Hair Follicle Mesenchyme-Associated PD-L1 Regulates T-Cell Activation Induced Apoptosis: A Potential Mechanism of Immune Privilege

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The immune privilege (IP) of hair follicles (HFs) has been well established in previous studies. However, whether cultured HF cells still exhibit IP properties, the individual factors involved in this process, and the detailed mechanisms that drive and maintain IP, are largely unidentified. We found preferential expression of IP-associated genes in cultured HF dermal papilla and dermal sheath cup cells (DSCCs) compared with non-follicular fibroblasts (FBs) at passage 4, suggesting a potential for functional IP. Notably, programmed cell death 1 ligand 1 (PD-L1) was significantly upregulated in DSCCs and dermal papilla cells relative to FBs. IFNγ secretion from peripheral blood mononuclear cells (PBMCs) co-cultured with histoincompatible DSCCs was significantly lower than with FB and higher percentages of early apoptotic, Annexin V^+ cells were observed in PBMC co-cultured with DSCCs. Knockdown of PD-L1 translation by silencing interfering RNA in DSCCs enabled increased IFNγ secretion by PBMCs, whereas transfection of pCMV6-XL4/hPD-L1 in FB significantly reduced IFNγ secretion and increased apoptosis in co-cultured PBMCs. We also found that apoptosis in allogeneic T cells induced by DSCCs was largely dependent on the mitochondrial pathway. Our study suggests IP properties are exhibited in cultured DSCCs in part through expression of negative co-signaling molecule PD-L1.

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INTRODUCTION

Immune privilege (IP) is a dynamic state that induces and maintains immune tolerance in critical organs for survival under physiological circumstances, inflammatory conditions, and foreign cell attack (Hunt, 2006; Simpson, 2006; Forrester *et al.*, 2008). The notable IP sites in the body include the testis, the placenta, the brain, and the eye. The definition of IP was first given by Medawar (1948), who made an assumption of immunological ignorance in prolonged survival of foreign tissue grafts placed into the anterior chamber of the eye. However, multiple active, rather than passive, IP mechanisms for preventing the induction and expansion of the innate and adaptive immune responses have subsequently been discovered by different groups (Suter *et al.*, 2003; Ito *et al.*, 2008). These mechanisms can include downregulated expression of major histocompatibility complex class I and II; expression of non-classical regulatory major histocompatibility complex I molecules such as HLA-G; physical barriers to immune surveillance; deletion of auto- or allo-reactive T cells by inducing apoptosis such as via the Fas/FasL pathway; modulation of co-stimulatory signals; and local production of potent secretory immunosuppressants (Paus *et al.*, 1994; Griffith *et al.*, 1995; Christoph *et al.*, 2000; McElwee *et al.*, 2003; Paus *et al.*, 2003; Carosella *et al.*, 2008). Secreted immunoregulatory cytokines providing IP are many and varied including transforming growth factor-β1/β2 (TGF-β1/β2), IL-10, calcitonin gene-related peptide, α-melanocyte-stimulating hormone, macrophage migration inhibitory factor, red/IK, and others (Guleria *et al.*, 2005; Hori *et al.*, 2006; Meyer *et al.*, 2008; Cheng *et al.*, 2009; Kinori *et al.*, 2012).

Although an understanding of tissue IP has only recently emerged, survival and growth of hair follicles (HFs) transplanted between different individuals is indicated in historical medical literature (Dieffenbach, 1822). The IP of HFs was first

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Abbreviations: DP, dermal papilla; DS, dermal sheath; DSCC, dermal sheath cup cell; FB, fibroblast; HF, hair follicle; IP, immune privilege; PBMC, peripheral blood mononuclear cell; PD-L1, programmed cell death 1 ligand 1; qPCR, quantitative real-time RT-PCR; siRNA, silencing interfering RNA; TGF-β1/β2, transforming growth factor-β1/β2

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examined by Billingham who demonstrated the ability of melanocytes from guinea pigs to survive and produce pigment when the cells were transplanted into allogeneic host albino HFs (Billingham and Silvers, 1971). In humans, tissue from primary HF bulbs was successfully transplanted to an immunocompetent, HLA-mismatched donor (Reynolds *et al.*, 1999). Available data using primary HFs have suggested several IP mechanisms may be active (Reynolds *et al.*, 1999; Paus *et al.*, 2005; Breitkopf *et al.*, 2013). However, whether cultured HF cells, which can be potentially used in the treatment of human hair loss and/or allogeneic organ/tissue transplantation, exhibit IP has largely remained unexplored (Ito *et al.*, 2004; Paus *et al.*, 2005; Kang *et al.*, 2010; Breitkopf *et al.*, 2013). In addition, the detailed mechanisms and factors in HF IP have yet to be fully defined.

We therefore investigated IP in HF dermal papilla (DP), dermal sheath (DS), and DS cup cells (DSCCs) compared with non-follicular fibroblasts (FB). The preliminary data showed that cultured DP and DSCC exhibited IP. DSCC elicited hypo-responsiveness from allogeneic T cells and inhibited T-cell activation and proliferation by inducing apoptosis of activated T cells. We found that programmed cell death 1 ligand 1 (PD-L1, also named B7-H1, or CD274) was upregulated in DSCC versus FB. The suppression was reversible following knockdown of PD-L1 in DSCC. In contrast, overexpression of PD-L1 in FB promoted hypo-responsiveness and apoptosis induction in peripheral blood mononuclear cell (PBMC). PD-L1 might represent a unique HF expressed factor conferring IP with a potential role in inflammatory hair loss, autoimmune diseases, and graft rejection.

RESULTS

Cell culture characterization

The cell cultures were observed to be almost exclusively mesenchymal cells (Supplementary Figure 1 online) as demonstrated by expression of vimentin (Wu *et al.*, 2005; Satelli and Li, 2011). Cell cultures were negative for $K14^+$ keratinocytes and $gp100^+$ melanocytes (data not shown) as well as $CD31^+$ endothelial cells (Supplementary Figure 1 online). DSCC and DP cells, but not DS or FB cells, were partially versican positive (Supplementary Figure 1 online), a marker of hair follicle inductive ability (Soma *et al.*, 2005). DSCC, DP, and DS, but not FB cells, were alpha smooth muscle actin positive (Jahoda *et al.*, 1984). Cultured DP, DSCC, DS, and FB populations were negative for $CD4^+$ and $CD8^+$ cells (data not shown).

IP-related genes are expressed in cultured HF cells

We first examined IP-related gene expression in DSCC, DP, and DS cells cultured for four passages relative to FB by quantitative real-time RT-PCR (qPCR; Figure 1). Class I *HLA-A* and *HLA-B* were significantly downregulated in both DSCC and DP compared with FB (Figure 1). In addition, *CCL2* and *IL1RA* were downregulated in DP and DSCC, respectively. In contrast, *TGFB1*, *TGFB2*, and *PDL1* (PD-L1) were significantly upregulated in DSCC and DP; *HLAG*, *INHBA* (Activin-A), *MICA*, and *IK* (red/IK) was significantly upregulated in DSCC. Other potential IP-related genes exhibited no significant difference (Supplementary Figure 2 online).

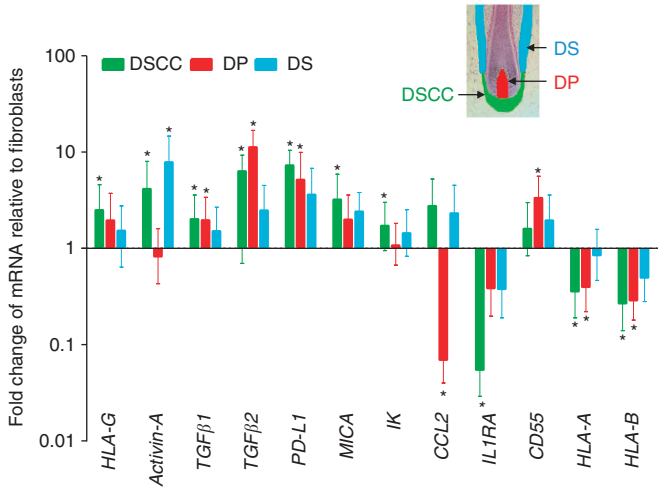

Figure 1. The expression pattern of IP-related genes in HFs. Hair follicle DSCC, DP, DS, and non-follicular FBs were dissected and cultured. Passage 4 cells were examined by qPCR for IP-related gene expression relative to non-follicular FB. The fold changes were generated from duplicated values of four healthy donors of both males and females aged on average 62.0 ± 6.6 years and expressed as the $2^{-\Delta\Delta CT}$ value. *Indicates $P \leqslant 0.05$. *CCL2*, chemokine (C-C motif) ligand 2; *CD55*, complement decay-accelerating factor; DP, dermal papilla; DS, dermal sheath; DSCC, DS cup cell; FB, fibroblast; HF, hair follicle; *IL1RA*, IL-1 receptor antagonist; IP, immune privilege; *PD-L1*, programmed cell death 1 ligand 1; qPCR, quantitative real-time RT-PCR; TGF-β*1*, transforming growth factor beta 1; *TGF-β2*, transforming growth factor beta 2.

PD-L1 is overexpressed in DSCC

Gene profiling showed that PD-L1 was highly upregulated in both DSCC and DP cells (Figures 1a and 2a). By western blot, the expression of PD-L1 protein in DSCC was 2.3-fold higher than in FB (Figure 2b), $P = 0.030$ (Figure 2b, $P = 0.030$). In primary tissue, we observed expression of PD-L1 in the lower DSCC, but diminished expression along the distal HF, and almost absent expression in non-bulbar DS. An estimated 40–60% of DP cells were also positive for PD-L1 (Figure 2c). Given expression of PD-L1 was highest in DSCC and lowest in FB (Figure 2b), these cell populations were selected for subsequent studies. The expression of PD-L1 was downregulated by 60–70% at 10 nM or 20 nM of silencing interfering RNA (siRNA; Figure 2d), but not other proteins such as TGF-β1, TGF-β2, or HLA-G (data not shown). On the other hand, the mRNA and protein expression of PD-L1 in FB was upregulated by transfection (Figure 2e and f).

DSCC and DP stimulate lower allogeneic immune responses relative to FB

We next evaluated the cells' ability to stimulate allogeneic immune responses using IFNγ as a marker of pro-inflammatory cell activation (Danzer *et al.*, 1996). The amount of IFNγ secretion from histoincompatible PBMCs co-cultured with DSCC or DP was significantly lower than with DS or FB cells after 5-day incubation (Figure 3a, 24.0, 28.4, 49.5, and 55.9 pg ml^{-1} in DSCC, DP, DS, and FB, respectively. FB vs. DSCC, $P = 0.004$; FB vs. DP, $P = 0.021$; FB vs. DS, $P = 0.832$; DSCC vs. DP, $P = 0.672$). We further identified which T-cell

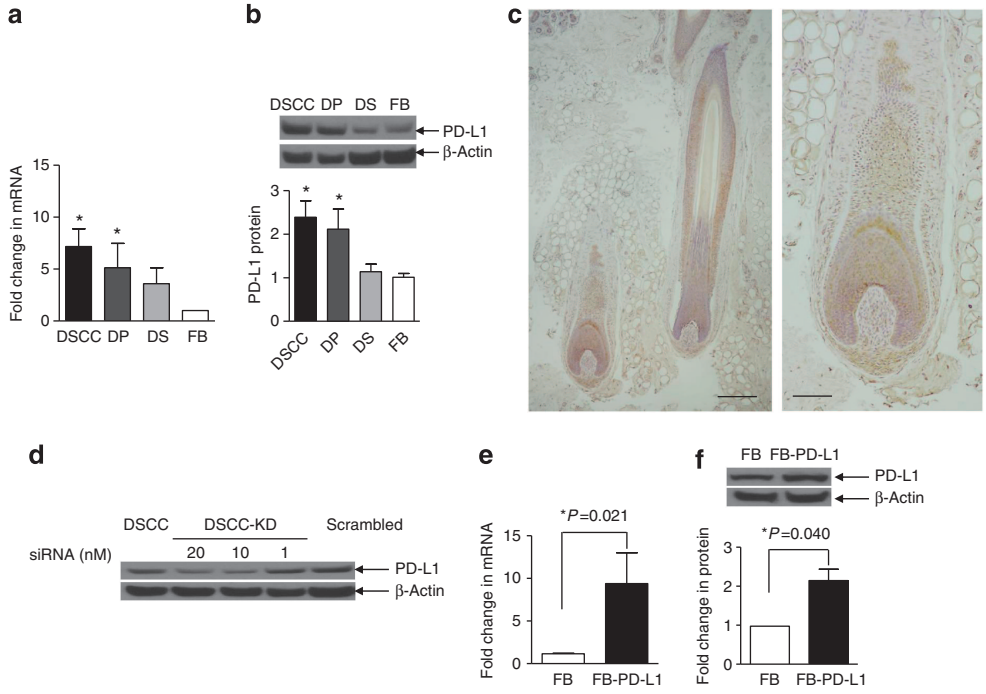

Figure 2. Programmed cell death 1 ligand 1 (PD-L1) is overexpressed in dermal sheath cup cells (DSCCs). The relative expression of PD-L1 mRNA (**a**) and protein (**b**) in DSCC at passage 4 are shown in **a** and **b**, respectively. (**c**) Immunohistochemistry staining of hair follicle (HF) representative of three different individuals, both males and females, aged on average 53.0 ± 11.2 years. PD-L1 positive cells (in brown) were present in the lowest region of DSCC and 40–60% of dermal papilla (DP) cells, but almost absent in dermal sheath (DS). Scale bar = 200 μm (left) 100 μm (right). (**d**) The efficiency of silencing interfering RNA (siRNA) knockdown of PD-L1 was tested by titration of different siRNA concentrations. The fold change of PD-L1 at the mRNA and protein level was tested by quantitative real-time RT-PCR (qPCR) (**e**) and western blot (**f**) after transfection, respectively. Scrambled RNA and empty vector was used as controls for knockdown and overexpression experiments, respectively. We used 10 nM of siRNA in subsequent assays. Results represent three individual experiments in duplicated repeats from six healthy male and female donors aged on average 47.5 ± 20.0 years. *Indicates $P \leqslant 0.05$. FB, fibroblasts; KD, knockdown.

subsets produced IFNγ in response to DSCC and FB cells. The percentage of both $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ was reduced in the presence of DSCC as compared with FB (Figure 3, FB vs. DSCC, $P = 0.004$ and $P = 0.047$ in $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ subsets, respectively), indicating that the ability of DSCC to promote T-cell activation was significantly lower than that of FB.

We next examined the proliferation of CD4 versus CD8 subsets stimulated by DSCC or FB. We found that the percentage of Ki-67^+ cells in both T-cell subsets was reduced in the presence of DSCC compared with FB (Figure 4, FB vs. DSCC, $P = 0.047$ and $P = 0.012$ in $CD4^+$Ki-67^+ and $CD8^+$ Ki-67^+ subsets, respectively), suggesting that the capacity of T-cell proliferation was impaired in the presence of DSCC. Furthermore, the percentage of $CD4^+Foxp3^+$ cells was statistically significantly increased in co-culture with DSCC as compared with FB (Supplementary Figure 3 online).

DSCC induce early apoptosis in activated T cells

We examined whether the hypo-responsiveness induced by DSCC in co-culture might result from T-cell apoptosis. A significantly higher percentage of apoptotic cells were found in both $CD4^+$ and $CD8^+$ subsets in the presence of DSCC (Figure 5, 9.7% vs. 15.0% $CD4^+$Annexin V^+ cells, $P = 0.011$; 10.1% vs. 17.2% $CD8^+$Annexin V^+ cells, $P = 0.025$). Caspases 8 and 9 are initiators for the extrinsic and intrinsic apoptotic pathways respectively; they activate effector caspase 3 (Holtzman *et al.*, 2000; Chen *et al.*, 2007). DSCC induced a 56% increase in activated caspase 3 in T cells compared with FB (Figure 6a, 3.7% vs. 6.8%, $P = 0.037$), suggesting a key role for the caspase-dependent pathway in T-cell apoptosis induction by DSCC. Caspase 8 was higher, but not significantly, in T cells co-cultured with DSCC compared with FB (Figure 6b). In contrast, caspase 9 was significantly higher in T cells co-cultured with DSCC (Figure 6c). JC-1 green, which indicates early apoptotic cells involving the mitochondrial pathway (Green and Reed, 1998), was also significantly higher in T cells co-cultured with DSCC (Figure 6d), $P = 0.001$, confirming that the intrinsic pathway is important in this effect.

The effect of PD-L1 knockdown in DSCC on allogeneic immune responses

We investigated how expression of PD-L1 in DSCC affected allogeneic immune responses by siRNA knockdown (DSCC-KD). The secretion of IFNγ was significantly higher in DSCC-KD compared with DSCC co-cultured with allogeneic PBMCs (Figure 3a, 38.4 vs. 24.0 pg ml^{-1}, $P = 0.037$), indicating that knockdown of PD-L1 expression in DSCC partially removed T-cell hypo-responsiveness in the allogeneic immune responses.

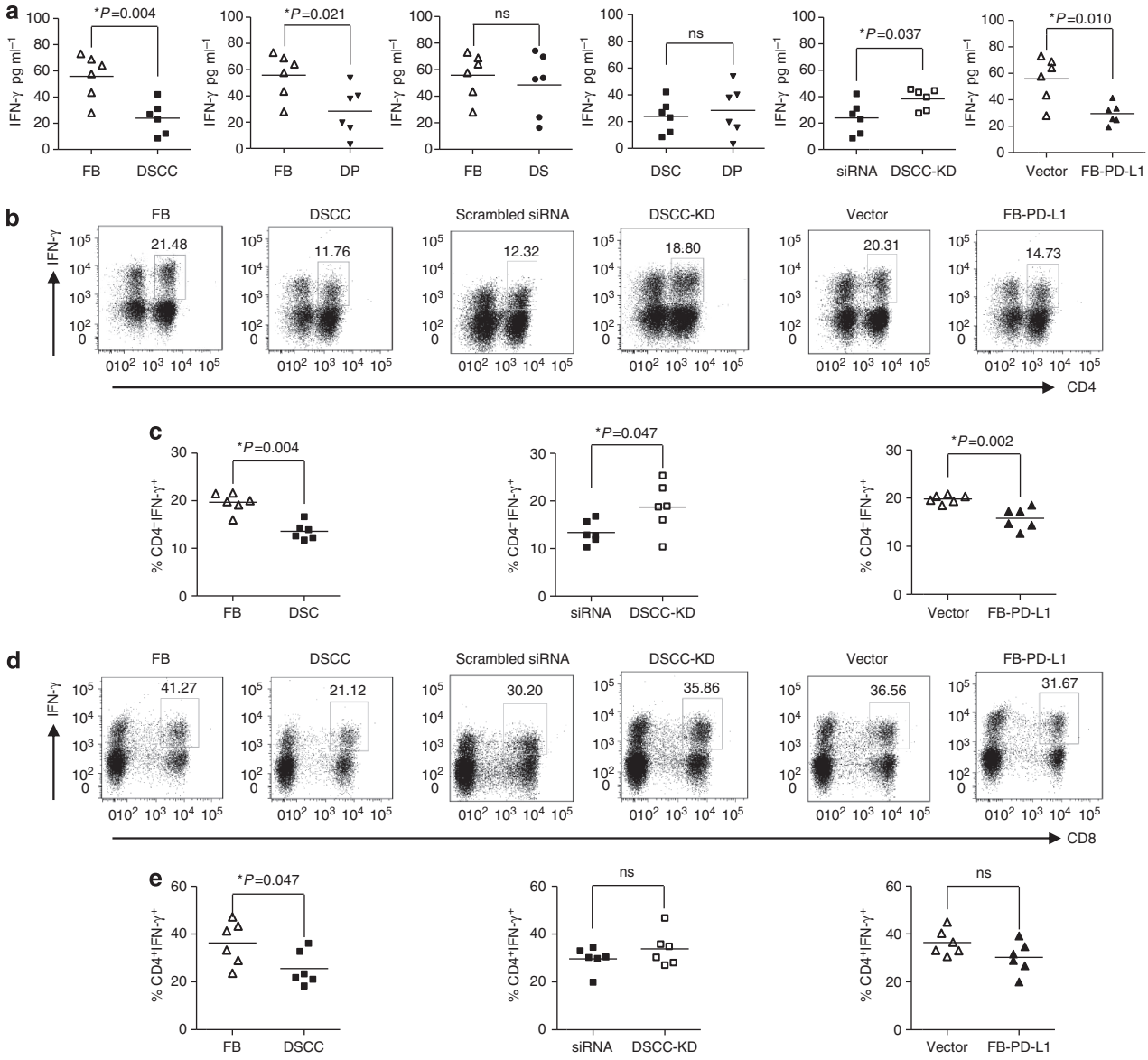

Figure 3. Dermal sheath cup cells (DSCCs) significantly inhibit allogeneic T-cell activation. Non-follicular fibroblast (FB) or DSCC, dermal papilla (DP), or dermal sheath (DS) cells (2×10^4) were co-cultured with allogeneic peripheral blood mononuclear cells (PBMCs; 2×10^5) for 5 or 7 days. (**a**) IFNγ secreted from PBMC co-cultured with DSCC or DP was significantly lower than from FB-PBMC co-culture. IFNγ secreted from PBMC co-cultured with DSCC-knockdown (KD) or FB-PD-L1 was significantly higher and lower than that of controls, respectively. (**b**) Percentage of IFNγ production in $CD4^+$ or $CD8^+$ subsets after 5 days co-culture was presented by flow cytometric analysis. Representative dot plots (**b**, **d**) and accumulated data (**c**, **e**) from $CD4^+IFN\gamma^+$ (**b**, **c**) or $CD8^+IFN\gamma^+$ (**d**, **e**) cell populations are shown. Scrambled silencing interfering RNA (siRNA) and empty vector were used as controls for programmed cell death 1 ligand 1 (PD-L1) knockdown and overexpression studies, respectively. Data represent three independent experiments with duplicates in each test using six samples for each setup from a total of 10 healthy male and female donors aged on average 55.5 ± 19.0 years. *Indicates $P \leqslant 0.05$. ns, no significance.

The percentage of $CD4^+IFN\gamma^+$ cells was significantly increased (Figure 3b and c, 13.6% vs. 18.7%, $P = 0.047$), but not significantly changed for $CD8^+IFN\gamma^+$ cells (Figure 3d and e, 25.6% vs. 33.8%, $P = 0.082$), in the presence of DSCC-KD compared with DSCC. The percentage of $CD8^+$Ki-67^+ cells was significantly increased (Figure 4c and d, 2.4% vs. 4.8%, $P = 0.047$), but not in $CD4^+$Ki-67^+ (Figure 4a and b, 2.7% vs. 4.8%, $P = 0.062$), in the presence of DSCC-KD compared with DSCC, indicating that reduction of PD-L1 expression in DSCC enabled T-cell activation and proliferation.

In addition, apoptosis was also reduced for both $CD4^+$ and $CD8^+$ cells when co-cultured with PD-L1-deficient DSCC. However, a statistically significant difference was only observed in the $CD4^+$ subset (Figure 5, 15.0% vs. 10.8% $CD4^+$Annexin V^+ cells, $P = 0.028$; 17.2% vs. 11.9% $CD8^+$Annexin V^+ cells, $P = 0.072$). The effects of PD-L1 on T-cell activation, proliferation, and apoptosis was further confirmed by a PD-L1 neutralizing antibody and results were observed to be similar (Supplementary Figure 4 online).

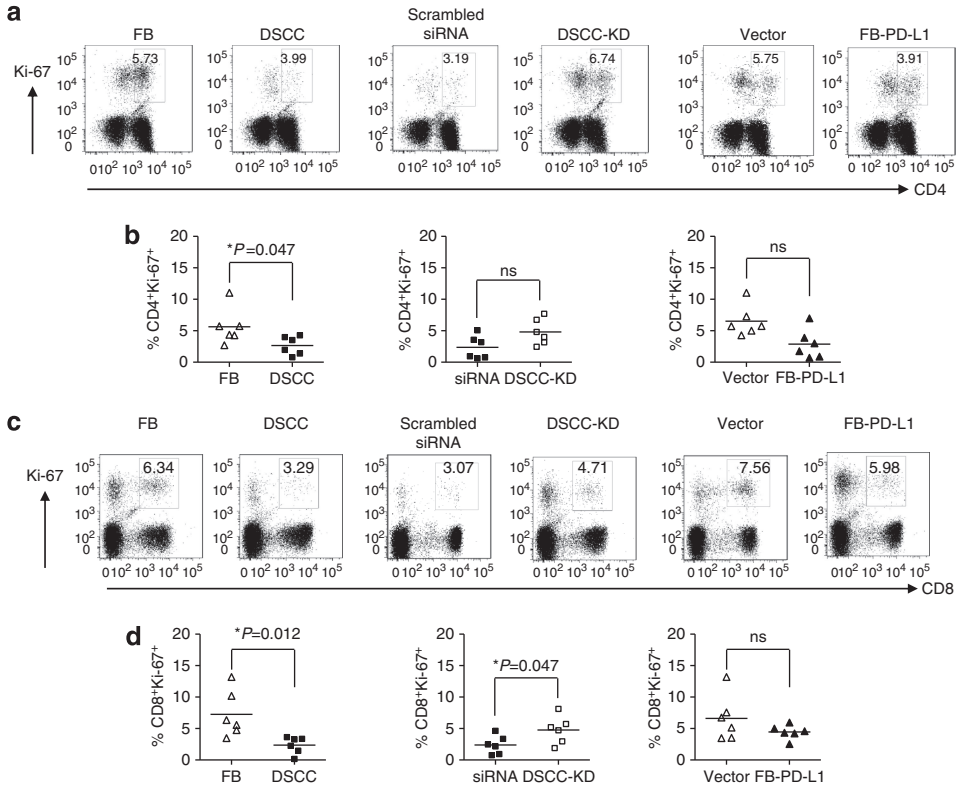

Figure 4. Dermal sheath cup cells (DSCCs) significantly inhibit allogeneic T-cell proliferation. Non-follicular fibroblast (FB) or DSCC cells (2×10^4) were co-cultured with allogeneic peripheral blood mononuclear cells (PBMCs; 2×10^5) for 5 or 7 days. Percentage of Ki-67 expression in $CD4^+$ (**a**, **b**) or $CD8^+$ (**c**, **d**) subsets after 7 days co-culture was examined by flow cytometric analysis. Representative dot plots (**a**, **c**) and accumulated data (**b**, **d**) from $CD4^+Ki\text{-}67^+$ (**a**, **b**) or $CD8^+Ki\text{-}67^+$ (**c**, **d**) cell populations are shown. Scrambled siRNA and empty vector were used as controls for programmed cell death 1 ligand 1 (PD-L1) knockdown and overexpression studies, respectively. Data represent three independent experiments with duplicates in each test using six samples for each setup from a total of 10 healthy male and female donors aged on average 55.5 ± 19.0 years. *Indicates $P \leqslant 0.05$. ns, no significance.

The effect of PD-L1 overexpression in FB on allogeneic immune responses

We next investigated how transfection of PD-L1 into FB (FB-PD-L1) affected allogeneic immune responses. The expression of PD-L1 in FB at both mRNA and protein levels was upregulated by transfection (Figure 2e and f). IFNγ secretion from allogeneic PBMCs was significantly lower with FB-PD-L1 compared with FB co-culture (Figure 3a, 36.3 vs. 29.5 pg ml^{-1}, $P = 0.010$), indicating that overexpression of PD-L1 in FB reduced allogeneic immune responses.

The percentage of $CD4^+IFN\gamma^+$ cells was significantly decreased (Figure 3b and c, 19.7% vs. 15.8%, $P = 0.002$) but not for $CD8^+IFN\gamma^+$ cells (Figure 3d and e, 36.3% vs. 30.2%, $P = 0.082$) in the presence of FB-PD-L1 compared with FB. The percentage of both $CD4^+Ki\text{-}67^+$ (Figure 4a and b, 5.6% vs. 2.9%, $P = 0.102$) and $CD8^+Ki\text{-}67^+$ cells (Figure 4c and d, 7.3% vs. 4.5%, $P = 0.125$) was decreased, but not significantly, in the presence of FB-PD-L1 compared with FB, indicating that PD-L1 overexpression in FB reduced allogeneic immune responses.

Apoptosis in both $CD4^+$ and $CD8^+$ PBMC subsets was also significantly increased when co-cultured with PD-L1-over-expressing FB (Figure 5, 9.7% vs. 15.1% $CD4^+$Annexin V^+ cells, $P = 0.029$; 10.1% vs. 17.2% in $CD8^+$Annexin V^+ cells, $P = 0.030$), consistent with apoptosis induced by DSCC via PD-L1.

DISCUSSION

We confirmed that the cultured DSCC, DP, DS, and FB cells were mesenchymal cells and not endothelial cells, melanocytes, or keratinocytes (Supplementary file online). Furthermore, DSCC and DP cultures expressed versican; a marker of hair inductive ability (Soma *et al.*, 2005). We investigated the potential for these cell populations to exhibit IP function. IP involves diverse immune evasion strategies (Hunt, 2006; Simpson, 2006; Forrester *et al.*, 2008). Previous HF studies have primarily evaluated IP in epithelium and undoubtedly, epithelium IP is a key component of HF protection (Paus *et al.*, 2005; Meyer *et al.*, 2008; Kinori *et al.*, 2011). However, in this study, we found that HF mesenchymal cells, primarily DSCC and DP, may also exhibit IP. Although many IP-related genes were not shown to be differentially expressed in HF mesenchyme, 12 of them were significantly differentially expressed in cultured DSCC and/or DP compared with FB.

Consistent with previous reports, we found downregulation of *HLA-A* and *HLA-B* in cultured DSCC and DP (Paus *et al.*, 1993, 2003; Breitkopf *et al.*, 2013). In contrast, non-classical

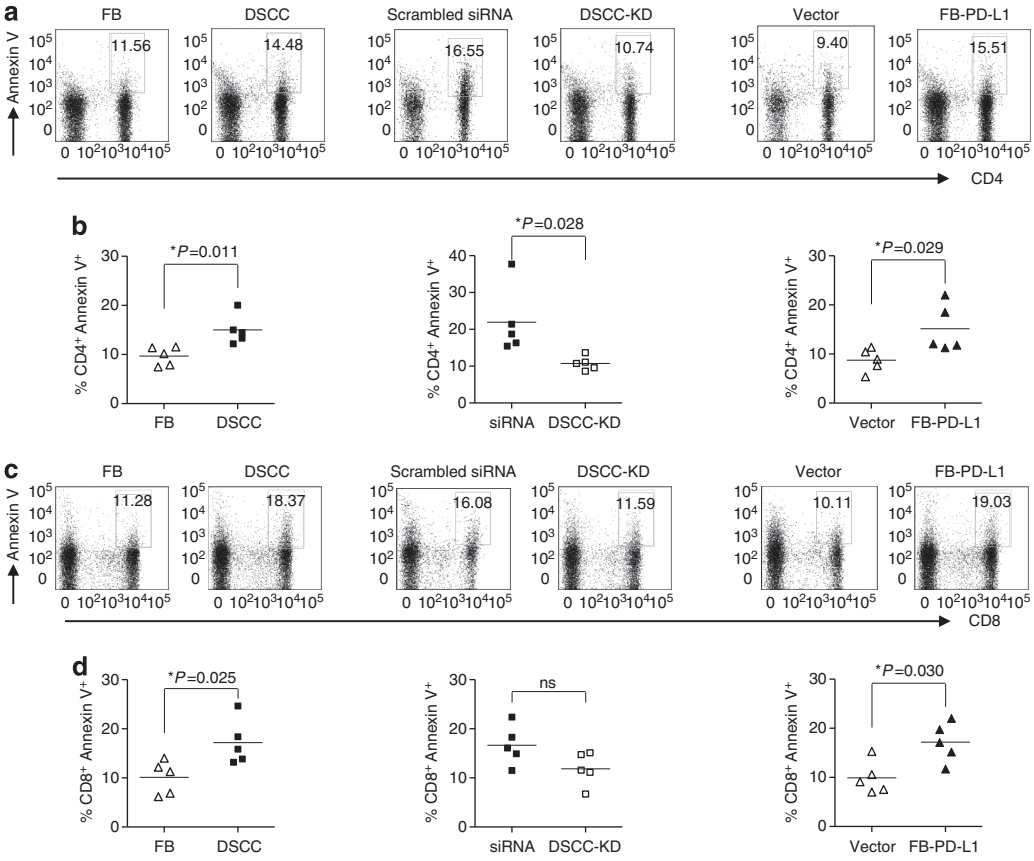

Figure 5. Dermal sheath cup cells (DSCCs) induce early apoptosis. Human peripheral blood mononuclear cells (PBMCs; 2×10^5) were co-cultured with allogeneic fibroblast (FB) or DSCC cells (2×10^4) for 5 days. Percentage of Annexin V^+ cells in $CD4^+$ subsets (**a**, **b**) and Annexin V^+ cells in $CD8^+$ subsets (**c**, **d**) was examined by flow cytometric analysis. Representative dot plots (**a**, **c**) and accumulated data (**b**, **d**) from $CD4^+$Annexin V^+ (**a**, **b**) or $CD8^+$Annexin V^+ (**c**, **d**) cell populations are shown. Scrambled silencing interfering RNA (siRNA) and empty vector were used as controls for programmed cell death 1 ligand 1 (PD-L1) knockdown and overexpression studies, respectively. Data represent three independent experiments with duplicates in each test using six samples for each setup from a total of 10 healthy males and female donors aged on average 55.5 ± 19.0 years. *Indicates $P \leqslant 0.05$. KD, knockdown; ns, no significance.

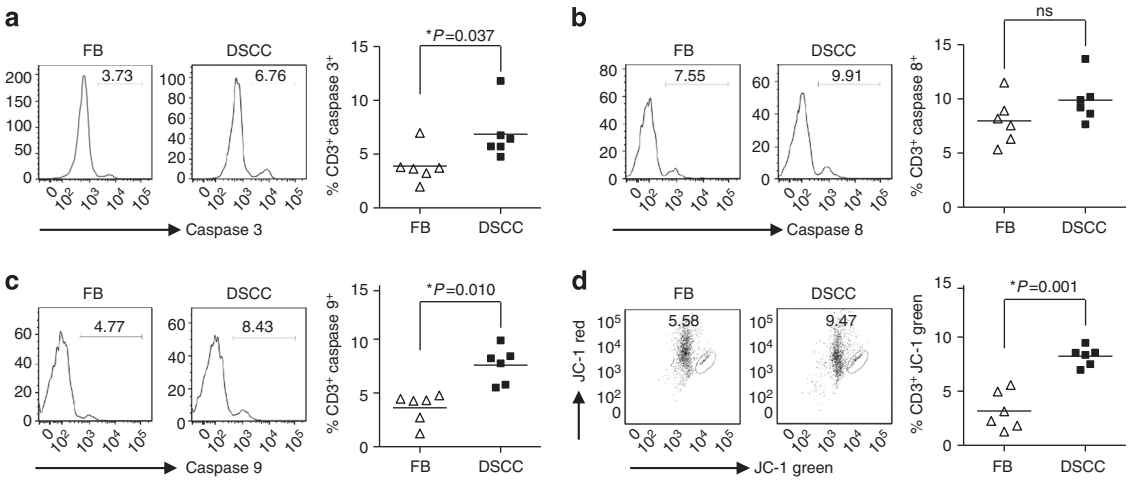

Figure 6. Dermal sheath cup cells (DSCCs) induce early apoptosis via the intrinsic pathway. Human peripheral blood mononuclear cells (PBMCs; 2×10^5) were co-cultured with allogeneic fibroblast (FB) or DSCC cells (2×10^4) for 5 days. (**a**) Percentage of activated caspase 3. (**b**) Percentage of active caspase 8. (**c**) Percentage of active caspase 9; and (**d**) JC-1 mitochondrial membrane potential (JC-1 green represents cells undergoing apoptosis via the intrinsic pathway) in $CD3^+$ T cells was examined by flow cytometric analysis. Representative dot plots and accumulation data are shown on the left and right, respectively. Data represent three independent experiments with duplicates in each test using six samples for each setup from a total of 10 healthy male and female donors aged on average 55.5 ± 19.0 years. *Indicates $P \leqslant 0.05$. KD, knockdown; ns, no significance.

class I gene *HLA-G* was significantly upregulated. HLA-G has properties that are known to maintain T-cell tolerance, including maternal–fetal immune tolerance, through inhibiting effector and NK cells, or through generating regulatory $CD4^+$ T cells (Lila *et al.*, 2002; Hunt *et al.*, 2005; Castellaneta *et al.*, 2011). Secretory cytokine *TGFB1* and *TGFB2* mRNA were significantly upregulated in DSCC. TGF-β molecules can regulate processes such as immune function, proliferation, and epithelial–mesenchymal transition. For example, TGF-β1 and TGF-β2 can convert effector T cells into suppressive T regulatory cells (Abbas *et al.*, 2013). Activin A, also a member of the TGF-β superfamily, was upregulated in DSCC. This molecule can regulate cell growth and proliferation via stimulating transcription of cell cycle inhibitors, such as cyclin-dependent kinase inhibitor 1A p21 (Burdette *et al.*, 2005). These results suggest that IP mechanisms in HF are complex; individual factors that contribute to the IP of HFs can vary between tissue/cell types.

IFNγ is predominately produced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T lymphocyte effector T cells in antigen-specific responses. IFNγ augments helper T-cell differentiation and expansion, modulates macrophage effector functions, influences isotype switching and secretion of immunoglobulins by B cells, and upregulates major histocompatibility complex class I and II expression (Schoenborn and Wilson, 2007). Therefore, IFNγ can be used as a surrogate marker for allogeneic T-cell activation (Danzer *et al.*, 1996; Breitkopf *et al.*, 2013). We showed a 57% inhibition of IFNγ secretion from PBMCs co-cultured with allogeneic DSCC compared with FB, demonstrating the ability of cultured DSCC to stimulate allogeneic immune responses continued to be compromised at passage 4; 12–14 population doublings.

Several IP strategies have been characterized in primary HF and other tissues (Gilhar *et al.*, 1998; Streilein, 1988; Paus *et al.*, 2003, 2005, 2006; Caspi, 2006; Breitkopf *et al.*, 2013). Here we describe our observation that PD-L1 is expressed in DSCC and promotes hypo-responsiveness in T cells. The mechanisms regulating PD-L1 expression in cells are not completely known. However, PD-L1 is expressed in several tissues and the expression level is associated with IP status (Guleria *et al.*, 2005; Hori *et al.*, 2006; Cheng *et al.*, 2009). PD-L1 delivers an inhibitory signal through PD-1 on T cells to induce cell anergy (Butte *et al.*, 2007; Azuma *et al.*, 2008; Park *et al.*, 2010). The PD-1/PD-L1 pathway functions as a cellular checkpoint to suppress ongoing immune responses and inflammation (Butte *et al.*, 2007; Azuma *et al.*, 2008; Park *et al.*, 2010). The constitutive expression of PD-L1 in the testis is an important mechanism underlying testicular IP and long-term survival of intra-testicular islet allografts (Cheng *et al.*, 2009). PD-L1-expressing corneal tissue also has IP (Hori *et al.*, 2006). Overexpression of PD-L1 in placenta contributes to feto-maternal tolerance while PD-L1 deficiency reduces allogeneic fetus survival (Guleria *et al.*, 2005; Habicht *et al.*, 2007; Ritprajak *et al.*, 2010). Although our results are generated from *in vitro* studies, we suggest that PD-L1 could also have an important role in HF IP *in vivo*.

The role of PD-L1 activity in autoimmunity is implicated from several lines of evidence. PD-1 gene polymorphisms have been correlated to autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, and multiple sclerosis (Fife and Pauken, 2011). PD-1-deficient rodent models develop glomerulonephritis, autoimmune cardiomyopathy, and severe collagen-induced arthritis (Okazaki and Honjo, 2006; Raptopoulou *et al.*, 2010). PD-L1-deficient mice develop experimental autoimmune encephalomyelitis (Carter *et al.*, 2007). In non-obese diabetic mice, PD-L1 expression in pancreatic islets retards lymphocyte invasion while non-obese diabetic mice deficient for PD-1 exhibit rapid islet infiltration by lymphocytes and diabetes onset (Okazaki and Honjo, 2006). Injection of anti-PD-1 or anti-PD-L1 accelerates spontaneous diabetes in non-obese diabetic mice (Ansari *et al.*, 2003). Similarly, neutralizing antibody exacerbates experimental autoimmune encephalomyelitis (Salama *et al.*, 2003). Stimulation of PD-1 using PD-L1 fusion proteins significantly reduces autoimmune activity in induced rat experimental autoimmune glomerulonephritis (Reynolds *et al.*, 2012). Further, PD-L1 expression was statistically significantly lower in alopecia areata affected mice compared with controls (Duncan *et al.*, 2013).

We found that DSCC cells have much higher expression levels of PD-L1 than non-follicular FB. Both $CD4^+$ and $CD8^+$ cell functions were impaired in the presence DSCC. Reduced IFNγ secretion from PBMC co-cultured with PD-L1 over-expressed in FB indicates PD-L1 expression can confer IP. In contrast, lower expression of PD-L1 in DSCC resulted in higher levels of IFNγ secretion from co-cultured PBMCs, indicating PD-L1 is an important factor for maintaining IP in DSCC. Changes in IFNγ secretion in both $CD4^+$ and $CD8^+$ cell subsets were observed, but the data suggest PD-L1 has a relatively greater influence on $CD4^+$ cells.

Induction of T-cell apoptosis has been suggested as one of the mechanisms for induction and maintenance of IP in placenta, testis, and cornea (Guleria *et al.*, 2005; Hori *et al.*, 2006; Cheng *et al.*, 2009). We found that DSCC induced a higher percentage of early apoptosis in both $CD4^+$ and $CD8^+$ T cells compared with FB. Decreased apoptosis was found when PD-L1 was knocked down in DSCC; in contrast, a significant increase in apoptosis occurred when PD-L1 was overexpressed in FB. The data confirm that PD-L1-expressing DSCC maintain the ability to suppress immune responses in part through induction of early apoptosis.

Apoptosis can be initiated by two main pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Caspase 3 is considered to be the most important effector caspase and is activated by initiator caspases 8 or 9 depending which pathway dominates; the extrinsic and intrinsic pathways activate caspases 8 and 9, respectively (Holtzman *et al.*, 2000; Askenasy *et al.*, 2005; Orbach *et al.*, 2007). In this study, DSCC-induced apoptosis in T cells associated with increased caspase 3 activation. Furthermore, DSCC increased caspase 9 expression and JC-1 mitochondrial membrane potential rather than caspase 8, suggesting that apoptosis induced by DSCC is mainly triggered via the intrinsic pathway.

In summary, the data suggest a mechanism for HF IP. PD-L1 may represent a predictive marker for induction and

maintaining IP status in tissues per cells. We also show that cultured DSCC exhibit IP, consistent with potential for use in treatment of inflammatory hair loss, autoimmune diseases, and allogeneic transplantation.

MATERIALS AND METHODS

Microdissection of human HF cells and isolation of PBMCs

Tissues were obtained from men and women aged from 20 to 60 undergoing cosmetic surgery with University Clinical Research Ethics Board approval and maintaining Declaration of Helsinki Principles. Consent was not required as tissues obtained from surgical procedures are considered discarded under Canadian law. PBMCs, from 14 healthy men and women aged on average 57.4 ± 16.4 years who gave informed consent, were isolated by Ficoll-paque (GE Life Science, Baie d'Urfe, Quebec, Canada). PBMCs were cultured in 1640 RPMI complete medium containing 10% heat-inactivated FBS (Invitrogen, Burlington, Ontario, Canada). DS cup, DP, non-bulbar DS tissues, and non-follicular dermal tissue containing FB were dissected. Briefly, epidermis, dermis, and fat were removed as well as the HF infundibulum. The HFs were cut to separate the lower bulb region, the bulb was then inverted and cut into the lowest portion of ''cup'' and DP. The DS was removed from the non-bulbar root sheaths and hair shaft. The individual tissues were cultured in AmnioMax C-100 (Invitrogen). The passage number is defined as each de-attachment using trypsin (Invitrogen) and re-attachment to the culture flasks. Passage 4 cells were used in all assays. In co-culture experiments, half of RPMI 1640 complete medium and half of AmnioMax were used in a u-bottomed plate.

ELISA

IFNγ secretion was determined by a standard sandwich ELISA using a kit (eBioscience, San Diego, CA) according to the manufacturer's instructions and detected at 450 nm with a correction of 540 nm ($n=3$ per group; in triplicates per group). Standard curves were created by optical density readings and sample values were calculated by this standard curve.

RNA isolation, complementary DNA synthesis, and quantitative RT-PCR

Total RNA from cultured DSCC, DP, DS, and FB ($n=4$ per group) were extracted by a standard protocol, as previously described, using the RNeasy Mini Kit (Qiagen, Mississauga, Canada) with its on-column RNase-free DNase I procedure. Complementary DNA was then synthesized by Superscript III Reverse Transcriptase (Invitrogen) in 20 μl reaction including 0.5 μg RNA and 150 ng random primer in addition to other kit components according to the manufacturer's protocol. RNaseH was utilized to remove complementary RNA. QPCR was performed in duplicate using DyNAmo HS SYBR Green qPCR kit (New England Biolabs, Mississauga, Canada) in a 20 μl volume containing 1 ng μl^{-1} of complementary DNA and 0.4 μM of each primer (Breitkopf *et al.*, 2013). Relative expression level was expressed as $2^{-(CT18s-CTgene)}$ (where CT is the cycling threshold) with 18S RNA as the endogenous control for normalization.

Immunohistochemistry

Scalp tissues ($n=3$) were formalin fixed, paraffin embedded, and sections cut at 5 μm. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide followed by incubation with rabbit anti-human PD-L1 (Abcam, Toronto, Canada), horseradish peroxidase-conjugated secondary anti-rabbit antibody (BD, Mississauga, Canada), 3,3′-diaminobenzidine substrate, and counterstained with hematoxylin (Sigma, Oakville, Canada).

Western blot

In all, 25 μg of each cell lysate from cultured HF cells or FBs ($n=3$ samples per group) were loaded onto a 12% separating Bis-Tris gel. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, Canada). The membrane was blocked in phosphate-buffered saline containing 5% skim milk for 1 hour followed by incubation with the primary antibody rabbit anti-mouse PD-L1 (Abcam) or mouse anti-actin at a concentration of 2.5 μg ml^{-1}. Secondary antibodies for PD-L1 and actin were goat anti-rabbit and goat anti-mouse (BD) conjugated with horseradish peroxidase, respectively. The blot was developed with Enhanced Chemiluminescence Plus Developer (Pierce, Nepean, Canada). The protein expression level was calculated using ImageJ software (Schneider *et al.*, 2012).

Flow cytometric analysis

For each sample, 10^6 cells were stained with anti-CD4, anti-CD8, Ki-67, anti-CD3, Annexin V, 7AAD, or isotype control rat IgG (all eBioscience). Intracellular staining of IFNγ was performed after 4 hours stimulation with phorbol 12-myristate 13-acetate and ionomycin (Sigma). Activated caspase 3 (BD #557091, polyclonal rabbit anti-active caspase 3), active caspase 8 (eBioscience #88-7005, fluorescein-conjugated IETD-FMK), caspase 9 (eBioscience #88-7006, fluorescein-conjugated LEHD-FMK), and JC-1 (eBioscience #65-0851; Green and Reed, 1998) was stained according to the manufacturer's instructions. A total of 20,000 live gated events were acquired on FACScan (BD), and FlowJo software (Tree Star, Ashland, OR) was used to analyze relevant populations.

Knockdown and overexpression of PD-L1

The expression of PD-L1 was knocked down by RNAiMax transfection (Invitrogen) or electroporation (Bio-Rad) of duplex siRNA-27 (rGrCrArArUrArUrGrArCrArArUrUrGrArArUrGrCrArArATT, rGrCrCrGrArCrUrArCrArArGrCrGrArArUrUrArCrUrGrUGA), and overexpression of PD-L1 was achieved by lipofectamine LTX & plus transfection (Invitrogen) of pCMV6-XL4/hPD-L1 (Dong *et al.*, 2002). The expression of mRNA and protein were examined by qPCR and western blot at 48 and 72 hours, respectively ($n=3$ per group; in duplicates per sample). Universal scrambled negative control siRNA duplex (OriGene Technologies, Rockville, MD) was used for siRNA control along with the empty vector without PD-L1 sequence.

Statistics

qPCR, ELISA, and flow cytometry data were analyzed by two-tailed Student's *t*-tests. For all figures, we state no significance if *P*-value is >0.05. Differences are considered significant if $P\leqslant 0.05$.

CONFLICT OF INTEREST

KJM is Chief Scientific Officer, and KJM and JS are founding shareholders, of Replicel Life Sciences. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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