecting the semi-allogeneic fetus from maternal immune attack and subsequent rejection [45]. Multiple isoforms of the HLA-G gene are generated by alternative splicing of a single messenger giving rise to four membrane-bound isoforms (HLA-G 1 through -4) and three soluble isoforms (HLA-G5, -G6, and -G7), generated by the inclusion of intron 4, which encodes a premature stop codon [46,47]. The soluble isoforms therefore lack the exons encoding for both the transmembrane and cytoplasmic domains. HLA-G isoforms were upregulated at least four times as deduced from microarrays of the DC/USSC cocultures.

Interestingly, previous published data by Lila et al. show that soluble HLA-G5, but not the membrane-bound HLA-G isoform, is able to suppress the allogeneic proliferative T cell response [48]. Further, addition of soluble HLA-G5 to MLRs showed an inhibitory effect on T cell proliferation in 53% of the cases [49]. Later on, a functional role for soluble HLA-G antigens in immune modulation mediated by MSCs was shown [43].

Recently, the implication of HLA-G in the inhibitory effect mediated by MSCs was investigated [44]. Although there was no significant difference in level of HLA-G expression in culture supernatant of MSC/MLR compared with MSCs cultured alone, using a neutralizing HLA-G antibody it was possible to partly counterbalance MSC-immunosuppressive effects [44]. By using the same neutralizing HLA-G antibody in MLR cultures in the presence or absence of USSCs, we observed a slight, yet not significant, restoration of T-lymphocyte proliferation (Fig. 5). This again indicates that the mechanism used by MSCs to inhibit T-lymphocyte proliferation differs from that used by USSCs.

GM2 ganglioside activator

The gene GM2 ganglioside activator (GM2A) is a cofactor for hexosaminidase A (HEXA) and hexosaminidase B

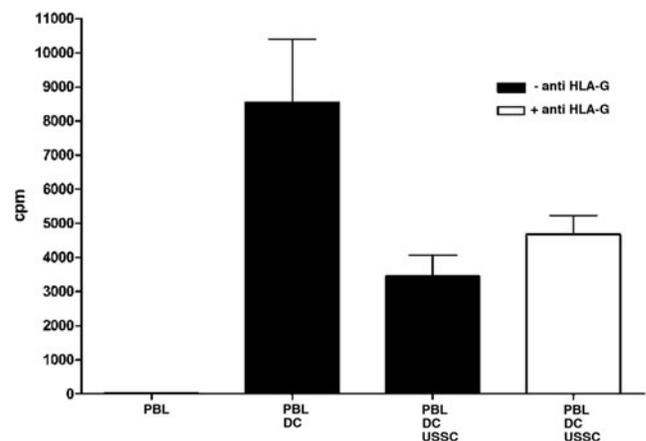


FIG. 5. Partial restoration of proliferation after neutralizing HLA-G. Results are expressed as mean values \pm SEM.

(HEXB), which together constitute the lysosomal enzyme hexosaminidase. This complex converts ganglioside GM2, a sialic-acid-containing glycosphingolipid, into ganglioside GM3 and N-acetyl-D-galactosamine. GM3 is implicated in the induction of cell differentiation, modulation of cell proliferation, and maintenance of fibroblast morphology, signal transduction, and integrin-mediated cell adhesion. Importantly, GM3 has been shown to inhibit immune function, and is produced at high levels by melanoma and other cancers [50–52]. The microarray experiments indicated that the four probe sets for GM2A were six times highly expressed in the DC/USSC cocultures. Reverse-transcription quantitative PCR was performed on 48-h cocultures of DCs with USSCs from two different donors, confirming the microarray data. Since it is still possible that GM3 is secreted to a higher extent in cocultures, we tested supernatants of cocultures in MLRs in combination with the antibody against GM3. However, the involvement of GM3 in USSC-mediated immunosuppression was excluded based on the results of those experiments.

Miscellaneous mechanisms

Cortisol prevents proliferation of T-lymphocytes by rendering the IL-2-producer T-lymphocytes unresponsive to IL-1, and unable to produce the T-lymphocyte growth factor [53]. However, no major difference in expression levels of cortisol was observed between MLR and MLR/USSC cultures, 5.59 versus 4.35 nM, respectively. Nitric oxide synthase inhibitors showed no effect excluding NO as the cause of the inhibition.

Microarray data showed that arginase and IDO were not upregulated by coculture of DCs with USSCs excluding those enzymes as the cause of the immune suppression. Recent data for murine MSCs also exclude IDO as the cause of the immune suppression [54].

Comparison of the microarrays revealed that 146 proteins were at least five times upregulated. Osteopontin was upregulated more than 50 times as deduced from the microarray results. Neither recombinant osteopontin nor neutralizing antibodies to osteopontin affected the MLR.

Hydroxyprostaglandin dehydrogenase 15-(NAD) was upregulated 5.7 times. This enzyme is involved in the breakdown of PGE2. Downregulation of this enzyme is found in inflammatory bowel disease leading to higher PGE2 levels [55]. The enzyme catalyzes the first step in the catalysis of PGE2 to 15-keto PGE2. Further catalysis is performed by serum albumin to 15-keto PGA22 and 15-keto PGB2 [56].

To earmark the cell that is producing the inhibiting factor, USSCs were mildly fixed with paraformaldehyde. Those fixed cells were unable to inhibit the immune response (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Those results are best explained by assuming that upon contact of DC with USSC the latter cell produces the inhibiting factor.

Lipid metabolites

Supernatant was fractionated using filters with different molecular weight cutoffs. The flow through was tested in DC-T cell cultures. The soluble factor appeared to be very small because the flow through of the 5-kDa filter still contained the suppressive entity. Supernatant from DCs

cultured with T-cells in the presence and absence of USSCs was analyzed for lipid metabolites.

The concentration of 86 metabolites is shown in Fig. 6. Prostaglandin B2 is not detected in the supernatant of DCs cocultured with allogeneic T-cells. When those cells were cocultured with USSCs over 60 nM of prostaglandin B2 was detected (Fig. 6).

Next we performed DC-T cell cultures in the presence and absence of prostaglandin B2. Indeed a dose-dependent inhibition of T cell proliferation was found (Fig. 7).

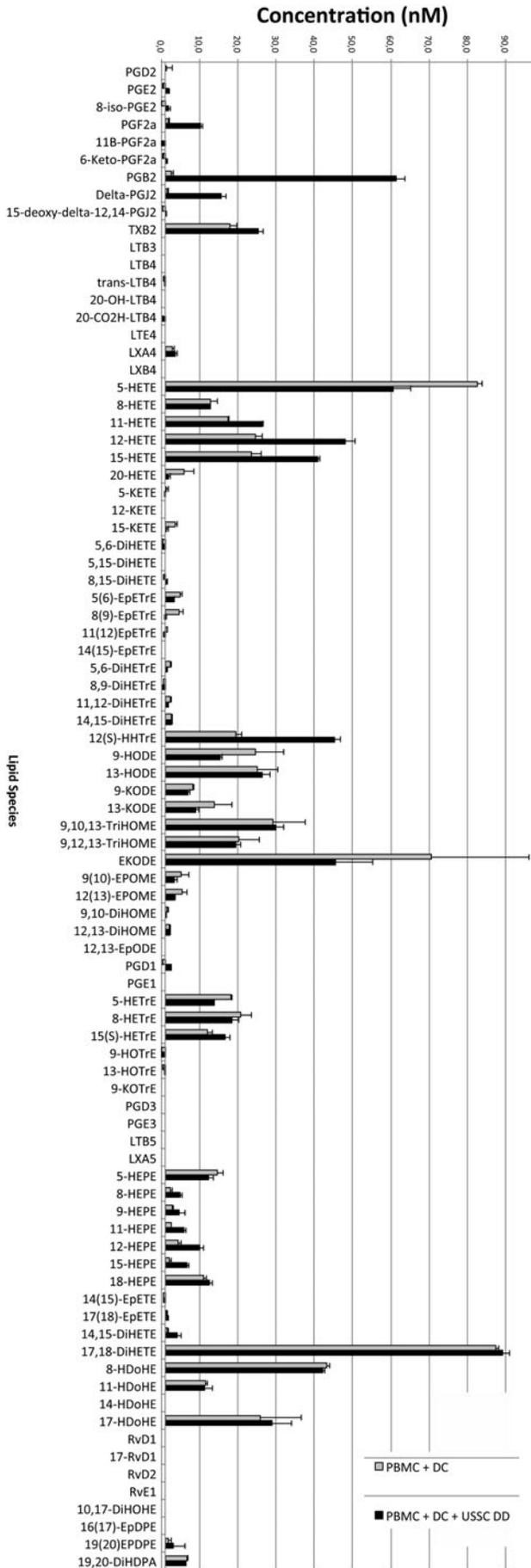
To further prove the involvement of PGB2, the putative receptor Thromboxane receptor A2 was blocked using four different antagonists: L655,240; ICI 192,605; L670,596; and AA 2414. The results are shown in Fig. 8. As is evident by blocking the Thromboxane receptor A2, the suppressive effect of PGB2 on the proliferation is abrogated. Different results were found for the different antagonists and also dependent on the T cell donor. L655,240 and L670,596 were found in all donors to abrogate the suppressive effect, while ICI appeared unable to block the suppressive effect of prostaglandin B2.

Discussion

Various reports in literature suggested that MSCs inhibit T-lymphocyte activation and induce immune tolerance. However, the precise mechanism underlying this phenomenon is still unclear. While one study suggested that MSCs interfere with contact between T-lymphocyte and APC [12], another study suggested that the MSCs themselves act as veto cells [57]. An indirect inhibitory effect of MSCs mediated by the APCs was proposed [37] or the involvement of IFN- γ in the induction of immune suppression [58].

Here, we report for the first time the characterization of the immunosuppressive properties of USSCs. We show that human USSCs do not induce an alloantigen-specific primary T-lymphocyte proliferative response. Like MSCs, they actively suppress T-lymphocyte proliferation induced by allogeneic cells. This suppression is hardly observed when USSCs are separated from the target cells by a TW membrane, indicating that the suppression is in part mediated by contact. Our data suggest that this direct contact leads to the activation of a soluble factor, resulting in a strong inhibition of T-lymphocyte proliferation. Further, the levels of IL-2 and macrophage migration inhibitory factor (MIF; data not shown), both factors known to induce T-lymphocyte proliferation [59,60], are upregulated in MLR/USSC cultures as compared with MLR cultures, indicating that the soluble factor responsible for the observed reduction of T-lymphocyte proliferation mediated by USSCs is an extremely potent inhibitor. It was described that CD90-positive MSCs reduced lympho-proliferative response in PHA-activated PBMCs [61]. On the other hand MSCs show a significantly lower expression for CD90 antigen, responsible for a lympho-proliferative allogeneic response in PHA/PBMCs [61]. These data suggest that the CD90 molecule may be considered a novel predictive marker for MSC inhibitory ability. CD90 is also highly expressed on USSCs [4], which we describe here as very potent inhibitors of lymphocyte proliferation.

TGF- β and IL-10 are well known for their immunosuppressive properties. Therefore we quantified IL-10 expression in MLR supernatants cultured with or without USSCs. Equal



amounts of IL-10 expression were observed in both cultures, indicating that IL-10 is not responsible for the inhibition of T-lymphocyte proliferation by USSCs. Previous studies using BM-MSCs showed that addition of an anti-TGF- β neutralizing antibody did not significantly reverse the BM-MSC suppressive effect [12,13,62]. Only one study reported that the addition of anti-TGF- β antibodies to MLR in the presence of BM-MSCs could restore T-lymphocyte proliferation, although the actual TGF- β level was not quantified in these supernatants [11]. Recent analysis of cytokine production of USSCs and BM-MSCs showed that TGF- β is produced in both cell types at high levels, 797.4 ± 78.0 and 489.5 ± 188.5 pg/mL, respectively [8]. In addition, activation of conditioned USSC medium, a method to activate latent TGF- β [28], resulted in a significant inhibition of T-lymphocyte proliferation. Therefore, activation of TGF- β during MLR/USSC is a prime candidate. To this purpose we performed MLR/USSC cocultures in the presence of anti-TGF- β neutralizing antibodies. However, addition of these antibodies did not restore T-lymphocyte proliferation, indicating that TGF- β is not involved in this process. This was confirmed by the determination of the levels of active TGF- β in supernatants of PBL/DC/USSC cocultures. Although there seems to be an increase of active TGF- β levels as compared with the PBL/DC coculture, addition of neutralizing anti-TGF- β antibody did not diminish this level. Despite the fact that this system is specifically designed to measure active TGF- β levels in supernatant, it is not a solid quantification method. These results are indicative for another soluble factor that binds to a cellular receptor and triggers the luciferase reporter responsible for the increase in luciferase activity.

Lipid metabolites of supernatants of normal MLR and USSC MLR showed a great similarity. Only a few metabolites differed. The prostaglandin B2 is an unusual finding. Only a few reports are available about this compound. PGB2 is obtained after dehydration of PGE2 [63]. Remarkably, PGB2 enhances the proliferation of Jurkat cells [64]. PGB2 is reported to bind to the thromboxane A2 receptor causing pulmonary hypertension [65]. More importantly naive T cells express the thromboxane A2 receptor while DCs produce thromboxane. An agonist of the thromboxane receptor impairs DC-dependent proliferation [66]. The binding of PGB2 to the thromboxane receptor explains the suppression found here in DC-T cell cultures. It is unexpected that cord-blood-derived MSCs express this prostaglandin. However, PGB2 was found in early ovine embryos, which makes it more plausible that PGB2 is found in umbilical cord MSCs [67]. Moreover, the enzyme-converting PGE2 was found to be upregulated. Further degradation is accomplished by serum albumin. During delivery, spontaneous closure of umbilical blood vessels occurs. Of the six naturally occurring prostaglandins, PGB2 was the most potent prostaglandin found in umbilical cord in constriction of UCB veins [68]. Also fetal membranes are metabolizing PGE2 among other metabolites into PGB2 [69].

FIG. 6. Lipid metabolites found in the supernatant of cultures of DCs+T cells and DCs+T cells+USSCs. The analysis of 86 metabolites is shown. Analysis was performed three times. Error bars show standard deviation.

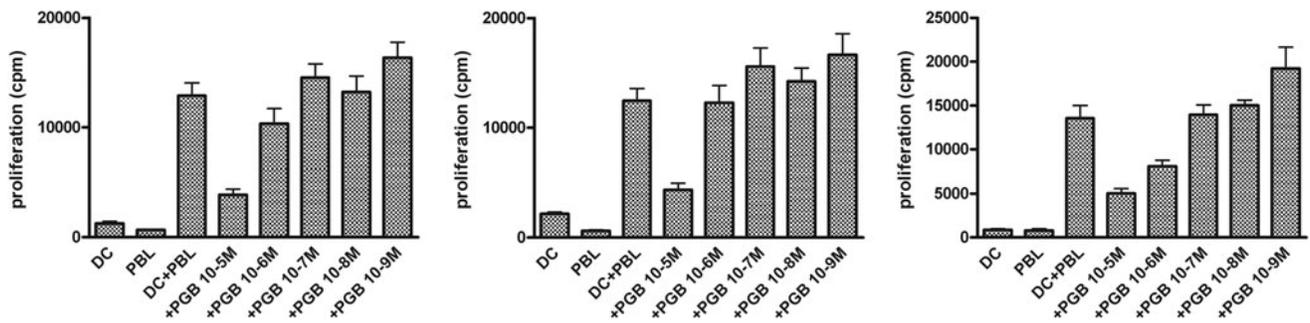


FIG. 7. Proliferation of DC-T cell cocultures in the presence of prostaglandin B2 for three T cell donors. Results are expressed as mean values ± SEM.

Conclusions

Cord blood MSCs suppress T-cell proliferation induced by dendritic cells. This suppressive effect is not caused by the known mechanisms as found for bone marrow MSCs as deduced from microarray studies and functional tests. Only blocking HLA-G was able to partially

restore T-cell proliferation. Prostaglandin B2 was found in supernatants of MLR in the presence of USSCs and able to inhibit T-cell proliferation. Besides its role in closure of cord blood vessels during delivery, it could also play a role in dampening the immune response in the feto-maternal interface contributing to the tolerance of the fetus.

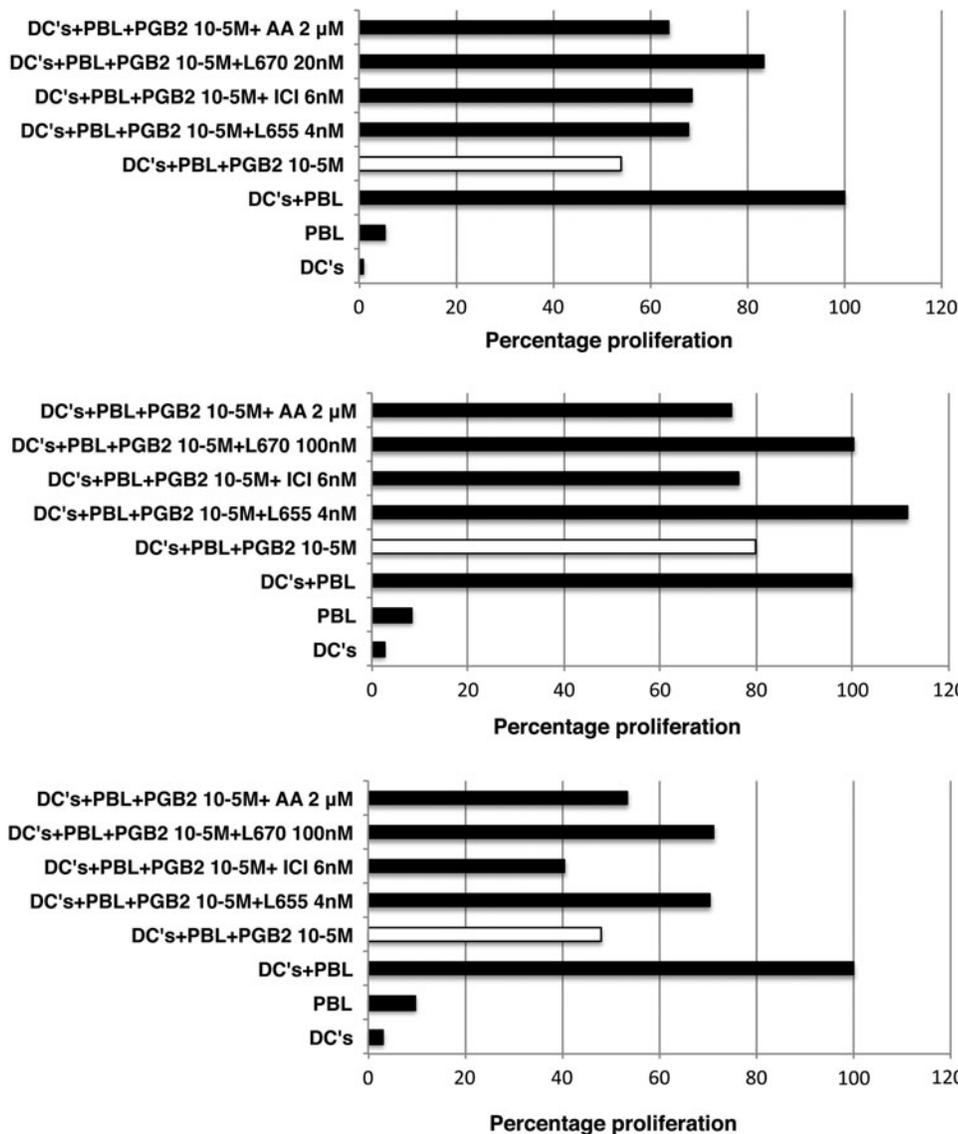


FIG. 8. The suppressive effect of prostaglandin B2 is reversed by thromboxane A2 receptor antagonists. L655,240 was used at a concentration of 4 nM; ICI 192,605 at 30 nM; L670,596 at 20 nM; and AA 2414 at 2 μM. Three donors were tested. To normalize the data between donors, the proliferation of DCs and PBLs was set at 100%. The open bar shows the inhibition caused by PGB2.

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Author Disclosure Statement

The authors declare that they have no competing financial interests.

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