OPEN

Clinical & Translational Immunology (2014) 3, e14; doi:10.1038/cti.2014.5

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ORIGINAL ARTICLE

Human placenta-derived adherent cells induce tolerogenic immune responses

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Human placenta-derived adherent cells (PDAC cells) are a culture expanded, undifferentiated mesenchymal-like population derived from full-term placental tissue, with immunomodulatory and anti-inflammatory properties. PDA-001 (cenplacel-L), an intravenous formulation of PDAC cells, is in clinical development for the treatment of autoimmune and inflammatory diseases. To elucidate the mechanisms underlying the immunoregulatory properties of PDAC cells, we investigated their effects on immune cell populations, including T cells and dendritic cells (DC) *in vitro* and *in vivo*. PDAC cells suppressed T-cell proliferation in an OT-II T-cell adoptive transfer model, reduced the severity of myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalomyelitis and ameliorated inflammation in a delayed type hypersensitivity response model. *In vitro*, PDAC cells suppressed T-cell proliferation and inhibited Th1 and Th17 differentiation. Analysis of tissues derived from PDAC cell-treated animals revealed diminished CD86 expression on splenic DC, suggesting that they can also modulate DC populations. Furthermore, PDAC cells modulate the differentiation and maturation of mouse bone marrow-derived DC. Similarly, human DC differentiated from CD14$^+$ monocytes in the presence of PDAC cells acquired a tolerogenic phenotype. These tolerogenic DC failed to induce allogeneic T-cell proliferation and differentiation toward Th1, but skewed T-cell differentiation toward Th2. Inhibition of cyclo-oxygenase-2 activity resulted in a significant, but not complete, abrogation of PDAC cells' effects on DC phenotype and function, implying a role for prostaglandin E2 in PDAC-mediated immunomodulation. This study identifies modulation of DC differentiation toward immune tolerance as a key mechanism underlying the immunomodulatory activities of PDAC cells.
Clinical & Translational Immunology (2014) **3,** e14; doi:10.1038/cti.2014.5; published online 2 May 2014

Keywords: antigen presenting cells; dendritic cells; mesenchymal stromal/stem cell; placenta-derived adherent cells; prostaglandin E2; tolerogenic

INTRODUCTION

During pregnancy, a fetus that expresses HLA antigens allogeneic to the mother is tolerated by the maternal immune system. Maternal tolerance during pregnancy not only protects the fetus from allograft rejection but also has been associated with increased rates of remission of several autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.[1,2] Placental tissue has an important role in immune regulation at the maternal–fetal interface, functioning as an immune-tolerant barrier, creating a privileged site, without immunocompromising the maternal host. Although the mechanisms of maternal tolerance induction are not fully understood, immunomodulatory cells within the placenta appear to be critical in maintaining fetomaternal tolerance during pregnancy.[3–9]

Placenta-derived adherent cells (PDAC cells) are a culture expanded, undifferentiated mesenchymal-like population derived from full-term placental tissue.[10] Genotypically, these cells are solely derived from the newborn. The cells display features characteristic of mesenchymal stromal cells (MSC), but with a unique phenotype associated with their placental origin. Human MSC, composed of heterogeneous multi-potent progenitor cells, have attracted interest as a therapeutic option for the prevention and treatment of immune-mediated diseases as well as regenerative medicine applications.[11–13] It is reasonable to hypothesize that cells isolated from the placenta may have unique immunomodulatory properties that could potentially provide therapeutic benefits particularly for inflammatory and autoimmune disorders. PDAC cells are being developed for such therapeutic indications; PDA-001 (cenplacel-L) is an intravenous (i.v.) formulation currently in clinical development.[14]

In this study, we have characterized the mechanisms of PDAC cell activity in animal models of immune-mediated inflammation and autoimmune disease. We demonstrate that PDAC cells suppress T-cell proliferation, modulate T-cell differentiation and function, and suppress production of immunostimulatory and inflammatory cytokines in various immune cell types. Because of the critical role of

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Received 11 November 2013; revised 12 February 2014; accepted 13 March 2014

dendritic cells (DC) in shaping adaptive immunity and tolerance, we focused particularly on the effects of human PDAC cells on DC populations. We find that PDAC cells strongly modulate DC differentiation and maturation and also induce DC to express a tolerogenic phenotype, effects that appear to be partially mediated by prostaglandin E2 (PGE_2). These results suggest that PDAC cells can exert their immunomodulatory activity either directly by interfering with T-cell function or indirectly by regulating antigen-presenting cells (APC).

RESULTS

Characterization of human PDAC cells

PDAC cells were isolated from the full-term, postpartum human placenta and expanded for approximately 20 population doublings as an adherent cell culture before functional and immunophenotypic assessments. Figure 1a shows PDAC cell adherence to the plastic surface during *in vitro* expansion culture. Flow cytometric analyses demonstrated that PDAC cells expressed surface markers that are typical of MSC, including CD90, CD73 and CD105, and also expressed CD200, which is associated with placental cells (Figure 1b).[15] Additional immunophenotypic characterization indicated that PDAC cells were negative for the hematopoietic cell markers CD34, CD45, CD14 and HLA-DR, positive for HLA-ABC expression, and did not express APC costimulatory markers CD80 and CD86 (Figure 1b). PDAC cells exhibited limited differentiation to express markers of adipogenic (Oil red O positive), osteogenic (alizarin red positive) and chondrogenic (Alcian Blue and Sirius Red positive) lineages, respectively, under appropriate differentiation culture conditions (Figure 1c). These results indicate that PDAC cells meet the classification standards for an MSC-like progenitor cell.[16]

The immunoregulatory effects of PDAC cells *in vitro* on T-cell activation and differentiation and on function of APC were defined in a series of experiments. PDAC cells significantly suppressed proliferation of allogeneic $CD4^+$ and $CD8^+$ cells in a mixed leukocyte reaction (MLR) (Supplementary Online Figure 1a), and reduced TNF-α production by activated T cells stimulated with anti-CD3 and anti-CD28 coated Dynabeads (Supplementary Online Figure 1b). When PDAC cells were added to T cells cultured under conditions that induce Th1 and Th17 differentiation, inhibition of differentiation was also observed (Supplementary Online Figure 2). When cultured with immature monocyte-derived dendritic cells (MoDC), IL-1β-pretreated PDAC cells prevented lipopolysaccharide (LPS) and interferon (IFN)-γ-induced upregulation of CD86, HLA-DR and CD83 on DC, as well as LPS and IFN-γ-induced interleukin (IL)-12 and tumor necrosis factor (TNF)-α production, indicating suppression of DC maturation (Supplementary Online Figures 3 and 4). In addition, PDAC cells also inhibited LPS-induced peripheral blood mononuclear cells (PBMC) IL-23 production (Supplementary Online Figure 4c) and TNF-α production but enhanced PBMC IL-10 secretion (data not shown). These results suggest that PDAC cells can suppress T-cell

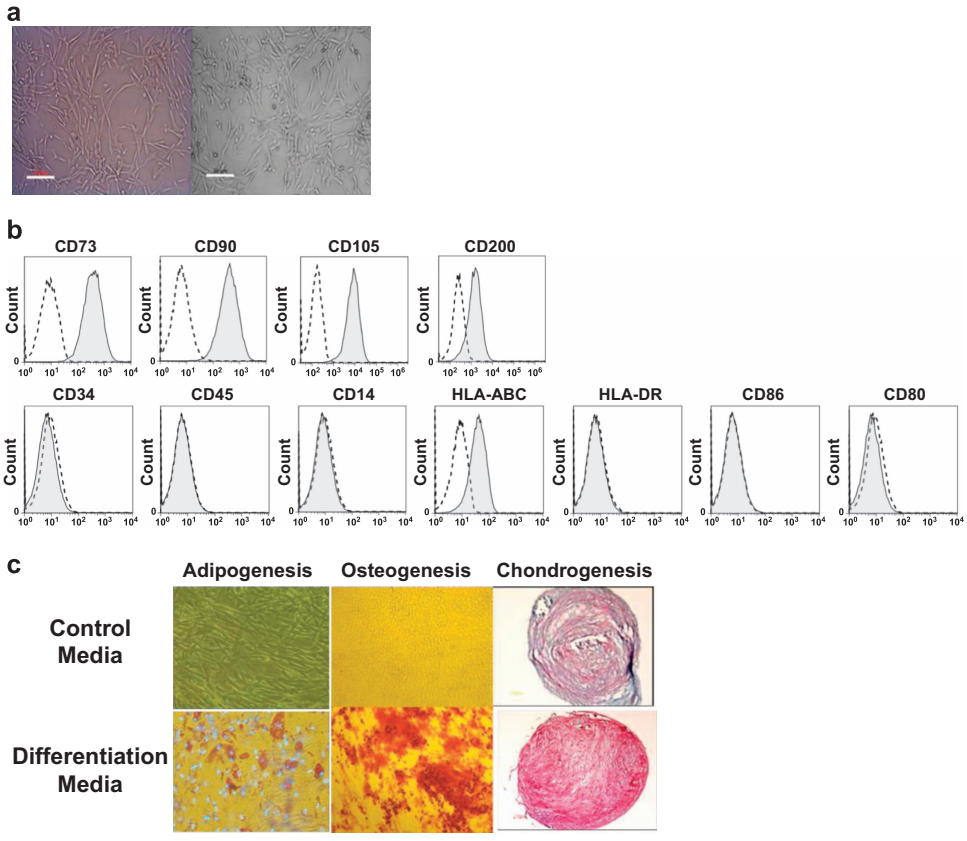


Figure 1 PDAC cells display MSC-like characteristics. (**a**) PDAC cells from two donors show spindle-shaped fibroblast morphology under phase contrast microscope after 6 passages of culture expansion *ex vivo*. (**b**) Flow cytometry analysis of PDAC-cell phenotype. Top row: expression of MSC markers (CD73, CD90, CD105) and placenta marker (CD200). Bottom row: expression of hematopoietic cell surface markers. Dotted lines indicate isotype-matched control antibody staining. Numbers indicate percent positive cells. Results from one of the six independent experiments are shown. (**c**) Adipogenic (Oil red O staining), osteogenic (alizarin red staining) and chondrogenic (Alcian Blue and Sirius Red staining) differentiation of PDAC cells under different lineage differentiation culture conditions.

activation either directly by interfering with T-cell functions or indirectly by exerting regulatory effects on APC.

PDAC cells suppress antigen-specific T-cell proliferation in an OT-II adoptive transfer model

Animal models of T-cell-mediated inflammation were used to determine whether PDAC cells could induce a tolerogenic response *in vivo*. In the first example, an OT-II transgenic mouse model, expressing the T-cell receptor specific for ovalbumin (OVA), was used to evaluate the effects of PDAC cells on antigen-specific $CD4^+$ T-cell proliferation. PDAC cells at doses of 0.3, 0.75 and 1.5×10^6 cells or vehicle were administered along with the adoptive transfer of $CD4^+$ T cells isolated from OT-II mice into recipient wild-type mice following OVA peptide immunization. No effects on animal body weight or toxicities were observed following PDAC cell treatment (data not shown), but PDAC cell treatment led to a dose-dependent decrease in the OVA-specific $CD4^+$ T-cell proliferative response in the spleen, as compared with vehicle-treated mice (Figure 2a). Coadministration of PDAC cells also resulted in an increase in the percentage of IL-10-producing splenic $CD4^+$ T cells in a dose-dependent manner (Figure 2b), indicating an induction of tolerogenic T-cell populations *in vivo*.

PDAC cells prevent delayed type hypersensitivity

PDAC cell-mediated immunomodulation was further investigated *in vivo* in a sheep red blood cell (sRBC)-induced DTH model. In the

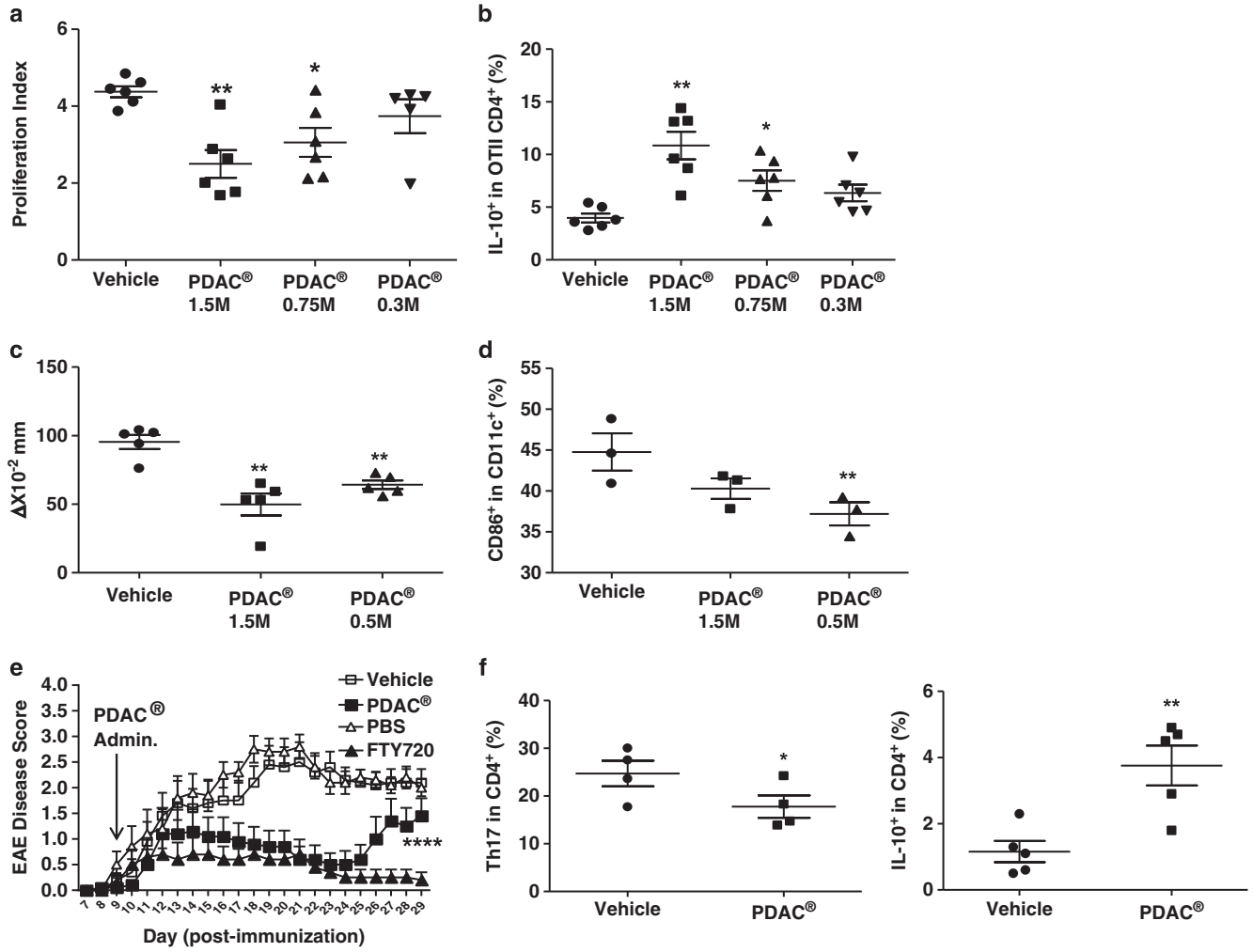


Figure 2 PDAC cells demonstrate immunoregulatory effects *in vivo* in three animal models. (**a**, **b**) OT-II Adoptive Transfer Model. PDAC cells at doses indicated and OT-II $CD4^+$ T cells (3.36×10^6) were coadministered into recipient mice. Following OVA peptide stimulation, spleens were isolated for analysis of (**a**) proliferation index and (**b**) percentage of IL-10-producing OT-II $CD4^+$ T cells. M, million cells. (**c**, **d**) DTH Model. Mice received PDAC cells or vehicle, as indicated, along with sRBC via separate tail veins. Mice were challenged with sRBC 4 days later by local injection with sRBC into the right paw. (**c**) Paw thickness, 24 h post challenge, expressed as the difference between right (sRBC challenged) and left paw. (**d**) Frequency of $CD86^+$ cells in $CD11c^+$ splenocytes. (**e**, **f**) EAE model. Nine days after immunization with MOG peptide, at the onset of EAE symptoms, mice received the treatments indicated. PDAC cells (1.5×10^6), vehicle and PBS were administered by tail vein injection; FTY720 was administered orally at 10 mg kg^{-1}. (**e**) Clinical scores, evaluated daily. The data are expressed as the mean ± s.e.m. of 10 mice per group. Mice received control FTY20 daily. In contrast, only a single dose of PDAC cells (arrow) was administered. (**f**) The frequency of Th17 cells (left) and IL-10-producing $CD4^+$-infiltrating T cells (right) in the spinal cord isolated from EAE mice, measured by flow cytometry. Results are expressed as mean ± s.e.m. of the percentage positive cells or proliferation index. Unless otherwise indicated, statistical significance for all parameters is denoted as $^*P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$, compared with appropriate vehicle control.

presence or absence of 0.5 or 1.5×10^6 PDAC cells, sRBCs were administered i.v. to mice to induce the DTH response. The right footpads of the mice were challenged with sRBCs 4 days later. All dosages of PDAC cells were well tolerated, with no effects on animal body weight or toxicities observed (data not shown). Assessed 24 h after challenge, mice that had received PDAC cells showed up to 50% reduction in paw swelling compared with vehicle controls (Figure 2c). This effect was associated with an observed reduction in $CD11c^+$ DC in the spleen (data not shown), and specifically a reduction in the $CD86^+$ $CD11c^+$ DC population (Figure 2d), demonstrating PDAC cell modulation of the DC population *in vivo*.

PDAC cells ameliorate EAE

The immunoregulatory properties of PDAC cells observed in the previous immune inflammatory models suggested that PDAC cells might be effective as a treatment for autoimmune disorders. The activity of PDAC cells was therefore evaluated in the EAE model of multiple sclerosis. PDAC cell treatment (1.5×10^6 cells, i.v.) at the onset of symptoms (day 9 after myelin oligodendrocyte glycoprotein (MOG) peptide immunization) was well tolerated and ameliorated the severity of encephalitis (Figure 2e). The efficacy of a single dose of PDAC cells was comparable to that achieved with daily treatment with the multiple sclerosis oral therapy fingolimod (FTY720) ($10\,mg\,kg^{-1}$ p.o), through day 26 of the study. The rebound in disease score suggests that, in this system, response durability is $\sim$2 weeks. In separate experiments, animals treated with PDAC cells or vehicle control at EAE disease onset were killed on day 16 post immunization, the previously observed peak of clinical symptoms, and the spinal cord inflammatory infiltrate was analyzed by $CD4^+$ T-cell intracellular cytokine detection. Consistent with results in the OT-II model, the EAE infiltrate from PDAC cell-treated animals contained a twofold higher frequency of IL-10-producing $CD4^+$ T cells, compared with the vehicle control group (Figure 2f). As Th17 cells are thought to have a pivotal role in the pathogenesis of EAE,[17] the effect of PDAC cell treatment on the Th17 T-cell population was also analyzed in this study. Spinal cords from PDAC cell-treated animals contained fewer Th17-infiltrating T cells than the vehicle control group at EAE disease peak, indicating the anti-inflammatory skewing of the T-cell response, which could explain the observed effect of PDAC cells on the severity of clinical disease (Figure 2f).

PDAC cells modulate differentiation of mouse bone marrow-derived dendritic cells (BMDC)

As 'professional APC', DC are endowed with the ability to prime naive T cells, and therefore to shape subsequent adaptive immune responses. On the basis of our *in vivo* observations, we hypothesized that PDAC cells may influence the T-cell response, at least in part, by modulating the mouse DC activity. To test this hypothesis, we conducted *in vitro* coculture studies with mouse BMDC to investigate whether PDAC cells could modulate DC differentiation and maturation. Mouse bone marrow cells (BMC) were cultured in the presence of GM-CSF to differentiate into immature BMDC and were then activated with LPS to induce BMDC maturation. The effects of PDAC cell coculture were tested during the BMDC differentiation process, or separately during the LPS-induced maturation. Mouse BMDC differentiated in the presence of PDAC cells showed a reduction in the $CD86^{hi}$ and MHC I-A/I-E^{hi} BMDC population in a PDAC cell dose-dependent manner (Figure 3a). Furthermore, the expression of a tolerogenic DC marker PD-L1 was enhanced, whereas the further increase in the mature $CD86^{hi}$ and MHC I-A/I-E^{hi} subset of BMDC induced by LPS was suppressed by PDAC cell coculture in a dose-dependent manner (Figure 3b). This effect did not require cell–cell contact, as BMDC incubated with PDAC cells separated by transwell membranes (Figure 3c) or with PDAC cell conditioned medium (PDAC-CM) (data not shown) also resulted in a reduction in the mature BMDC population. The BMDC stimulated with LPS in the presence of PDAC cells produced significantly lower levels of IL-12 but higher levels of IL-10 (Figure 3d). Taken together, these results confirmed the ability of PDAC cells to re-program DC differentiation and functional maturation toward a tolerogenic phenotype and implicated species-independent soluble factor(s) as mediators of this activity.

Human MoDC differentiated in the presence of PDAC cells or PDAC-CM exhibit a tolerogenic phenotype

The effects of PDAC cells on DC differentiation were further examined using human DC. Immature DC (iDC) were differentiated from human peripheral $CD14^+$ monocytes in medium containing GM-CSF and IL-4, and cultured in the presence or absence of PDAC cells or PDAC-CM. iDC were then activated to develop into mature DC (mDC) by subsequent stimulation with LPS and IFN-γ. DC differentiation, indicated by the upregulation of DC markers and costimulatory molecules and downregulation of monocyte markers, was inhibited in the presence of PDAC cells. PDAC cells (Supplementary Online Figure 5a) or PDAC-CM (Figure 4a) reduced conventional DC (cDC) marker CD1a expression and enhanced expression of the plasmacytoid DC (pDC) marker CD123 and the monocyte lineage marker CD14 on iDC in a dose-dependent manner. DC differentiated in the presence of PDAC cells (PDAC-iDC) (Supplementary Online Figure 5a) or PDAC-CM also demonstrated dose-dependent reduction of costimulatory molecule CD86 expression and enhancement of PD-L1 expression (Figure 4a). Mature DC that were originally differentiated in the presence of PDAC cells or PDAC-CM (PDAC-mDC) were further impaired in their ability to express CD86 and induce the human DC maturation marker CD83, in response to LPS and IFN-γ stimulation (Supplementary Online Figure 5b and Figure 4b). Moreover, intracellular staining revealed reduced production of the Th1-polarizing cytokine IL-12 (Supplementary Online Figure 5b and Figure 4b). Secretory IL-12 and IL-23 production was also reduced along with an increase in IL-10 production in the culture supernatant of PDAC-mDC induced from PDAC-iDC that were originally differentiated in the presence of PDAC cells (Supplementary Online Figure 5c) or PDAC-CM (Figure 4c). Taken together, these findings suggest that PDAC cells can influence human MoDC differentiation toward a tolerogenic phenotype.

Tolerogenic DC differentiated in the presence of PDAC-CM fail to stimulate allogeneic T-cell proliferation and differentiation toward Th1 but promote Th2 polarization

The capacity of 'tolerogenic' PDAC-mDC to prime T cells was further evaluated in an MLR using allogeneic naive $CD4^+$ T cells as responder cells. As depicted in Figure 4d, PDAC-mDC failed to stimulate allogeneic T-cell proliferation, whereas significant T-cell proliferation was observed with mDC differentiated in the absence of PDAC-CM. Furthermore, PDAC-mDC were also impaired in their ability to promote naive T-cell differentiation toward the $CD4^+$IFN-γ^+ Th1 cells and instead induced T-cell polarization toward the $CD4^+$IL-4^+ Th2 cells (Figure 4e). These results demonstrate that DC differentiated in the presence of PDAC-CM function as tolerogenic APC in *in vitro* assays of T-cell priming.

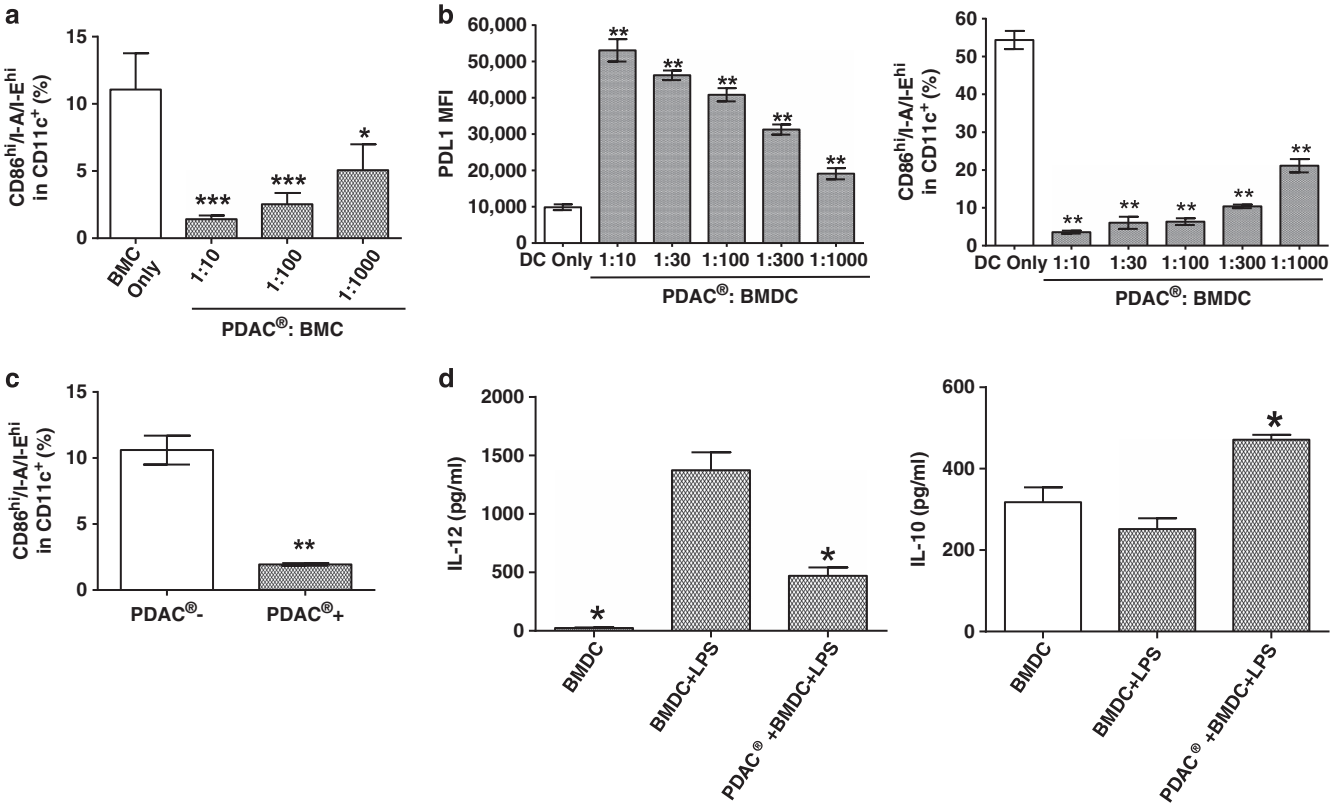


Figure 3 PDAC cells induce tolerogenic mouse BMDC *in vitro*. (**a**) Mouse BMC (1×10^6) were incubated with GM-CSF for 8 days, in the presence or absence of PDAC cells. CD86-high and I-A/I-E-high expression by immature BMDC derived from BMC cultured alone or in the presence of PDAC cells; PDAC cell:BMC ratios are indicated in the figure. $^*P<0.05$, $^{***}P<0.001$, compared with BMC-only control. (**b**–**d**) BMC-derived immature BMDC were cocultured with PDAC cells at ratio of PDAC cell:BMDC as indicated, with LPS stimulation for 4 h. (**b**) Mean fluorescence intensity (MFI) of PD-L1 expression on mature BMDC (left). Percentage of the BMDC population with CD86-high and I-A/I-E-high expression in mature BMDC (right). (**c**) CD86-high and I-A/I-E-high expression by mature BMDC (in Transwell cultures) alone or with PDAC cells at a ratio of 1:300 PDAC cell:BMDC after 4-h stimulation with LPS. Results are expressed as mean ± s.e.m. ($n=3$). $^{**}P<0.01$, compared with DC-only control. (**d**) IL-12 and IL-10 levels in supernatant collected from BMDC in the absence or presence of PDAC cells at a ratio of 1:30 PDAC cell:BMDC, with LPS stimulation for 4 h as indicated. Results are expressed as mean ± s.e.m. of cytokine levels in BMDC (1.0×10^6 cells per ml), from one of the three independent experiments. $^*P<0.05$, compared with BMDC with LPS control.

PGE_2 contributes to PDAC cell-mediated induction of tolerogenic DC

Results from PDAC-CM and PDAC cell coculture studies in transwells with both mouse and human DC (Figures 3 and 4) indicate that soluble factor(s) with cross-species activity secreted by PDAC cells mediates the regulatory effect of PDAC cells on DC differentiation. PGE_2, an eicosanoid derived from fatty acids, is known to modulate DC function[18] and is conserved across many species and immune cell types, making it a promising candidate as a mediator of immunomodulation by PDAC cells. To evaluate the role of PGE_2 in PDAC cell-induced DC modulation, PGE_2 production by PDAC cells and the effect of PGE_2 inhibition on PDAC cell immunomodulatory activity was investigated.

PDAC cells constitutively produced significant amounts of PGE_2 over a 24-h period (Figure 5a) that progressively increased over 72 h of culture (data not shown). Addition of 5 μM NS-398, a selective COX-2 inhibitor, markedly suppressed the synthesis of PGE_2 (Figure 5a). The effects of PDAC-CM on DC maturation and differentiation were either partially or completely reversed by the inclusion of NS-398 during the generation of PDAC-CM. Differentiation of MoDC in the presence of PDAC-CM containing NS-398 resulted in partial reversal of CD1a downregulation, and partial reversal of CD123 and CD14, and PD-L1 upregulation. PDAC-CM-mediated downregulation of $CD86^{hi}$ was completely reversed (Figure 5b). Inclusion of PDAC-CM prepared in the presence of NS-398 during DC differentiation also inhibited the phenotypic and functional changes observed upon LPS stimulation. PDAC-CM prepared in presence of NS-398 partially restored the LPS-induced DC $CD86^{hi}$ population and CD83 expression, decreased IL-10 secretion and partially restored IL-12 and IL-23 production. Collectively, these findings indicate the involvement of PDAC cell-derived PGE_2 in tolerogenic DC induction by PDAC-CM.

DISCUSSION

Human PDAC cells are a novel MSC-like population with potent immunomodulatory properties. PDA-001, an i.v. formulation of PDAC cells, is currently in development for the treatment of diseases of immune dysregulation.[14] Previously, PDAC cell immunomodulatory activity has been documented in a neuropathic pain model.[19] In this study, we describe the basic characteristics of these human placenta-derived cells and investigate the mechanisms underlying their biological activity in models of autoimmune disease and immune-mediated inflammation. Our mechanism of

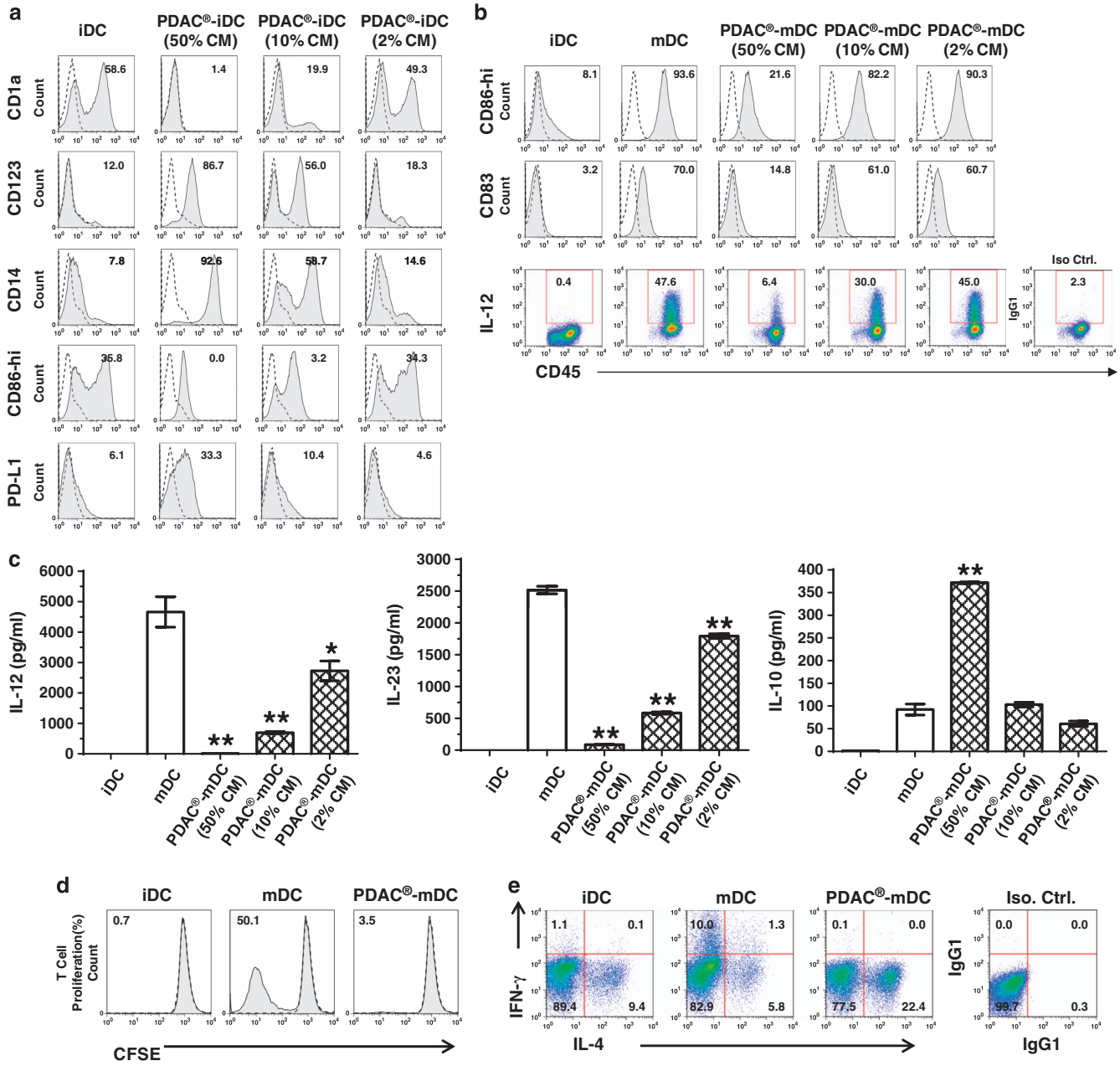


Figure 4 PDAC-CM can induce tolerogenic human DC *in vitro*. (**a**) Flow cytometry analysis of cell surface expression of CD1a, CD123, CD14, CD86^{hi} and PD-L1 by iDC. iDC were generated from peripheral blood monocytes after 4 days in culture with GM-CSF and IL-4, in the presence or absence of various concentrations of PDAC-CM as indicated. (**b**, **c**) iDC generated in the presence or absence of various concentrations of PDAC-CM were treated with LPS plus IFN-γ and analyzed by flow cytometry for cell surface CD86 and CD83 expression, intracellular IL-12 production (**b**) and secretion of IL-12, IL-23 and IL-10 (**c**). iDC: before LPS and IFN-γ treatment; mDC: LPS and IFN-γ-stimulated iDC; PDAC-iDC (%CM): iDC generated in the presence of indicated concentration of PDAC-CM; PDAC-mDC (%CM): LPS plus IFN-γ-stimulated PDAC-iDC. Dotted lines indicate isotype-matched control antibody staining. Numbers indicate percent positive cells. Results are expressed as mean ± s.e.m. of cytokine levels in 1.5×10^5 ml^{-1} of mDC obtained from the analysis of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, compared with mDC control. (**d**, **e**) Effects of PDAC-mDC on T-cell proliferation and differentiation in MLR. iDC (before LPS plus IFN-γ treatment), mDC (LPS plus IFN-γ stimulated iDC) and PDAC-mDC (LPS plus IFN-γ stimulated PDAC-iDC differentiated with 50% of PDAC-CM) were cocultured with allogeneic CD4$^+$ naive T cells. (**d**) Percent proliferation of CFSE-labeled CD4$^+$ T cells, analyzed after 5-day culture with the DC:T at a ratio of 1:100. Dotted lines indicate CFSE-labeled T cells without DC. Numbers in top left corners indicate percent proliferated cells. (**e**) IFN-γ$^+$ and IL-4$^+$ production by CD4$^+$ allogeneic T cells analyzed via intracellular cytokine staining after a 6-day mixed culture with a DC:T ratio of 1:20. Plots are gated on CD4$^+$ cells and quadrant frequencies are indicated.

action studies focused on defining effects of PDAC cells on T-cell and DC differentiation and function.

PDAC cells express surface markers that are typical of MSC, and also express CD200, a marker associated with placental cells.[15] PDAC cells exhibit limited differentiation potential, exemplified by the ability to express markers of adipogenic, osteogenic and chondrogenic lineage, under specific lineage culture conditions. In both *in vitro* and animal models, PDAC cells modulate T-cell priming

and T-cell differentiation. *In vitro*, PDAC cells reduce T-cell activation as well as Th1 and Th17 differentiation. Inhibition of DC maturation was also observed. In an antigen-specific OT-II $CD4^+$ T-cell adoptive transfer model, PDAC cells inhibited T-cell proliferation and increased IL-10-producing T cells in the spleen of animals challenged with the OVA antigen. In an EAE model, PDAC cell-induced reduction of clinical symptoms after peptide immunization was associated with a decrease in Th17-producing T cells and an increase in IL-10-producing cells in the central nerve system. In a DTH study, we found that PDAC cell-mediated suppression of inflammation after immunization with sRBCs was associated with reduced numbers of $CD86^+$ DC in the spleen. These data demonstrating effects on both T-cell and DC populations suggested that PDAC cell modulation of inflammation could be mediated either by direct T-cell interaction or by indirect modulation of APC. We therefore sought to examine PDAC cell-mediated effects on DC differentiation in further detail.

Described as 'nature's adjuvant', DC are the most potent APC, with an exquisite capacity to interact with T cells and modulate their responses. DC comprise heterogeneous populations that can mediate not only immunogenic but also tolerogenic effects *in vivo*, dependent upon environmental signals.[20] Characterization of PDAC cell effects on DC both in mouse and human cell culture demonstrated the ability of PDAC cells to induce tolerogenic DC. PDAC cells, even at low ratios to DC precursor cells (1:1000), strongly promoted the generation *in vitro* of DC with phenotype $CD1a^-$, $CD123^+$, $CD14^+$, $CD86^-$, PD-$L1^+$, IL-12^-, IL-23^- and IL-10^+. The downregulation of immunostimulatory/costimulatory signals such as CD1a, CD86, IL-12 and IL-23 and upregulation of PD-L1 and IL-10 are characteristics of tolerogenic DC.[21] Tolerogenic DC promote tolerance through modulation of T-cell responses, which is consistent with the observed PDAC-mDC suppression of allogeneic T-cell proliferation and reduced Th1 and Th17 $CD4^+$ T-cell differentiation. The PDAC-mDC phenotype is very similar but not identical to that of the recently described subtype of tolerogenic DC, termed DC-10, characterized as $CD14^+$, $CD16^+$, $CD11c^+$, $CD11b^+$, HLA-DR^+, $CD93^+$, $CD1a^-$ and IL-10^+. This tolerogenic DC subtype has been shown to induce IL-10-producing type 1 regulatory T cells (Tr1).[22] Although not directly demonstrated, this is consistent with the observed induction of IL-10-producing $CD4^+$ T cells in PDAC cell-treated animals in both the OT-II and EAE models.

Taken together, these data provide further support for a mechanism of PDAC cell-mediated suppression of immunoinflammatory responses through modification of T-cell immunity both directly and indirectly via induction of tolerogenic APC.

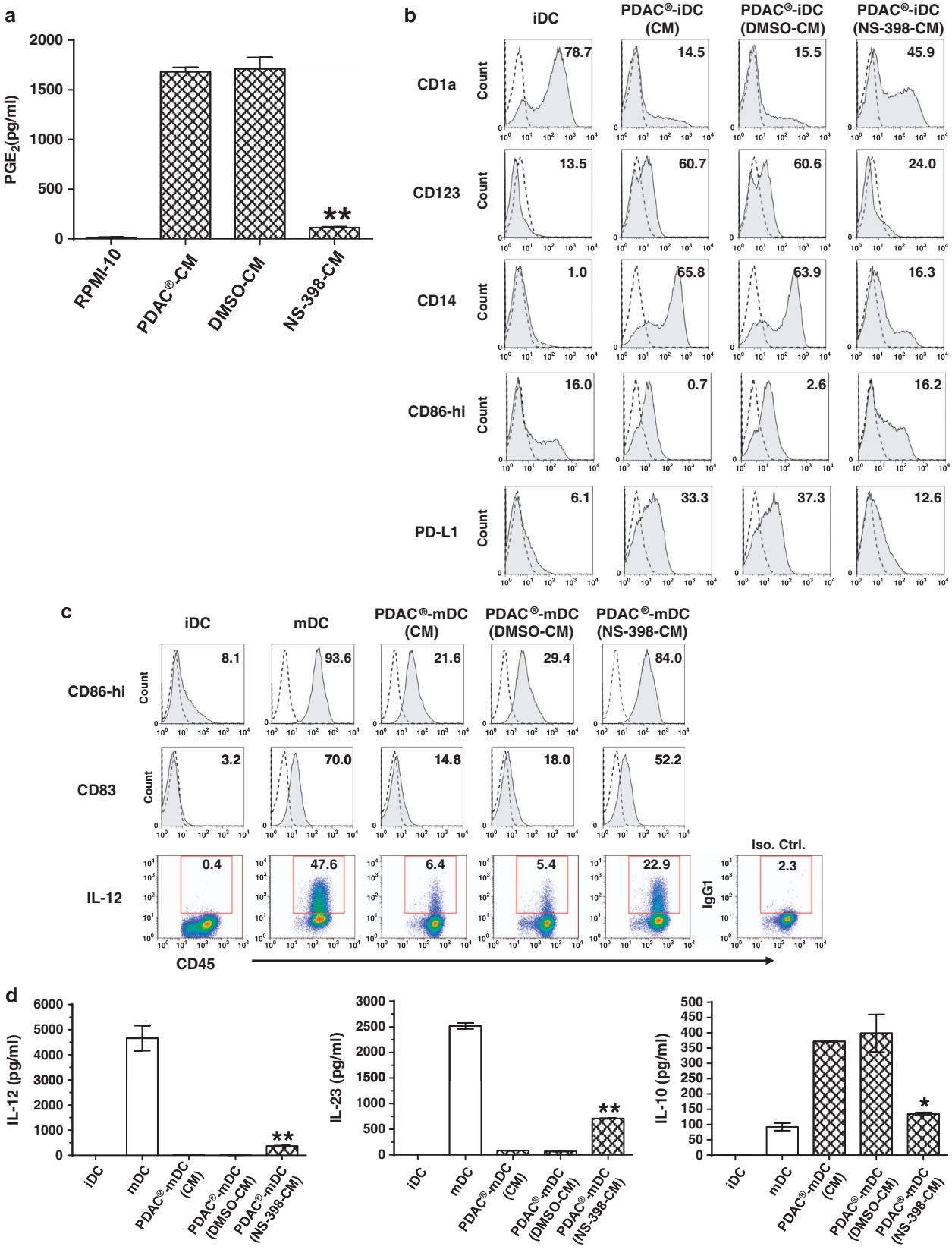
As PDAC cells are an MSC-like cell population, these immunoregulatory properties are similar to those observed for MSCs. Many *in vitro* studies have characterized MSC-mediated modulation of the phenotype and function of various immune cells, including T cells and DC.[11] Some MSCs have also been shown to modulate DC function, including effects on migration, maturation and antigen presentation, through multiple mechanisms.[23] Our study identifies modulation of DC function toward immune tolerance as a key mechanism underlying the ability of PDAC cells to suppress immune-mediated inflammation. To our knowledge, this is the first report showing that an MSC-like population induces PD-L1 expression by DC. A coinhibitory molecule belonging to B7 family, PD-L1, is critical for maintaining peripheral immune tolerance.[24] Failure of DC to maintain T-cell tolerance may contribute to the pathogenesis of autoimmune and inflammatory disorders.[25] Promoting tolerogenic DC could be exploited for various therapeutic purposes, with the advantage of promoting tolerance to specific pathogenic antigens without broad, systemic immunosuppression.

Our data comparing effects of PDAC cell cocultures and PDAC-CM on tolerogenic DC generation suggest that the immunoregulatory effects of PDAC cells are mediated via soluble factor(s). The observation that PDAC cells modulate mouse BMDC differentiation both in experiments of PDAC cells and BMDC coculture as well as PDAC cells and BMDC separated via a transwell further suggested that the soluble factor(s) was not species specific. PGE_2, a small molecule derivative of arachidonic acid, is well recognized as an immunoregulatory agent that can act across species.[18] In addition to the activity *in vitro*, PGE_2 or PGE_2 analogs show immunoregulatory efficacy in animal models, such as attenuation of EAE progression[26] and inhibition of intraerythrocytic Babesia microti-induced DTH.[27] A number of studies have implicated PGE_2 as an important molecular mediator underlying MSC-mediated suppression of DC maturation.[28] Our data support a role of PGE_2 secreted by PDAC cells as a mediator of the observed PDAC cell immunomodulatory effects on DC in culture. In the presence of PGE_2-deficient PDAC-CM, produced by the addition of the highly selective COX-2 inhibitor NS-398, the phenotypic and functional changes associated with PDAC cell-induced tolerogenic DC were significantly reduced. Instead, cDC development was mostly maintained, as indicated by the preservation of CD1a, CD86 and CD83 expression, loss of CD123, CD14 and PD-L1 expression and diminished IL-10 secretion by PDAC-iDC during maturation. As human MoDC are deficient in PGE_2 production (Zelle-Rieser *et al.*[29] and data not shown), NS-398 is presumed to act exclusively via suppression of PDAC cell-derived PGE_2.

Because only partial restoration of DC phenotype and function was observed in the presence of PGE_2-deficient CM, multiple mechanisms are likely involved in PDAC cell-mediated immunomodulation. *In vitro* studies that aimed to characterize the underlying molecular mechanisms of MSC-mediated immunosuppression identified a variety of contributing factors including IL-6,[30] galectin-1,[31] TGF-β1,[32] hepatocyte growth factor,[32–34] HLA-G,[35] IDO[36] and TNF-α-induced protein 6.[37] Such findings have not, however, been consistent in all studies,[34] and MSC species-specific and tissue of origin-specific effects have been suggested as possible explanations for such discrepancies. In addition to PGE_2, we also investigated the effects of PDAC cell-derived IL-6, galectin-1 and galectin-3 in coculture experiments, but found none to be critical to PDAC cell-mediated immunoregulation (data not shown). Furthermore, the presence of other soluble factors including TGF-β1, HLA-G and HGF in PDAC-CM was examined. We detected minimal TGF-β and HLA-G secretion but high levels of HGF in PDAC-CM (data not shown).

The therapeutic potential of human MSC in a xenogeneic setting has been explored in a variety of animal models.[38–40] However, it is still unknown how accurately findings in animal studies will predict clinical effects and which end points in the animal models will prove relevant to observations in humans. In this report, we demonstrated immunomodulation by PDAC cells *in vivo* in multiple animal models of immune-mediated inflammation and autoimmune disease. PDAC cells potently suppressed T-cell proliferation *in vivo* and significantly reduced the severity of Th1/Th17-driven EAE. PDAC cells ameliorated inflammation in a DTH response model in mice and reduced the number of $CD86^+$ DC in the spleen. Our studies have shown that these PDAC cell functions can be observed in both human and mouse cell cultures *in vitro*. The

concordance between our *in vivo* and *in vitro* findings, including those using human cells, suggests that certain animal models may be useful translational tools to better understand the pharmacodynamic effects and mechanisms of action of human PDAC cells, as well as to identify potential biomarkers of PDAC cell activity for use in the clinical setting.



CONCLUSION

In this study, we have demonstrated the ability of PDAC cells to inhibit immune-mediated inflammation in several animal models. This effect was associated with suppression of T-cell priming and differentiation, as well as modulation of DC maturation. Further characterization of the effects of PDAC cells on DC maturation and differentiation in mouse and human culture studies revealed modifications of DC phenotype characteristic of tolerogenic DC, which were mediated in part by PGE_2. Our findings strongly support modulation of DC function toward immune tolerance as a key mechanism underlying the immunomodulatory activity of PDAC cells. We are currently assessing the safety and therapeutic immunomodulatory activities of PDAC cells in human clinical trials.

METHODS

PDAC cell isolation and cell culture

PDAC cells were isolated by mechanical and enzymatic digestion of newborn membranes obtained exclusively from the normal, full-term, postpartum human placenta. PDAC cells were expanded and maintained in the PDAC cell expansion medium until passage 6. PDAC cell expansion medium is composed of Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) containing 2% (v/v) fetal bovine serum (Invitrogen), 0.001% (w/v) linoleic acid-albumin from bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), 0.1% (v/v) insulin, transferrin, selenium solution (Invitrogen), 50 μg ml^{-1} gentamycin (Invitrogen), 100 μM L-ascorbic acid (Sigma-Aldrich), 50 nM dexamethasone (Sigma-Aldrich), 1 ng ml^{-1} human platelet-human derived growth factor BB (R&D Systems, Minneapolis, MN, USA) and 1 ng ml^{-1} epidermal growth factor (R&D Systems). PDAC cells were cryopreserved for future use following passage 6 in freezing medium containing 5% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich), 10% (v/v) human serum albumin (SeraCare Life Sciences, Milford, MA, USA) and 5.5% (w/v) dextran (Hospira, Lake Forest, IL, USA).

PDAC cell differentiation assays

PDAC cells were plated at 1×10^4 cells cm^{-2} in 24-well plates in PDAC cell expansion medium and cultured until they reached ~80% confluence. For adipogenic and osteogenic differentiation, the PDAC cell expansion medium was changed to adipogenic (DMEM high glucose medium supplemented with 10% Fetal Calf Serum, 10 μg ml^{-1} insulin, 1 μM dexmethasone, 0.1 mM indomethacin, 0.5 mM isobutylmethylxanthine and 100 IU ml^{-1} Pen-Strep) or osteogenic induction medium (Cambrex, East Rutherford, NJ, USA). Evidence of osteogenesis was determined by the accumulation of calcium deposition in the extracellular matrix using Alizarin Red S (ARS) staining (GFS chemicals, Columbus, OH, USA) after 4 weeks of culture. Evidence of adipogenesis was determined by the presence of fat vacuoles using Oil-Red O staining with Oil-Red O Solution (Rowley Biochemical Institute, Danvers, MA, USA) after 5 weeks of culture. For chondrogenic differentiation, PDAC cells were trypsinized, washed twice in incomplete chondrogenesis medium (Cambrex) and resuspended at 5×10^5 cells per ml in complete chondrogenesis medium with TGF-β3 supplement added at 10 ng ml^{-1}. Aliquots of 500 μl were pipetted into 15 ml polypropylene centrifuge tubes, centrifuged at 300 *g* for 5 min to induce pellet formation, then incubated at 37 °C for 28 days, changing media every 3–4 days. On day 28, pellets were fixed in formalin for Alcian Blue and Sirius Red staining (Sigma-Aldrich).

Animal studies

Animal studies were conducted by contract research organizations or academic centers accredited by AAALAC (the Association for Assessment and Accreditation of Laboratory Animal Care), and studies were approved by the institutional IACUC and safety committees. Protocols adhered to Federal and State regulations and euthanasia protocols followed the guidance established by the American Veterinary Medical Association.[41]

Adoptive transfer of OT-II CD4$^+$ T cells and induction of antigen-specific proliferation

Four- to eight-week-old female C57BL/6-Tg (TcraTcrb) 425Cbn/J mice (Jackson Lab, Bar Harbor, ME, USA) were killed by isoflurane overdose, after which their spleens were removed. Single-cell suspensions were prepared from the spleens by mechanical dissociation using the gentle MACS-Octo Dissociator (Miltenyi Biotec, Cambridge, MA, USA). Red blood cells were removed by incubation in ACK lysis buffer (Life Technologies, Grand Island, NY, USA). The collected viable cells were washed twice in cold Hank's solution and filtered through 30-μm preseparation filters. The CD4$^+$ T cells were purified by negative selection, using an MACS system according to the manufacturer's protocol (Miltenyi Biotec). The purified CD4$^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) according to the CellTrace CFSE Kit instructions (Life Technologies).

For the primary stimulation, the purified CFSE-labeled CD4$^+$ T cells were resuspended in phosphate-buffered saline (PBS), and 3.36×10^6 cells per 0.3 ml of PBS were transferred into the recipient B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice via i.v. injection into the tail vein (Day 0). After 24 h, the recipient mice were immunized subcutaneously with 100 μg OVA peptide emulsified in complete Freund's adjuvant (CFA). Vehicle or PDAC cells at dosages of 0.3, 0.75 or 1.5×10^6 in 0.4 ml PBS were administered *via* tail vein injection 3 h after administration of OVA peptide. On day 8, the recipient B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice were killed as described above, and single-cell suspensions were prepared from the spleens for flow cytometry analysis as described below. The adoptively transferred OT-II CD4$^+$ T-cell population was identified *ex vivo* by expression of the congenic marker CD45.2. The proliferation of adoptively transferred T cells was analyzed by flow cytometric analysis as described. The health status of the mice was examined daily until the time of euthanasia.

Delayed type hypersensitivity

Four- to eight-week-old (BALB/c X C57BL/6) male F1 mice (Jackson Lab) were housed in the animal facility of University of Medicine and Dentistry of New Jersey (Newark, NJ, USA). Sheep red blood cells (sRBCs) (Colorado Serum Company, Denver, CO, USA) were used as an antigen. Two hundred microliters of sRBC suspension (1×10^6 cells per ml) in PBS was injected via tail vein into each mouse. In another tail vein, each mouse also received 0.5×10^6 or 1.5×10^6 PDAC cells, or PBS vehicle. To induce DTH response,

Figure 5 PGE_2 partially mediates the immunomodulatory effect of PDAC cells. (**a**) PGE_2 synthesis by PDAC cells. PGE_2 detected by ELISA in culture medium alone (RPMI-10), PDAC-CM, PDAC-CM + DMSO vehicle (DMSO-CM) and PDAC-CM + NS-398 (NS-398-CM), after 24 h. (**b**) Flow cytometry analysis of surface marker expression by immature DC generated in the presence of PDAC-CM, with or without NS-398. PDAC-CM, DMSO-CM and NS-398-CM were diluted at 1:1 with RPMI-10 medium before addition to MoDC culture. (**c**, **d**) Flow cytometry analysis of surface marker expression and intracellular IL-12 production (**c**) and IL-12, IL-23 and IL-10 secretion (**d**) by PDAC-iDC generated in the presence of PDAC-CM with or without DMSO or NS-398, following maturation induced by LPS. iDC: MoDC before LPS stimulation; mDC: mature DC induced by LPS; PDAC-mDC (CM): iDC generated in the presence of PDAC-CM, after maturation in response to LPS; PDAC-mDC (DMSO-CM): iDC generated in the presence of PDAC-CM containing DMSO, after maturation in response to LPS; PDAC-mDC (NS-398-CM): iDC generated in the presence of PDAC-CM containing NS-398, after maturation in response to LPS. Dotted lines indicate isotype-matched control antibody staining. Numbers indicate percent positive cells. Cytokine levels are expressed as mean ± s.e.m. in 1.5×10^5 cells per ml. Results from one of the three independent experiments are shown. $^*P<0.05$, $^{**}P<0.01$, compared with CM-mDC control.

mice were challenged 4 days later with 50 µl sRBCs (2×10^9 cells per ml) in PBS injected into the right footpad. Right footpad swelling was measured by using an electronic digital caliper at 24 h after the challenge using paw thickness as the readout. The left footpad was used as the control. Mice were killed on day 5, and spleens were collected for *ex vivo* analysis.

Induction of EAE and isolation of single-cell suspensions from the spinal cord

To induce EAE, 4- to 8-week old C57Bl/6NTac mice (Taconic Farm, Germantown, NY, USA) were immunized subcutaneously with 200 µg of MOG-35–55 peptide emulsified in CFA in combination with intraperitoneal (i.p.) injections of 400 ng of pertussis toxin (PTX) 24 h after immunization. All reagents were purchased from Sigma (Sigma-Aldrich). Nine days post immunization, 400 µl of PBS, vehicle and 1.5×10^6 PDAC cells in 400 µl of vehicle were administered to the mice through tail vein injection. Fingolimod (FTY720), a daily oral therapy for multiple sclerosis (10 mg kg^{-1}), was given by intragastric gavage (in 200 µl PBS) daily from day 9 through 31 as an internal comparator. Each group had 10 mice that were examined daily for clinical signs of EAE and overall health status. In this EAE model, the severity of the encephalitis was assessed using a predetermined clinical scoring system according to a 0–5 scale: 0 = normal behavior; no neurological signs; 0.5 = partial tail weakness; 1.0 = tail weakness or waddling gait with tail tonicity; 1.5 = waddling gait with partial tail weakness; 2.0 = waddling gait with limp tail (Ataxia); 2.5 = ataxia with partial limb paralysis; 3.0 = full paralysis of one limb; 3.5 = full paralysis of one limb with partial paralysis of second limb; 4.0 = full paralysis of both Limbs; 4.5 = moribund; and 5.0 = death.

On day 16 post immunization, at the peak of clinical EAE symptoms, spinal cords were collected and single-cell suspensions were prepared by mechanical dissociation using gentle MACS-Octo Dissociator (Miltenyi Biotec). Myelin was removed using Myelin Removal beads II (Miltenyi Biotec) before performing *ex vivo* flow cytometry analyses, as described below.

Mouse BMDC

Femurs and tibia from male C57BL/6 5- to 7-week old mice (Taconic) were collected, and bone marrow was collected from each bone and pooled. Red blood cells were lysed and BMC were suspended at 1×10^6 cells per ml in RPMI-10 medium (RPMI-1640 medium (ATCC, Manassas, VA, USA) supplemented with 10% (v/v) fetal bovine serum and 100 IU ml^{-1} penicillin-streptomycin (Invitrogen). One ml of bone marrow suspension was added to each well of a 24-well plate and incubated in the presence or absence of PDAC cells, at ratios of 1:10 to 1:1000 PDAC cell:BMC. Cultures were incubated for 8 days in the presence of 20 ng ml^{-1} recombinant mouse GM-CSF (Peprotech, Rocky Hill, NJ, USA) for BMDC differentiation[42] with medium changed every 2 days. PDAC cell transwell cultures of BMDC were maintained for 8 days. On day 8, the suspension fraction, containing the majority of immature BMDC, was collected. A fraction of the immature BMDC was analyzed by flow cytometry. To induce BMDC maturation, collected cells were washed and resuspended at 1×10^6 cells per ml in RPMI-10 medium containing 0.1 µg ml^{-1} LPS (from the *Escherichia coli* 0111:B4 strain; Sigma-Aldrich) and incubated for 4 h, then analyzed by flow cytomtery. When indicated, BMDC maturation was performed in the presence of PDAC cell coculture. For maturation of BMDC in the presence of PDAC cells separated by transwells, PDAC cells (3×10^4) were seeded onto each filter chamber of a Transwell-six-well plate (0.4 µm pore size) (Corning, Tewksbury, MA, USA), and BMC (1×10^6) were seeded into each culture well below. For analysis of the effect on DC differentiation and maturation, DC cell surface phenotype and cytokine secretion profile are characterized as described below.

Mouse cytokine multiplex analysis

Culture supernatants were collected from immature BMDC cultured in the presence or absence of PDAC cells at a ratio of 1:30 PDAC cell:BMDC, after 4 h of LPS stimulation. IL-10 and IL-12 levels were measured in duplicate using a Cytokine Mouse 20-plex Panel (Invitrogen) following the manufacturer's instructions.

Generation of MoDC

PBMC were separated from healthy donor whole blood-derived buffy coats (Blood Center of New Jersey, East Orange, NJ, USA) using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ, USA). The CD14$^+$ monocytes were positively selected from PBMC using the CD14 MicroBead MACS (Miltenyi Biotec), according to the manufacturer's instructions. The purity of isolated monocytes was determined by flow cytometry. Then $4–6 \times 10^6$ monocytes were cultured in 100×15 mm petri dishes (Fisher Scientific, Pittsburgh, PA, USA) in 10 ml of RPMI-10 medium with 20 ng ml^{-1} recombinant GM-CSF (PeproTech) and 40 ng ml^{-1} recombinant human IL-4 (R&D Systems). After 4 days, the iDC were collected by gentle rinse and analyzed by flow cytometry to determine iDC phenotype. To generate mDC, 3×10^5 iDC were re-seeded at day 4 onto six-well tissue culture plates and incubated for 24 h with 0.5 µg ml^{-1} of LPS (from the *Escherichia coli* 0111:B4 strain; Sigma-Aldrich) alone or in combination with 10 ng ml^{-1} of recombinant human IFN-γ (Becton Dickinson, San Jose, CA, USA).

MoDC differentiation in PDAC cell-conditioned medium

PDAC-CM was generated by culturing PDAC cells at 8×10^4 cells per ml in 10 ml of RPMI-10 medium for 24 h in a T75 CellBIND flask (Corning). In selected flasks, 5 µl of 10 mM NS-398 (Cayman Chemical, Ann Arbor, MI, USA) stock solution in DMSO was added (final concentration 5 µM NS-398) to inhibit PGE_2 production. In certain experiments, PDAC-CM was mixed with sufficient volumes of fresh RPMI-10 medium to yield PDAC-CM fractions ranging from 2 to 50% in 10 ml of total medium per petri dish. Monocytes ($4–6 \times 10^6$ per petri dish) were resuspended in this mixed medium and cultured, each in 10 ml total medium, as described above.

Isolation and CFSE labeling of human naive CD4$^+$ T cells

PBMC were separated as described above. Human CD4$^+$ naive T cells were negatively selected using a Human Naïve CD4$^+$ T Cell Isolation Kit II (Miltenyi Biotec). The purified naive CD4$^+$ T-cell population was labeled with CFSE at a final concentration of 5 µM according to the CellTrace CFSE Kit instructions (Life Technologies). CFSE-labeled cells were cryogenically stored in FBS containing 10% DMSO for future use.

Mixed leukocyte reactions

Two thousand mDC or iDC differentiated in the presence or absence of PDAC-CM were mixed with 2×10^5 allogeneic CFSE-naive CD4$^+$ T cells in individual wells of a 96-well culture plate and cultured for 5 days. T-cell proliferation was assessed using flow cytometric analysis of CFSE dilution in previously labeled T cells. In T-cell polarization studies, 5×10^4 DC from various conditions, as indicated, were mixed with 1×10^6 allogeneic CD4$^+$ T cells in individual wells of a 24-well culture plate. Cells were collected following 6 days of culture for intracellular cytokine staining.

Flow cytometric analyses

All antibodies for flow cytometry staining were purchased from BD Biosciences (Biosciences, San Jose, CA, USA) except anti-human-CD45-PerCP-Cy5.5, anti-CD14-PE, anti-HLA-DR-PE, anti-CD83-PE, anti-IL-12-PE that were purchased from eBioscience (San Diego, CA, USA) and anti-CD105-Alexa Fluoro647 that was purchased from AbD Serotech, Kidlington, UK.

For surface marker expression analysis, cells (PDAC cells, DC and T cells) or single-cell suspensions from EAE and OT-II models were washed with FACS wash buffer (PBS with 2% FBS) and labeled with antibodies by incubation at 4 °C for 30 min. The surface staining of cells for intracellular cytokine staining was performed 5 h after the addition of Golgi-stop (BD Biosciences). After two washes, DC and T cells of single-cell suspensions were treated with BD Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences) for 20 min at 4 °C. Cells were subsequently washed with BD PermWash buffer (BD Biosciences) and incubated with antibodies against intracellular cytokines.

Flow cytometry analysis was performed on a FACS Canto II (BD Biosciences). Data were acquired with FACSDiva software (BD Biosciences) and analyzed using FlowJo flow cytometry software (Tree Star, Ashland, OR, USA).

In vivo proliferation of the adoptively transferred CFSE-labeled T cells was analyzed by calculating the proliferative index, an average number of cell divisions (over the 8 day period) in the OT-II CD4 T-cell population, using the cell proliferation analysis tool in the FlowJo software.

Cytometric bead arrays

Supernatants from DC cultures were collected after 24-h incubation with no addition, LPS or LPS plus IFN-γ, as indicated above. The IL-12, TNF-α and IL-10 levels in undiluted supernatants were quantified in duplicate using the BD cytometric bead array Human Inflammatory Cytokines Kit (BD Biosciences) following the manufacturer's instructions. The data were analyzed with FCAP Assay software (Soft Flow, Pecs, Hungary).

Enzyme-linked immunosorbent assay

IL-23 was measured in undiluted DC culture supernatants in duplicate using the human IL-23 sandwich ELISA Ready-SET-Go! Set (eBioscience). The PGE_2 level in the conditioned medium was measured using a prostaglandin E_2 EIA kit (Cayman Chemical). ELISA assays were conducted according to the manufacturer's instructions.

Statistical analysis

Statistical significance was determined by 2-tailed Student's *t*-test in all *in vitro* studies. *P*-values of less than 0.05 were considered statistically significant. Two-way analysis of variance (ANOVA) with repeated measures was used to analyze differences between groups of mice *in vivo*. When significant treatment related effects were observed (ANOVA; $P<0.05$), pair wise least significant difference *post-hoc* analyses were performed.

CONFLICT OF INTEREST

The authors are employees of Celgene Corporation.

ACKNOWLEDGEMENTS

We thank Erica Giarritta and Kathy Karasiewicz for technical assistance. We thank Dr David Valacer and Dr Michael Lotze for helpful suggestion on the manuscript. The work was supported by Celgene Corporation internal research funding.

Author contributions: WL, BL and RH: conception, design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. VJ, SH, XL, QY, KL and H-JC: design, collection and/or assembly of data, data analysis and interpretation. AM, JS and XZ: collection and/or assembly of data, data analysis. UH, VRA and SEA: design and manuscript preparation.

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