Although there were four samples with discordant result in dual/X4 samples, FPR of these samples were low (range: 14.7–23.6%).

Discussion

We developed a quick, safe and sensitive HIV-1 PTA utilizing double split proteins (DSP-Pheno) and validated the specificity of the assay using laboratory strains with known co-receptor usage. We recognize several limitations of this preliminary study, but the results nevertheless are promising. We assayed bulk envelope genes amplified from plasma from HIV-1-infected patients, rather than cloned envelope genes, and our sample only included subtype B HIV-1. Future studies are necessary to demonstrate the usefulness of the DSP-Pheno.

One caveat of the DSP-Pheno assay is that it is a cell-fusion system, and cell–cell fusion may differ in significant details from virus–cell fusion. For example, recent studies have shown that HIV-1 virions carry fewer surface glycoproteins than previously assumed [24]. The DSP-Pheno assay uses neuroglyoma cell-derived NP-2 cell lines with overexpressed CD4 and co-receptors. Although these NP-2-derived cell lines have been characterized extensively [16,17], some unknown cell surface molecules may be involved in the fusion process. The DSP-Pheno assay is a gag-free system and requires only the assembly of reporter proteins pre-formed in the fusion partner, but infection by a retrovirus requires that the entire gag particle pass through the fusion pore. Careful comparison between DSP-Pheno and in-house pseudoviral assay or GTA using clonal clinical isolates is under way.

GFP portion is necessary as a module of DSP to compensate weak self-association of split RL [15]. Although RL would be more suitable for quantitative assay, GFP may prove single clear positive fusion in the sample with very low RL readout. This feature of DSP-Pheno incorporating two different assays may be useful for certain scientific purposes.

Although several issues remain to be clarified, DSP-Pheno has multiple advantages over the conventional pseudoviral PTA: (i) the turnaround time for DSP-Pheno is short, with results available in as few as 5 days, starting from patients' plasma; (ii) DSP-Pheno is a virus-free assay that does not require a special biosafety facility, making it particularly appealing for in-house use; and (iii) the RL assay in DSP-Pheno has high sensitivity and specificity and compares favourably with the best pseudoviral PTA published in the detection of minor X4 populations using laboratory strains. Trofile™ (Monogram Biosciences Inc., CA, USA) is currently the only commercially available PTA approved for clinical use, and the latest version, "Enhanced Trofile™," detects X4 minor populations present in concentrations as low as 0.3% [25]. A pseudoviral PTA described by Soda and colleagues had 1% detection threshold for X4 viruses [16]. Although the RL assay in DSP-Pheno could detect X4 laboratory strains present in concentrations as low as 0.3%, further studies are needed to apply the assay for the clinical use. DSP-Pheno may also be useful for the comparison of with GTA to improve the algorithm for the co-receptor usage of non-B subtypes.

Conclusions

We described a new cell-fusion-based, high-throughput PTA for HIV-1, which would be suitable for in-house studies. Equipped with a two-way reporter system, RL and GFP, DSP-Pheno is sensitive and offers a short turnaround time. Although maintenance of cell lines and laboratory equipment for the assay is necessary, it provides a safe assay system without infectious viruses. With further validation against other conventional analysis, DSP-Pheno may prove to be a useful laboratory tool. The assay may be useful especially for the research on non-B subtype HIV-1 whose co-receptor usage has not been studied much.

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Competing interests

AI has received grant support from Toyama Chemical Co. Ltd., Astellas, ViiV Healthcare KK, MSD KK, Baxter through the University of Tokyo. AI has received speaker's honoraria/payment for the article from Eiken Chemical Co. Ltd., Astellas, Toyama Chemical Co. Ltd, Torii Pharmaceutical Co. Ltd., Takeda Pharmaceutical Co. Ltd. and MSD.
For the remaining authors, there are no competing interests.

Authors' contributions

PT, NH and AI planned the experimental design. PT and NH did the experiments. NK, ZM and HH provided the materials. ZM, HH, AK-T and GFG and joined the discussion. TF, TK, HN, MK and AI were responsible for the patient care and provided clinical information. PT, NH and AI wrote the article. PT and NH contributed equally to the work.

Acknowledgements

This work was supported in part by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for Program of Japan Initiative for Global Research Network on Infectious Diseases (10005010) (AI); Global COE Program (Center of Education and Research for Advanced Genome-Based Medicine – For personalized medicine and the control of worldwide infectious diseases) of MEXT (F06) (AI); JSPS KAKENHI (25293226) (AKT); JSPS KAKENHI (24790437) (NH); Grants for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H24-AIDS-IPPAN-008) (AKT); Grants for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H25-AIDS-IPPAN-006) (NH); Research on international cooperation in medical science, Research on global health issues, Health and Labour Science Research Grants, the Ministry of Health, Labor, and Welfare of Japan (H25-KOKUI-SITEI-001) (AI).
The authors thank Ms. Barbara Rutledge for discussion and editing English.

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Journal of Reproductive Immunology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm

Human decidual macrophages suppress IFN-γ production by T cells through costimulatory B7-H1:PD-1 signaling in early pregnancy

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ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form 29 July 2013
Accepted 2 August 2013

Keywords:
Costimulation
B7-H1
Decidual macrophage
Human pregnancy

ABSTRACT

In human pregnancy, CD14+ decidual macrophages (DMs) are the dominant professional antigen-presenting cells in the decidua, comprising 20–30% of the local leukocyte population. Although the relevance of DMs to feto-maternal immune tolerance has been described, the molecular mechanisms underlying these functions have not been fully elucidated. B7-H1, a costimulatory ligand in the B7 family, negatively modulates T cell activity by binding to its corresponding receptor, PD-1. The present study aimed to investigate the functional significance of costimulatory interactions between DMs and T cells, with a particular focus on B7-H1:PD-1 signaling. An analysis of the expression profile of B7 ligands on human DMs revealed that B7-H1 was present on DMs isolated from early but not term pregnancies. B7-H1 was not expressed on the peripheral monocytes (PMs) of pregnant women. In response to IFN-γ, B7-H1 expression was induced on PMs and was enhanced on DMs, suggesting that this cytokine might be a key factor in the control of B7-H1 expression in the decidua. The majority of decidual T cells were noted to exhibit robust expression of PD-1, whereas the expression was limited to a small subpopulation of circulating T cells. Functional assays demonstrated that DMs are able to suppress T cell IFN-γ production via B7-H1:PD-1 interactions. This suppressive property was not observed for PMs, which lack B7-H1. B7-H1 on DMs may function as a key regulator of local IFN-γ production and thereby contribute to the development of appropriate maternal immune responses to the fetus in early pregnancy.

1. Introduction

The uterine decidua is the interface at which maternal immunocompetent cells encounter paternally inherited fetal antigens. The evasion of potentially harmful alloresponses by the maternal immune system is required for the maintenance of pregnancy. Although the mechanisms involved in the prevention of excessive maternal immune activation are still incompletely understood, it is hypothesized that the development of feto-maternal immune tolerance is multi-faceted and redundant and involves humoral factors and cell-to-cell interactions (Trowsdale and Betz, 2006).

In human pregnancy, CD14+ myelomonocytic cells called decidual macrophages (DMs) comprise 20–30% of the total leukocyte population in the decidua (Trundley et al., 2006). This percentage remains stable until it decreases at term, implying that these cells might play a role in the maintenance of local homeostasis. Macrophages are categorized as professional antigen-presenting cells (APCs) and are therefore important contributors to the initial immune response to external pathogens and alloantigens (Mizuno et al., 1994). The tissue-specific activities of local macrophages are determined by the

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0165-0378/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jri.2013.08.001

Please cite this article in press as: Sayama, S., et al., Human decidual macrophages suppress IFN-γ production by T cells through costimulatory B7-H1:PD-1 signaling in early pregnancy. J. Reprod. Immunol. (2013), http://dx.doi.org/10.1016/j.jri.2013.08.001

characteristics of the surrounding cytokine milieu and via receptor-ligand-induced signaling after direct contact with other local cells (Miller and Hunt, 1996). Although recent studies using gene expression arrays revealed that DMs display unique phenotypic attributes, such as showing M2 polarity (Gustafsson et al., 2008; Houser et al., 2011), their functional relevance to local immune regulation has not been fully elucidated.

Typical antigen presentation to T cells is mediated by primary interactions between major histocompatibility complex (MHC) molecules on APCs and the T cell receptors (TCR). Secondary or costimulatory signaling modifies the effects of primary ligand receptor binding and involves the binding of a second ligand on APCs to a corresponding receptor on the surface of T cells (Mueller et al., 1989). Costimulatory interactions can positively or negatively modulate primary signaling and will ultimately determine the type and quality of T cell responses such as clonal proliferation, cytokine production, and functional differentiation. The B7 family is a major costimulatory ligand group. B7.1 and B7.2, the first members of this family to be described, bind to their common corresponding receptors, CD28 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (Freeman et al., 1991, 1993). Ligation with CD28 induces activation of T cells, whereas interactions with CTLA-4 generate inhibitory signaling. In the last decade, several new members have been added to the B7 costimulatory ligand group, including B7-H1 (Freeman et al., 2000), B7-DC (Youngnak et al., 2003), B7-H2 (Yoshinaga et al., 1999), B7-H3 (Sun et al., 2002), B7-H4 (Ishitani et al., 2003), and B7-H6 (Brandt et al., 2009). B7-H1 and B7-DC act as inhibitory costimulatory ligands by binding to their common receptor, programmed death-1 (PD-1), which is expressed on activated but not on resting T cells (Freeman et al., 2000). Involvement of the PD-1 pathway in peripheral tolerance is suggested in PD-1-deficient mice that develop a lupus-like autoimmune disease (Nishimura et al., 1999) and autoimmune dilated cardiomyopathy (Lucas et al., 2008).

Accumulating evidence implicates B7-mediated signaling in feto-maternal immune regulation. We have previously shown that decidual stromal cells constitutively express B7-H1 and B7-DC, and that interactions between B7-H1 and B7-DC with their ligands are likely involved in the suppression of excessive T cell activation in the decidua (Nagamatsu et al., 2009). Enhanced expression of inducible costimulator (ICOS), a receptor for B7-H2 (Nagamatsu et al., 2011) and PD-1 (Taglauer et al., 2008) has been confirmed by us and others on a majority of the T cells residing in the decidua. Taken together, these findings imply that B7 costimulatory pathways may be critical in the fine tuning of T cell activity at the feto-maternal interface.

The primary aim of this study was to clarify the association of B7-mediated costimulatory signaling system in immune regulatory interactions between DMs and T cells at the feto-maternal interface. We examined the expression profiles of B7 ligands on DMs and of their corresponding receptors on T cells in the human decidua.

The types and expression level of costimulatory ligands on local macrophages are controlled by locally produced inflammatory cytokines and determine specific immunological characteristics of these antigen-presenting cells (Nagamatsu and Schust, 2010). IFN-γ is known as a key inflammatory cytokine that modulates a wide variety of immunological processes, including costimulatory signaling. The production of this cytokine in the early decidua has been described (Ashkar et al., 2000). In this study, we confirmed specific B7-H1 expression on DMs in the early stages of pregnancy and the expression is controlled by IFN-γ. Additionally, we defined the possible impact of DM-derived costimulatory signaling via B7-H1 ligation with T cell-expressed PD-1 on IFN-γ production by T cells.

2. Materials and methods

2.1. Monoclonal antibodies

Purified mouse anti-human CD3 monoclonal antibody (mAb) (clone name: OKT3), FITC-conjugated mouse anti-human CD14 (HCD14) mAb, PE-conjugated mouse anti-human B7-H1 (MIH1), B7-DC (MIH18), B7-H2 (MIH12), B7.1 (2D10), B7.2 (IT2.2), PD-1(CD279) mAbs, and allophycocyanin-conjugated mouse anti-human HLA-DR (L243) mAb were purchased from Biolegend (San Diego, CA, USA). FITC-conjugated anti-human CD4 and APC-conjugated anti-human CD8 antibodies were purchased from e-Bioscience (San Diego, CA, USA).

2.2. Sample collection

Human first-trimester decidual samples were collected from elective pregnancy termination cases performed at 7 to 11 weeks' gestation ($n = 10$). Human term decidual samples were collected from elective cesarean section cases without maternal or fetal complications ($n = 10$). Peripheral blood was collected from each subject prior to initiation of the termination procedure or cesarean section. Informed consent was obtained and the use of human tissues was conducted under the IRB approval of the University of Tokyo.

2.3. Isolation of decidual macrophages, peripheral monocytes, and T cells

Decidual tissues from early pregnancy were isolated by removing macroscopic blood clots and trophoblast villi. Term decidual samples were prepared by carefully separating the decidual layer from the fetal membranes (decidua parietalis) and the maternal surface of the placentas (decidua basalis). Decidual samples were minced and enzymatically digested in 1 mg/ml type I collagenase and 300 U/ml DNAse I (Sigma Aldrich, St. Louis, MO, USA) for 20 min at 37 °C. Cell suspensions were filtered through a 40-μm cell strainer (BD Bioscience, San Jose, CA, USA) and centrifuged at 300 × g for 10 min. The resultant cell pellet was re-suspended in phosphate-buffered saline (PBS) and layered on Percoll Plus™ (GE Healthcare) diluted to a concentration of 35% with PBS. Ficoll-Paque Plus™ (GE Healthcare Japan, Asahigaoka, Tokyo) was then layered beneath the Percoll layer. The prepared tube containing three liquid layers was centrifuged at

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500 × g for 40 min. Cells floating between the middle and lower layers were collected. DMs were purified from these decidual mononuclear cells (DMCs) using immunomagnetic positive selection with anti-CD14 Ab-coated magnetic beads (MACS; Miltenyi Biotec, Auburn, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples using standard density gradient centrifugation over Ficoll-Paque Plus™. Peripheral monocytes (PMs) were isolated from the PBMCs using anti-CD14 immunomagnetic beads in a method similar to that performed to separate DMs from DMCs.

T cells in DMCs and PBMCs were isolated using immunomagnetic negative selection (pan T cell collection kit, MACS, Miltenyi Biotec) as previously described (Nagamatsu et al., 2011). Flow cytometric analysis was conducted to check the isolation purity by staining the collected cells with anti-CD3 antibody. The purity of both decidual T cells and peripheral T cells after immunomagnetic selection was constantly above 97%.

2.4. Flow cytometric analysis

Purified DMs and PMs were incubated in human serum for 5 min at 4 °C to block the Fcγ receptor. The cells were then exposed to PE-conjugated mAbs for B7 family ligands for 30 min at 4 °C. PE-conjugated isotype-matched mouse IgG was used as a negative control. After washing three times with PBS/1% bovine serum albumin (BSA), the cells were resuspended in 500 μl of PBS/1% BSA and analyzed by flow cytometry (FACS Caliber; Becton Dickinson, Mountain View, CA, USA). For experiments examining the effect of IFN-γ on B7H1 expression, DMs were stimulated in a 12-well plate with or without 100 ng/ml of IFN-γ for 48 h prior to immunostaining and analysis.

Multicolor flow cytometric analysis was performed to examine PD-1 expression on T cells in the decidua and peripheral blood. Following immunomagnetic beads selection, T cells were stained with FITC-conjugated anti-human CD4 and APC-conjugated anti-human CD8 antibodies together with PE-conjugated anti-PD-1 antibody. PD-1 expression level was investigated on histograms by gating a CD4-positive or CD8-positive subset.

2.5. Co-culture of DMs or PMs with T cells and antibody blockade

Peripheral blood mononuclear cells were isolated from the peripheral blood of healthy third-party donors by density gradient centrifugation using Ficoll-Paque™ Plus. T cells were purified from the PBMCs using immunomagnetic negative selection (pan T cell collection kit, MACS, Miltenyi Biotec). DMs and PMs were suspended in RPMI 1640 containing 10% fetal bovine serum (FBS) and seeded onto a 96-well plate (4×10^4 cells/well). DMs and PMs were incubated for 3 h either with anti-B7H1 mAb (M1H1) (5 μg/ml) or normal mouse IgG (5 μg/ml; negative control). After removing unbound antibodies by washing twice with culture media, freshly isolated allogeneic T cells (2×10^5 cells/well) and anti-CD3 mAb (OKT3) at a concentration of 200 ng/ml were added to each well to activate TCR-CD3 complex and to induce PD-1 expression on T cells. Culture supernatants from these co-cultures were collected at 72 h and stored at −80 °C for bulk cytokine measurement.

2.6. Cytokine measurement

For the analysis of the effects of B7-H1 signaling on T cell cytokine production in co-cultures, IFN-γ concentrations in culture supernatants were determined using ELISA (ELISA MAX, Biolegend). Supernatants were thawed and diluted 10x with PBS to adjust IFN-γ levels to lie within assay detection levels (8 pg/ml to 1000 pg/ml).

2.7. Statistical analysis

Wilcoxon signed-rank testing was used to compare cytokine concentrations in co-culture assays. $P < 0.05$ was considered significant.

3. Results

3.1. Purity of isolated DMs

We began by verifying the efficacy of our methods for isolation of DMs and PMs. Cells collected from decidual samples were selected using anti-CD 14 magnetic immunobeads. Since the antigenicity for CD14 on DMs and PMs was not saturated by positive immunomagnetic selection, DMs were identified as a double positive cell population by staining for CD14 and HLA-DR in flow cytometric analysis (Fig. 1). The percentages of the $CD14^+HLA\text{-}DR^+$ subset in the isolated cells from both early pregnancy and term decidual samples were constantly above 93%, whereas the isolation purity of PMs was above 95%.

3.2. Expression of B7 family ligands on DMs and PMs

Flow cytometric analyses for B7 family ligands on PMs and DMs from pregnant women in early pregnancy demonstrated that:

(1) B7-H1 and B7.2, but not B7-DC, B7-H2 or B7.1, were expressed on early pregnancy DMs, and
(2) High levels of B7.2, but not B7.1, B7-H1, B7-DC or B7-H2, were detected on the PMs, isolated from the same women.

To determine whether B7 ligand expression on DMs and PMs persisted over the course of human pregnancy, similar analyses of B7 ligand expression pattern were performed on DMs and PMs isolated from term decidual and peripheral blood samples. Term DMs expressed B7.1 and B7.2, but not B7-H1, B7-H2 or B7-DC, whereas no remarkable difference in expression pattern was observed on PMs between early and term pregnancy. These results indicate that, in contrast to PMs and term DMs, expression of B7-H1 is unique to DMs from early pregnancy (Fig. 2).

3.3. Expression of PD-1 on decidual T cells

Since cell surface expression of B7-H1 was limited to DMs from early gestation, we next analyzed the expression

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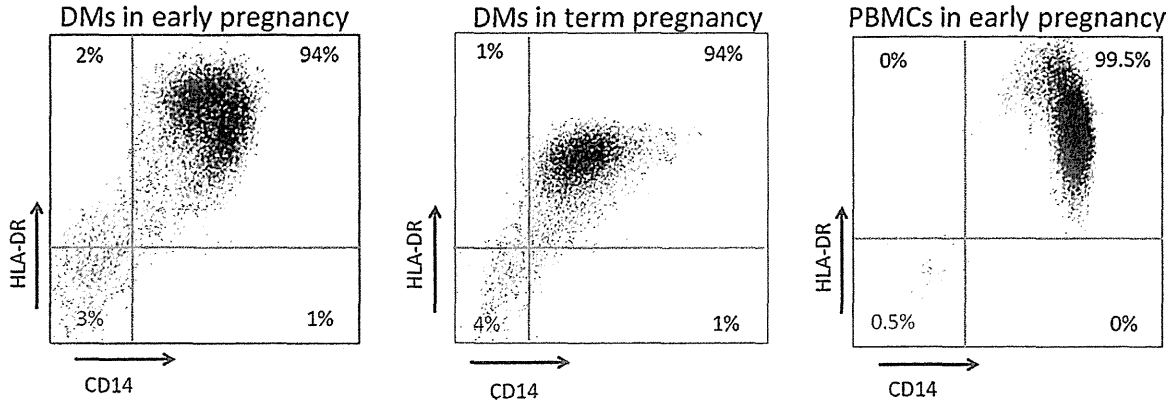

Fig. 1. Purity of the isolated decidual macrophages (DMs) and peripheral monocytes (PMs). DMs were isolated from the decidual samples in early and term pregnancy using immunomagnetic beads, as described in Materials and Methods. PMs were isolated from the peripheral blood of pregnant women in early pregnancy. Isolation purity was confirmed by flow cytometric analysis. DMs and PMs were identified as the $CD14^{+}HLA\text{-}DR^{+}$ cell population in a right-upper quadrant of each dot plot.

of the corresponding receptor, PD-1 on T cells isolated from decidual samples from early pregnancy. Flow cytometric analyses demonstrated that $65.7 \pm 8.7\%$ (mean ± S.E., $n = 5$) of the total T cell population in early decidual samples was positive for PD-1 staining. In the peripheral blood of the same pregnant women, PD-1 is expressed on only $12.5 \pm 4.0\%$ (mean ± S.E., $n = 5$) of T cells (Fig. 3A). The expression intensity of PD-1 was markedly higher on decidual T cells than on peripheral T cells (Fig. 3A). In the analysis for T cells isolated from term pregnancy samples, findings

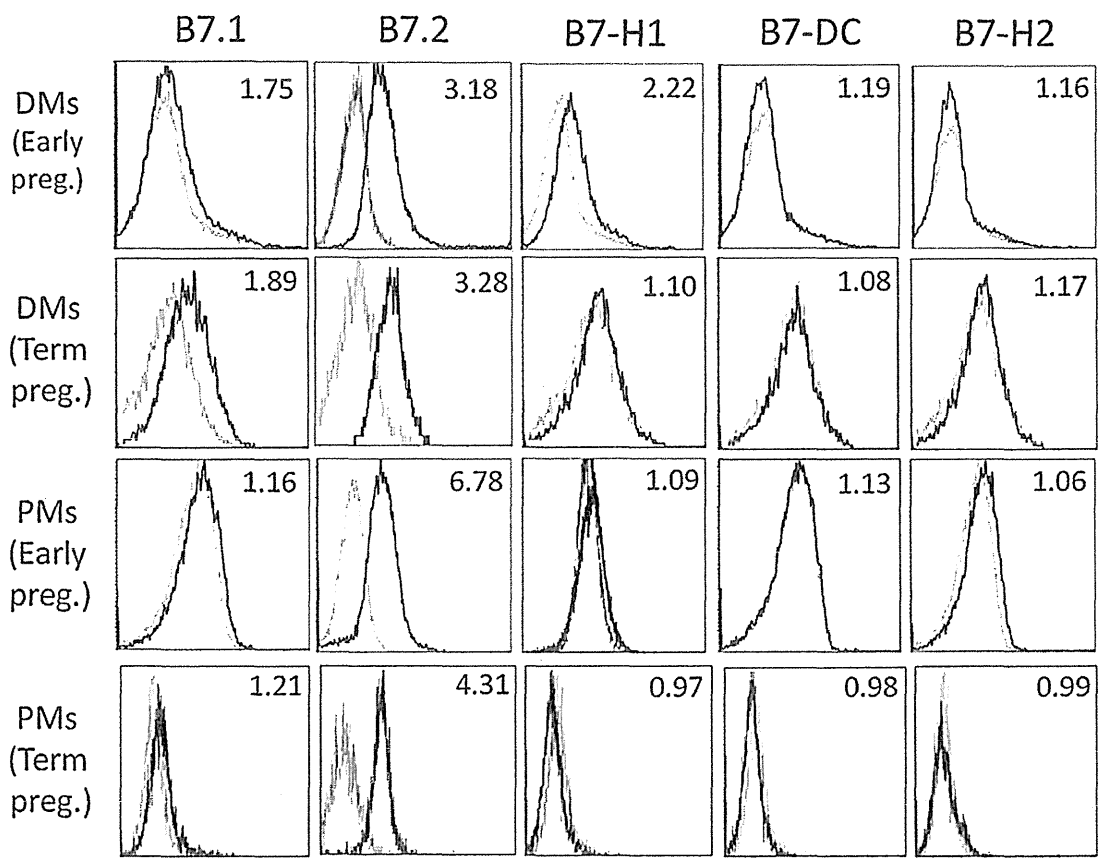

Fig. 2. B7 family ligand expression on DMs and PMs. DMs isolated from the early pregnancy and term pregnancy decidual samples and PMs from women in early and term pregnancy were stained with mAbs specific for B7.1, B7.2, B7-H1, B7-DC, and B7-H2. The expression patterns of B7 family ligands were analyzed on a flow cytometer. In each histogram, gray lines represent isotype controls and black lines represent specific immunostaining. The data shown are representative of five experiments using individual samples.

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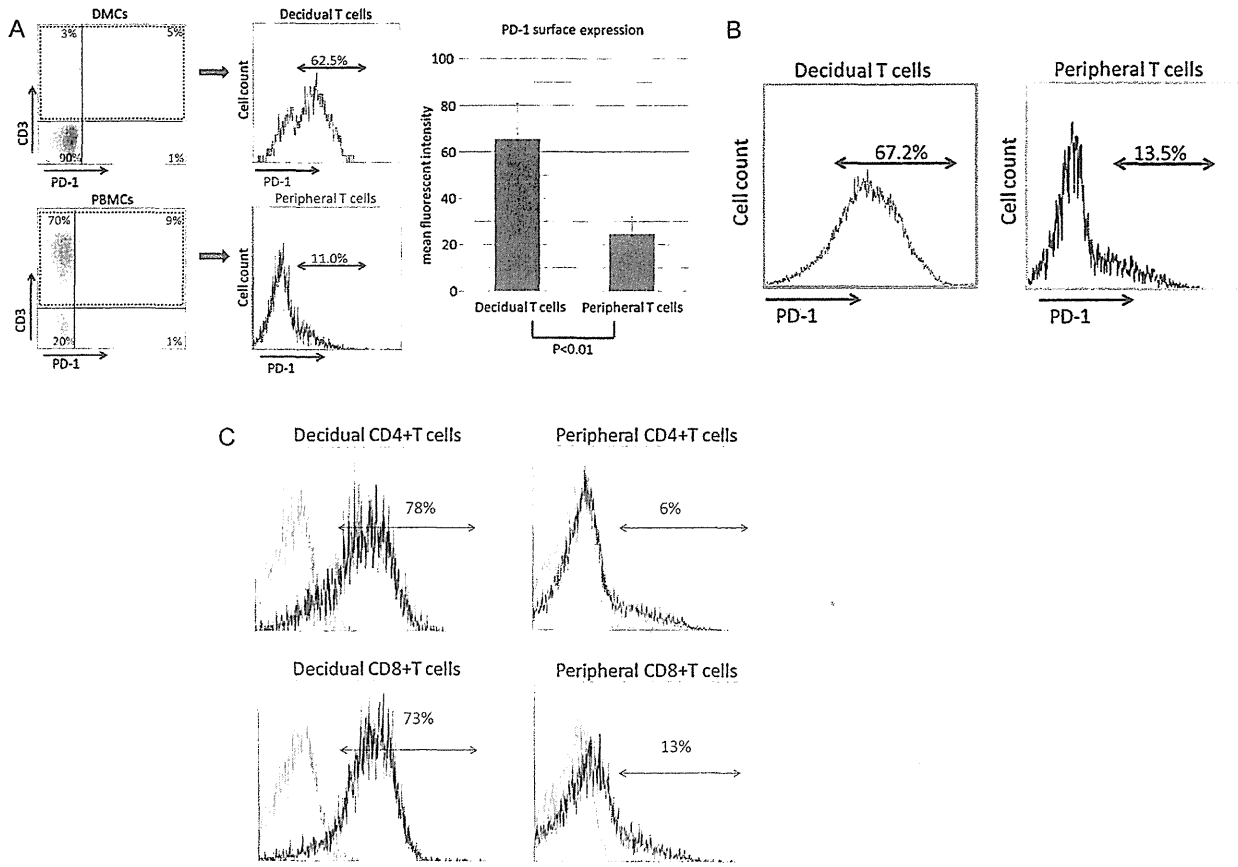

Fig. 3. PD-1 expression on peripheral T cells and decidual T cells. (A) Decidual mononuclear cells (DMCs) were isolated from early pregnancy decidual samples and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood obtained from the same pregnant individual. Both DMCs and PBMCs were stained for CD3, a marker for T cells, and PD-1 expression was examined on decidual and peripheral T cells. In each dot plot, the quadrant areas delimited as CD3- or PD1-positive and -negative were determined based on staining levels using isotype controls. Each histogram on the right presents the percentage of PD-1-positive cells among $CD3^+$ T cells that are included in dashed squares in the left dot plots. The bar chart summarizes mean PD-1 fluorescence intensity on CD3-positive cells (T cells) among DMCs and PBMCs. Error bars = SEM. (B) DMCs in the decidua and PBMCs in peripheral blood of term pregnancy were isolated, and PD-1 expression on T cells was analyzed using the same method as performed in (A). The value on each histogram represents the percentage of PD-1-positive population in total T cells. (C) T cells were isolated from DMCs and PBMCs by negative selection using immunomagnetic beads. T cells were stained for CD4 and CD8 together with PD-1 to examine PD-1 expression on different T cell subsets. PD-1 negative region was determined as an area containing 98% of isotype control, and staining level exceeding the negative region was considered PD-1-positive. The data in (A)–(C) are representative of at least three independent experiments using DMCs and PBMCs derived from individual samples.

obtained were similar to those observed on T cells in early pregnancy, showing contrasting PD-1 expression between peripheral blood and the decidua (Fig. 3B). We also assessed the PD-1 expression on distinct $CD4^+$ and $CD8^+$ T cell subsets using multicolor flow cytometry. $CD4^+$ and $CD8^+$ T cells in the early decidua display intense PD-1 expression with no marked differences noted between $CD4^+$ and $CD8^+$ cells. In contrast, PD-1 expression on $CD4^+$ and $CD8^+$ T cells isolated from the peripheral blood of women in early pregnancy was quite limited (Fig. 3C).

3.4. B7-H1 expression on DMs and PMs is affected by IFN-γ stimulation

IFN-γ is produced in the early decidua and is known to be involved in a variety of immunological processes at the feto-maternal interface. We investigated the effects of IFN-γ on the expression of B7-H1 on PMs and DMs. DMs isolated from early decidual samples were incubated with or without IFN-γ (100 ng/ml) for 48 h prior to flow cytometric analysis of B7-H1 expression. IFN-γ stimulation of PMs isolated from the peripheral blood of the same subject was similarly performed for comparison. Surface expression of B7-H1 was enhanced on IFN-γ-stimulated DMs compared with non-exposed DMs. More interestingly, B7-H1 expression was undetectable on PMs in the absence of IFN-γ, but induced on these cells upon IFN-γ exposure (Fig. 4).

3.5. Impact of B7-H1:PD-1 interactions on IFN-γ production by T cells

Costimulatory ligand expression analyses suggested possible B7-H1:PD-1 interactions between macrophages and T cells co-residing in the decidua during early pregnancy. Therefore, we next used allogeneic co-culture protocols to investigate the role of B7-H1 ligation to PD-1 in the regulation of T cell cytokine production by decidual macrophages. Peripheral T cells isolated from an unrelated

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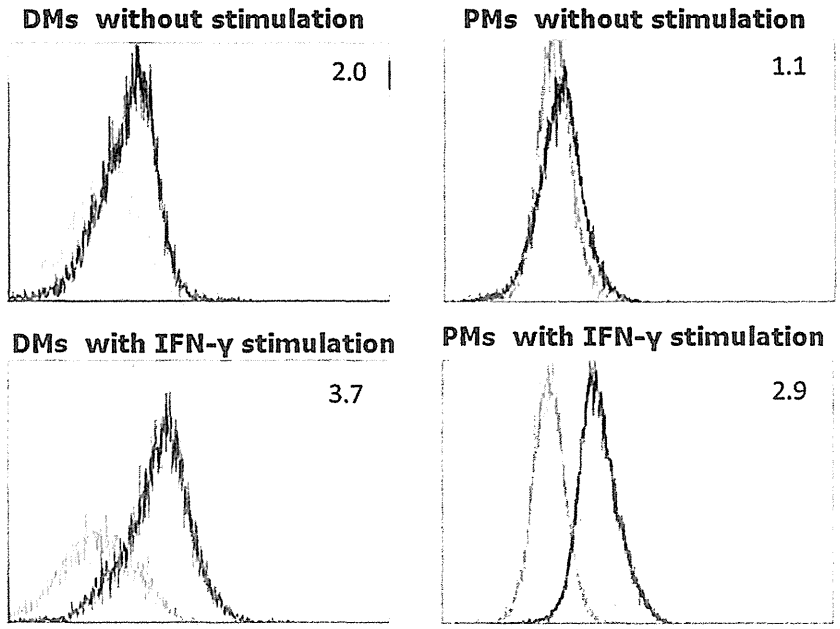

Fig. 4. The impact of IFN-γ stimulation on B7-H1 expression on PMs and DMs. DMs and PMs isolated from the decidua and the peripheral blood of the same woman in early pregnancy were cultured with or without IFN-γ (100 ng/ml) for 48 h. The cells were stained with an anti-B7-H1 mAb and alterations in B7-H1 expression levels were analyzed by flow cytometry. In each histogram, gray lines represent isotype controls and the black lines represent specific immune staining. The numbers in each histogram depict specific staining intensity relative to negative control. The data shown are representative of three experiments using individual samples.

non-pregnant donor were stimulated with anti-CD3 antibody and were co-cultured with early pregnancy DMs or PMs from the same pregnant woman. It has been described in previous studies that T cell activation by OKT-3 (anti-CD3 antibody) induces PD-1 expression on T cells (Ishida et al., 1992). In agreement with the reported fact, OKT-3-induced PD-1 expression was confirmed on T cells introduced into the co-culture in this study (Fig. 5A). This stimulation with anti-CD3 antibody works as a substitute for the activation of TCR-CD3 complex that occurs upon fetal antigen recognition by decidual T cells.

IFN-γ production by T cells under several co-culture conditions was measured using ELISA. IFN-γ production by DMs and PMs cultured in isolation was undetectable with or without anti-B7-H1 antibody, indicating that the source of IFN-γ in this co-culture system was the donor T cells. T cells cultured alone under OKT-3 stimulation produced a small but detectable amount of IFN-γ at baseline. This finding agreed with the known fact that T cell activation is not properly induced by primary signaling via TCR-CD3 complex activation alone without concurrent costimulatory interaction. IFN-γ production increased upon co-culture with both DMs and PMs. This enhancement was an expected reaction of T cells activated in this allogeneic co-culture setting. T cell IFN-γ production was significantly lower under co-culture with DMs compared with PMs, suggesting that DMs have a higher ability to suppress IFN-γ production via B7-H1:PD-1 interaction than PMs (Fig. 5B).

To determine whether the suppression of T cell IFN-γ production by DMs was associated with B7-H1:PD-1 interactions, B7-H1 ligation to PD-1 was blocked by pre-incubating DMs or PMs with anti-B7-H1 mAb prior to co-culture with PD-1$^+$ allogeneic T cells and subsequent ELISA for IFN-γ. IFN-γ production was significantly increased upon antibody-mediated blockade of B7-H1:PD-1 ligation compared with isotype-matched antibody controls when DMs were co-cultured with T cells. Treatment of PMs with anti-B7-H1 mAb prior to co-culture with PD-1$^+$ allogeneic T cells did not significantly increase allogeneic T cell IFN-γ production. These results indicate that DMs can suppress T-cell IFN-γ production through inhibitory interaction mediated by B7-H1:PD-1, whereas PMs without B7-H1 expression lack in such suppressive ability. However, it should be noted that B7-H1 blockade on DMs did not completely recover T cell IFN-γ production to the same level as in the co-culture with PMs (Fig. 5C).

4. Discussion

In the human decidua, CD14$^+$ HLA-DR$^+$ myelomonocytic cells, called DMs, are the predominant APC population throughout gestation (Bulmer and Johnson, 1984). Past studies have characterized this population, as a whole, as immunosuppressive, demonstrated by the abundant production of humoral mediators such as prostaglandin E2 and the anti-inflammatory cytokine, IL-10 (Heikkinen et al., 2003; Parhar et al., 1989). Our findings depict a new immunomodulatory mechanism for DMs that is mediated by inhibitory co-stimulatory signaling. The PD-1 signaling generated upon B7-H1 ligation downregulates phosphatidylinositol 3-kinase/AKT pathway signaling which consequently inhibits T cell activity (Parry et al., 2005). Accumulating evidence has associated in vivo PD-1 signaling with peripheral tolerance and cancer immune

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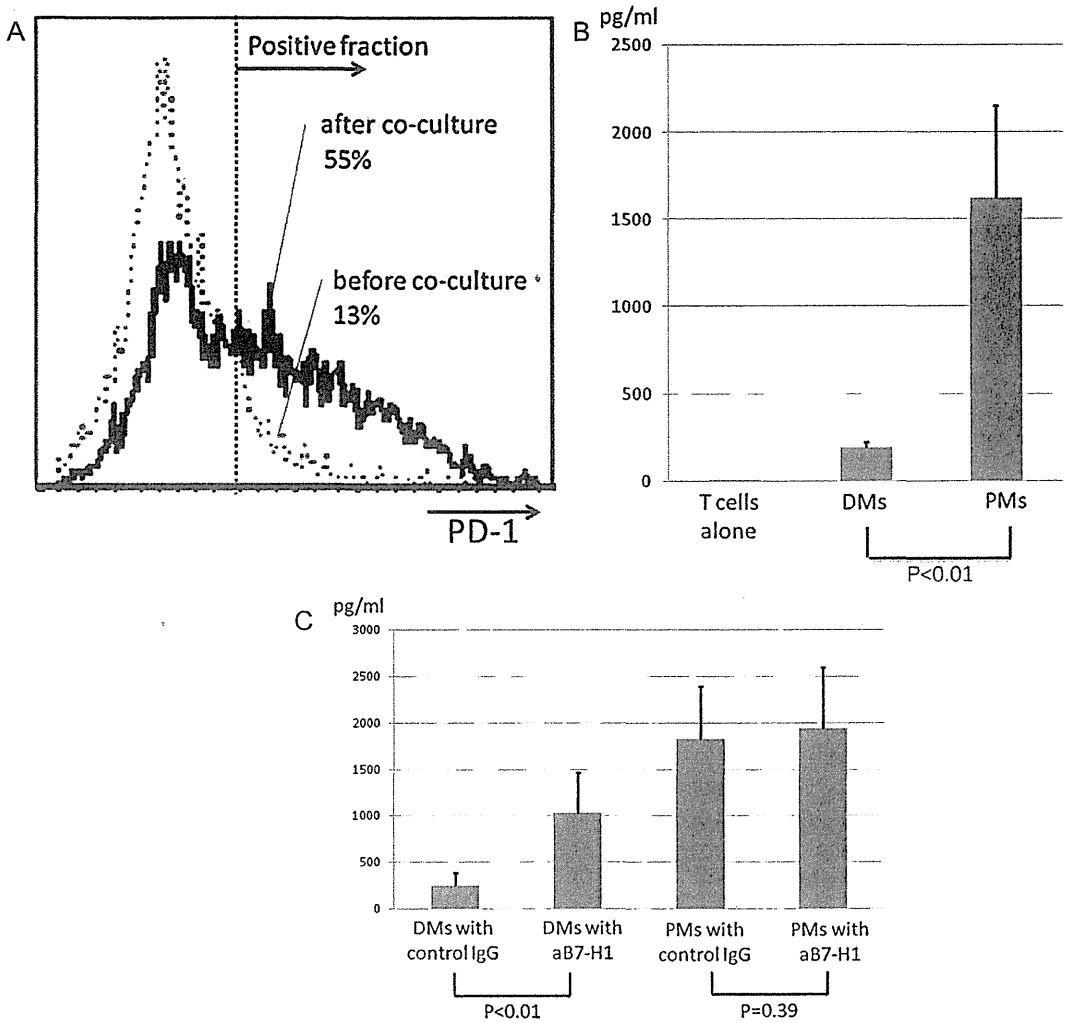

Fig. 5. *The effects of B7-H1:PD-1 signaling on IFN-γ production by T cell co-culture with PMs and DMs. T cells were isolated from the peripheral blood* of healthy non-pregnant donors. DMs and PMs were obtained from the deciduas and peripheral blood samples of the same women in early pregnancy. T cells activated with anti-CD3 antibody (OKT-3) were co-cultured with DMs or PMs for 72 h. (A) Alteration of PD-1 expression on T cells in the co-culture with OKT-3 stimulation was analyzed on flow cytometer. PD-1 expression levels on T cells before the co-culture (dotted line) and after the co-culture with OKT-3 stimulation (thick line) are shown in a histogram. The percentages of PD-1 positive T cells are presented in the histogram. The image shown is representative of five distinct experiments. (B) IFN-γ production by T cells were measured using ELISA. IFN-γ production levels were compared among three different settings: T cells cultured alone, co-cultured with DMs and co-cultured with PMs. (C) DMs and PMs were pre-incubated with anti-B7-H1 mAb to block B7-H1 ligation to PD-1. Incubations with normal mouse IgG served as negative controls. T cells were cultured with treated DMs or PMs as in (A) for 72 h. The effects of B7-H1:PD-1 blockade on IFN-γ production by T cells were analyzed by measuring IFN-γ concentrations in culture supernatants. Data in (B) and (C) summarize five experiments using samples derived from distinct individuals. Error bars = SEM.

evasion (Nishimura et al., 1999; Topalian et al., 2012). The present study shows that the expression pattern of B7 family ligands on DMs is altered depending on the gestational stage of the pregnancy. The fact that B7-H1 expression on DMs is limited to early pregnancy, when maternal immune cells initially encounter fetal antigens, suggests that B7-H1-mediated interactions may be important in the establishment of feto-maternal immune tolerance.

Monocytes in the peripheral circulation are the main source of tissue macrophages. Recruitment and phenotypical differentiation of monocytes are controlled by the chemokines and cytokines produced in the tissue microenvironment (Nagamatsu and Schust, 2010). In this study, B7-H1 was not detected on PMs isolated from women during early pregnancy, but B7.2 was commonly detected on both PMs and DMs. A possible role of B7.2 in myelomonocytic cells in the decidua for immune tolerance has been described (Miwa et al., 2005). They demonstrated that indoleamine 2,3-dioxygenase expression induced upon B7.2:CTLA-4 interaction in decidual dendritic cells is diminished in miscarriage cases compared with normal pregnancy. We also found that IFN-γ induced the expression of B7-H1 on PMs and enhanced the intensity of B7-H1 expression on DMs. In agreement with our observations, it has been reported that binding of the transcription factor, interferon regulatory factor-1 (IRF-1), to the B7-H1 promoter leads to the up-regulation of B7-H1 transcription upon IFN-γ stimulation (Seung-Jin

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et al., 2006). It is therefore plausible that PMs migrating into the decidua acquire a B7-H1-positive phenotype following exposure to local IFN-γ.

Fine tuning of local IFN-γ levels is key to successful pregnancy. Beneficial and harmful effects of this multifunctional cytokine on pregnancy have been described. Studies in a murine bone marrow transplantation model have previously demonstrated that IFN-γ produced by uterine natural killer cells is indispensable for vessel modification in the decidua (Ashkar et al., 2000). In contrast, excessively high IFN-γ levels in the microenvironment of the implanting pregnancy may upset the delicate local cytokine balance, as exemplified by Th1/Th2 polarity, and result in adverse pregnancy outcomes (Chaouat et al., 2004). We have demonstrated the possible functional role of DM-expressed B7-H1 in maintaining this balance during early pregnancy using co-culture assays to show that DM diminished IFN-γ production by T cells via B7-H1 ligation to PD-1. Supporting this concept, B7-H1 negative PMs were not able to suppress IFN-γ production by T cells. It is necessary, however, to be cautious about the interpretation of the findings, because there may be some discrepancy between the allogeneic co-culture setting introduced in our study and the physiological situation at the feto-maternal interface where syngeneic T cells recognize fetal antigen cross-presented by DMs. Moreover, the fact that PD-1 signaling blockade did not completely recover IFN-γ production by T cells co-cultured with DMs to the same level as T cells co-cultured with PMs suggests that DMs might have additional unidentified suppressive mechanisms other than B7-H1 expression. Taken together, our investigations support a paradigm in which PD-1:B7-H1 interactions negatively impact IFN-γ secretion from activated T cells in early human decidua. Since B7-H1 on DMs is up-regulated by IFN-γ exposure, DMs may be central to the control of decidual IFN-γ levels through negative feedback mediated by B7-H1 costimulatory signaling.

While some costimulatory receptors, like CD28, are expressed on all T cell types, others have more limited distribution patterns. For example, ICOS and PD-1 are only expressed on activated T cells (Coyle et al., 2000; Hutloff et al., 1999; Ishida et al., 1992). They are absent on resting naive T cells, but are rapidly induced upon antigen recognition (Freeman et al., 2000). We have previously reported that ICOS was highly expressed on the entire T cell population in human decidua (Nagamatsu et al., 2011). The present study confirmed that PD-1 was also present in the majority of decidual T cells, a finding previously reported in a detailed description of decidual PD-1 expression patterns by another group (Taglauer et al., 2008). These findings strongly support the concept that decidual T cells are in a persistently activated state, likely in response to antigen exposure, probably of fetal origin. Supporting our hypothesis, a substantial decrease in naïve CD8 cells and an increase in CD8 memory cells in the decidua were described by Tilburgs et al. (2010) B7-H1:PD-1 interactions between DMs and T cells may aid in the prevention of possibly harmful T cell activities at the feto-maternal interface. Supporting this in an animal model, blockade of murine B7-H1 signaling increased fetal rejection after allogeneic, but not in syngeneic matings (Guleria et al., 2005). There is still controversy over the physiological impact of B7-H1:PD-1 interaction on feto-maternal tolerance, since no significant alteration in fecundity was observed in an allogeneic pregnancy model using PD-1-deficient mice (Taglauer et al., 2009).

In summary, we have confirmed that B7-H1 expression on human DMs is restricted to early pregnancy and that its receptor, PD-1, is highly expressed on T cells co-residing in the decidua. We also found that IFN-γ stimulation of DMs enhances B7-H1 surface expression and that DMs can inhibit IFN-γ production by activated T cells via B7-H1:PD-1 interactions. Inhibitory signaling mediated by DM-expressed B7-H1 may help to optimize local IFN-γ levels and help to balance the maternal immune response to the fetus during early human pregnancy, although it should be noted that this co-stimulating reaction may not act exactly the same in vivo.

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