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PD-1 on Immature and PD-1 Ligands on Migratory Human Langerhans Cells Regulate Antigen-Presenting Cell Activity

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Abstract

Langerhans cells (LCs) are known as "sentinels" of the immune system that function as professional antigen-presenting cells (APCs) after migration to draining lymph node. LCs are proposed to have a role in tolerance and the resolution of cutaneous immune responses. The Programmed Death-1 (PD-1) receptor and its ligands, PD-L1 and PD-L2, are a co-inhibitory pathway that contributes to the negative regulation of T-lymphocyte activation and peripheral tolerance. Surprisingly, we found PD-1 to be expressed on immature LCs (iLCs) *in situ*. PD-1 engagement on iLCs reduced IL-6 and macrophage inflammatory protein (MIP)-1α cytokine production in response to TLR2 signals but had no effect on LC maturation. PD-L1 and PD-L2 were expressed at very low levels on iLCs. Maturation of LCs upon migration from epidermis led to loss of PD-l expression and gain of high expression of PD-L1 and PD-L2 as well as co-stimulatory molecules. Blockade of PD-L1 and/or PD-L2 on migratory LCs (mLCs) and DDCs enhanced T-cell activation, as has been reported for other APCs. Thus the PD-1 pathway is active in iLCs and inhibits iLC activities, but expression of receptor and ligands reverses upon maturation and PD-L1 and PD-L2 on mLC function to inhibit T-cell responses.

INTRODUCTION

Langerhans cells (LCs) are myeloid-derived dendritic cells (DCs) distributed along the stratified epithelia of tissues exposed to the environment such as skin and mucosa. LCs are known as "sentinels" of the immune system and form a network in the epidermis where they actively take up antigen by extending and retracting their dendrites. During migration to draining lymph nodes, LCs process antigen and upregulate the level of co-stimulatory molecules. In the lymph node, LCs function as professional antigen-presenting cells (APCs) (Merad *et al.*, 2008). In the steady state, LCs are proposed to have a role in tolerance and the resolution of cutaneous immune responses.

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CONFLICT OF INTEREST

GF has patents in the PD-1 field and receives royalties. Other authors state no conflict of interest.

Although DCs can function as inducers of T-cell activation, they also have a role in peripheral tolerance (Banchereau and Steinman, 1998; Steinman *et al.*, 2003). In murine models when DCs present antigen in the absence of inflammation and pathogenic stimulus, they can induce peripheral tolerance (Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002). There is increasing evidence for a role of LCs in tolerance (Merad *et al.*, 2008); however, most of these studies have been conducted using mouse models and the results are controversial. For instance, it has been suggested that epidermal LCs induce tolerance through stimulation of regulatory T cell development (Loser *et al.*, 2006). However, Langerin$^+$ cells that have migrated to the lymph node express high levels of CD80, CD86, CD40, and major histocompatibility complex class-II, as well as PD-1 ligand (PD-L)1 and PD-L2, suggesting that the function of these LCs in mice is not tolerogenic (Stoitzner *et al.*, 2005).

Here we explore the expression and function of a co-inhibitory pathway, PD-1 and PD-1 ligands, on LCs. PD-1 (CD279) is a CD28 family member expressed on activated T and B cells, as well as some myeloid cells (Okazaki and Honjo, 2007). PD-1 delivers a co-inhibitory signal upon binding to either of its two ligands PD-L1 (B7-H1; CD274) or PD-L2 (B7-DC; CD273) (Greenwald *et al.*, 2005). The PD-1 pathway has a role in peripheral tolerance and deficiency of either PD-1 or PD-L1 results in accelerated development of diabetes in NOD mice (Wang *et al.*, 2005; Keir *et al.*, 2006). The PD-1 pathway also functions to limit immune-mediated damage. Tumors and chronic infections have exploited the PD-1 pathway to evade eradication by the immune system (Greenwald *et al.*, 2005; Freeman *et al.*, 2006; Zou and Chen, 2008).

PD-L2 is expressed mainly on activated DCs and macrophages (Ishida *et al.*, 2002; Yamazaki *et al.*, 2002; Liang *et al.*, 2003) whereas PD-L1 is broadly expressed on many cell types. PD-L1 is upregulated after activation of B cells, T cells, DCs, and macrophages (Ishida *et al.*, 2002; Yamazaki *et al.*, 2002; Brown *et al.*, 2003; Liang *et al.*, 2003), and also expressed on various parenchymal cells (Freeman *et al.*, 2000; Ansari *et al.*, 2003; Brown *et al.*, 2003; Iwai *et al.*, 2003; Liang *et al.*, 2003; Salama *et al.*, 2003; Wiendl *et al.*, 2003). Blockade of PD-1 ligands on human DCs derived from monocytes enhances T-cell activation (Brown *et al.*, 2003).

Here we explore the expression of the immunoinhibitory PD-1/PD-L pathway in human epidermal LCs. Our studies show that PD-1 is expressed on immature (iLCs) *in situ* and that this expression is functionally significant. PD-1 on iLCs reduces IL-6 and macrophage inflammatory protein (MIP)-1α cytokine production in response to Toll-like receptor-2 (TLR2) signals. Expression of PD-1 declines on LCs with maturation, whereas expression of PD-1 ligands increases. PD-L1 and PD-L2 on migratory LCs (mLC) function to inhibit T-cell responses.

RESULTS

Phenotypic differences between iLCs and mLCs

Our goal was to compare the phenotype and function of iLCs and mLCs. As shown in Figure 1a, both iLCs and mLCs expressed the typical LC markers CD1a and CD207 (Langerin). The maturation marker, CD83, was not expressed on iLCs but was upregulated in mLCs (Figure 1b). The co-stimulatory protein, CD80, was not expressed on iLCs but strongly expressed on mLCs (Figure 1c). The co-stimulatory protein, CD86, was weakly expressed on a subpopulation of iLCs but strongly expressed on all mLCs (Figure 1d). These results show clear changes in surface marker expression after migration of LCs and suggest important differences in genetic program and function between iLCs and mLCs.

PD-1 expression on human LCs

As expression of co-stimulatory proteins changes with LC maturation, we examined the expression of co-inhibitory receptors and ligands. Flow cytometric analysis showed that the co-inhibitory receptor, PD-1, is present at moderate levels on the cell surface of iLCs but expression is much lower on mLCs (Figure 2a). To confirm this unexpected finding, expression of PD-1 was examined by reverse transcription–PCR (RT–PCR). Two preparations of iLCs expressed PD-1 mRNA as did the positive control of Jurkat cells transfected with PD-1 cDNA, but expression was not detected on mLCs or keratinocytes (Figure 2b). Localization of PD-1 on iLCs was examined by immunofluorescence using confocal microscopy. Both PD-1 and CD1a were primarily located on the cell surface (Figure 2c). Immunohistochemical analysis of serial sections of human skin showed expression of PD-1 together with CD207 on iLCs in the basal epidermis (Figure 2d). Double staining of frozen sections of skin with PD-1 and CD1a showed co-expression on LCs. These results indicate that PD-1 is expressed on iLCs and declines with LC migration due to decrease in gene expression.

PD-1 engagement on iLCs reduces TLR-mediated cytokine production

In T cells, PD-1 engagement by PD-1 ligands diminishes T-cell receptor (TCR)/CD28 signaling and PD-1 is described as a co-inhibitory receptor. However, the role of PD-1 signaling in iLCs is unknown and it is unclear whether PD-1 in iLCs signals directly or modifies the signal from another receptor. As TLR signals promote cytokine production by LCs, we examined whether PD-1 engagement affected the levels of TLR-induced LC cytokine production. We stimulated iLCs with a TLR2 agonist, Pam3Cys (Niebuhr *et al.*, 2009), as LCs are known to express TLR2 (Peiser *et al.*, 2008). To engage PD-1 on iLCs, we cultured the iLCs with beads coated with PD-L1-Ig, PD-L2-Ig, or an isotype IgG2a as control. We examined the production of 11 cytokines that have been reported to be produced by LCs after various treatments (Wang *et al.*, 1999). Pam3Cys induced the production of IL-6, IL-10, MIP-1α, and MIP-1β. PD-1 engagement significantly reduced Pam3Cys-induced production of IL-6 and MIP-1α in three separate experiments (Figure 3c). PD-1 engagement reduced the production of MIP-1β and IL-10, but this result did not reach statistical significance (data not shown). Other cytokines assayed, including IL-1α, IL-1β, tumor necrosis factor-α, IL-12p40, IL-15, IL-18, and RANTES were expressed at low/undetectable levels after treatment with Pam3Cys and levels were not significantly affected by PD-1 engagement. These results suggest that PD-1 can act as a co-inhibitory receptor in iLCs, attenuating TLR-mediated signals.

PD-1 engagement on iLCs does not affect the expression of the maturation markers CD83, CD80, and CD86

PD-1 engagement did not affect LC maturation, as judged by a high percentage and mean fluorescence intensity of CD83, CD80, and CD86 cell-surface maturation markers (Figure 3a and b, and Table 1). In addition, TLR2 engagement by Pam3Cys did not affect LC maturation and was not affected by PD-1 engagement. These results suggest PD-1-mediated co-inhibition is specific and not global.

PD-1 ligands are upregulated after migration of LCs

iLCs expressed low levels of PD-L1 and PD-L2; however, mLCs expressed high levels of both PD-L1 and PD-L2 (Figure 4). Quantitation of mRNA expression levels in iLCs and mLCs showed upregulation of PD-1 ligands in mLCs was due to an increase in mRNA levels (Figure 4e and f). Unstimulated keratinocytes did not express PD-L1 or PD-L2 mRNAs.

Blockade of the PD-1 pathway upregulates T-cell alloresponses to mLCs

As LC maturation results in the upregulation of both co-stimulatory (CD80, CD86) and co-inhibitory (PD-L1, PD-L2) molecules, we examined the effects of blockade of the PD-1 pathway on a CD4 T-cell alloresponse to mLCs in 6 day and in 40 hours cultures. Blockade of PD-L1 on mLCs significantly augmented CD4 T-cell alloresponses (Figure 4g and h). Blockade of PD-L2 significantly increased responses in the 40 hours culture, but the effect was not significant in the 6-day culture. Blockade of both together was more effective than either alone. Blockade of PD-1 on T cells also significantly augmented T-cell alloresponses. T cells or mLCs alone did not proliferate significantly. These results indicate that PD-L1 and PD-L2 on mLCs can engage PD-1 on T cells and downregulate T-cell responses.

Blockade of the PD-1 pathway upregulates T-cell alloresponses to DDCs and mLCs

We compared the effects of PD-1 pathway blockade on the capacity of mLCs and dermal dendritic cells (DDCs) to stimulate a T-cell alloresponse. DDCs were characterized as $CD11c^{+}CD14^{-}$ cells with low levels of CD1a (Figure 5a). DDCs expressed high levels of PD-L1 and PD-L2 mRNA (Figures 5b). Blockade of PD-L1 or PD-1 significantly augmented CD4 T-cell alloresponses to both mLC and DDC (Figure 5c and d). Blockade of PD-L2 alone did not significantly increase alloresponses. Blockade of PD-L1 and PD-L2 together was more effective than blockade of either alone. These results show that PD-1 ligand expression is significant in both populations of skin APCs.

DISCUSSION

Our work with human LCs shows constitutive expression of PD-1 on iLCs in human skin. PD-1 expression has been extensively studied in T cells and has recently been described in stimulated murine DCs but has not been examined in LCs. PD-1 expression can be induced on mouse splenic DC by inflammatory stimuli, including *Listeria monocytogenes* infection or TLR2, TLR3, TLR4, or NOD signaling (Yao *et al.*, 2009). PD-1-deficient DCs were more effective than wild-type DCs in innate protection of mice against *L. monocytogenes* lethal infection. PD-1 engagement on $PD\text{-}1^{+}$ splenic DCs downregulated IL-12 and tumor necrosis factor-α production. These results show an emerging role for PD-1 in the negative regulation of DC function during innate immune responses. Our results with human LCs contrast with the mouse DCs results in showing constitutive expression of PD-1 *in situ* rather than induced expression, underscoring the importance of our findings for immune responses in human skin.

In T cells, engagement of PD-1 by PD-L1 or PD-L2 results in phosphorylation of tyrosines in the PD-1 cytoplasmic domain and recruitment of phosphatases, particularly SHP2 (Latchman *et al.*, 2001; Chemnitz *et al.*, 2004; Sheppard *et al.*, 2004), resulting in attenuation of the TCR signal. This reduction in TCR signals also reduces the TCR-induced stop signal, resulting in briefer engagement of the T cell with the APCs (Fife *et al.*, 2009). On the basis of this co-inhibitory mechanism in T cells, we examined whether PD-1 engagement could inhibit the the consequences of TLR2 signaling in iLCs, as LCs express TLR2 and the TLR2 pathway is known to involve tyrosine phosphorylation (Gray *et al.*, 2006; Medvedev *et al.*, 2007; MacLeod *et al.*, 2008). We used a TLR2 agonist, Pam3Cys (Niebuhr *et al.*, 2009), to stimulate iLCs in the presence or absence of PD-L1-Ig or PD-L2-Ig. Pam3Cys induced the production of IL-6 and MIP-1α in iLCs; furthermore, engagement of PD-1 reduced the production of these cytokines. These results indicate that PD-1 in iLCs can act in a co-inhibitory manner. PD-1 did not globally inhibit LC maturation and had no effect on the expression of CD83, CD80, or CD86.

Cytokine production by LCs may regulate tolerance and immune responses. PD-1 engagement downregulated IL-6 and MIP-1α production by iLCs. IL-6 is a pleiotropic cytokine known to be involved in inflammatory and autoimmune diseases, and T-cell subset differentiation (Blanco *et al.*, 2008). MIP-1α is important for T-cell chemotaxis from the circulation to inflamed tissue. These results suggest that PD-1 on iLCs can regulate LC function and suggest a wider analysis of PD-1 function on LCs *in vivo*.

The cells that would naturally provide a PD-L1 or PD-L2 signal to PD-1 on iLCs remain to be determined. Previous work has shown high levels of PD-L1 and PD-L2 on keratinocytes during chronic inflammation (Freeman *et al.*, 2000; Youngnak-Piboonratanakit *et al.*, 2004). IFN-γ potently upregulates PD-L1 expression on keratinocytes.

Here we show that as LCs migrate from the epidermis and mature, expression of PD-1 declines and expression of PD-L1 and PD-L2 increases. This “reversal” of receptor/ligand expression during cell maturation is also seen for some other co-stimulatory pathways such as LIGHT/HVEM (Morel *et al.*, 2000; Tamada *et al.*, 2000). As seen with other types of APCs, we showed that blockade of either PD-L1 or PD-L2 on mLCs enhanced T-cell activation. Dual blockade of PD-L1 and PD-L2 led to the greatest increases indicating that both ligands were active.

Maturation of LCs leads to high-level expression of major histocompatibility complex class-II, co-stimulatory molecules such as CD80, CD86, CD40, and CD83, as well as co-inhibitory PD-L1 and PD-L2. This dual expression of co-stimulatory and co-inhibitory molecules on APCs seems paradoxical; however, T-cell populations differentially express receptors for co-stimulatory and co-inhibitory signals and will respond differently. For instance, naïve T cells express CD28 but not cytotoxic T lymphocyte antigen-4 or PD-1 (Greenwald *et al.*, 2005), whereas “exhausted” CD8 T cells or regulatory T cells express high levels of cytotoxic T lymphocyte antigen-4 and PD-1 (Barber *et al.*, 2006; Day *et al.*, 2006; Raimondi *et al.*, 2006). Expression of both types of ligand on an APC may permit fine-tuning of the T-cell response and expression of the co-inhibitory ligands would raise the threshold of TCR signal needed for T-cell activation. This would reduce the response to weak antigens and focus the response on antigens that deliver a strong TCR signal (Latchman *et al.*, 2001). Studies of the role of LCs *in vivo* in mice show a role in the resolution of cutaneous immune responses and inhibition of contact hypersensitivity and responses against skin commensal microorganisms and innocuous environmental antigens, and are reviewed by Kaplan *et al.* (2008); Obhrai *et al.* (2008); and Igyarto *et al.* (2009).

Consistent with previous studies showing that PD-1 engagement downregulates TCR or B-cell receptor signals in lymphocytes, our results show that PD-1 engagement can attenuate TLR signaling and downregulate cytokine production in iLCs. Our experiments have identified one function of PD-1 in LCs and further *in vivo* experiments are needed to identify the complete function of PD-1 in LCs. On the basis of our *in vitro* results, we speculate that PD-1 expression on iLCs in human skin could have a role in reducing the APC functions of LCs before migration and maintaining hyporesponsiveness in the steady state (Kaplan *et al.*, 2005; Obhrai *et al.*, 2008; Vesely *et al.*, 2009). The role of PD-1 on APCs is just beginning to emerge and recent work suggests that PD-1 on APCs has an important regulatory role.

MATERIALS AND METHODS

LC and DDC preparation

Normal human skin from reduction mammoplasties was acquired as discarded material from Brigham and Women’s Hospital (Boston, Massachusetts) after institutional IRB approval. LC extraction was performed following our published method (Pena-Cruz *et al.*, 2001).

After removing adipose tissue, skin was incubated in dispase grade-II (Boehringer-Mannheim, Indianapolis, IN) overnight. The epidermis was manually detached from the dermis. A single-cell suspension was prepared by digestion with 2.5 μg ml^{-1} trypsin (Sigma, St Louis, MO) and 100 U ml^{-1} DNase-I (Roche, Indianapolis, IN) in phosphate-buffered saline (PBS) at 37 °C for 30 minutes. Cells were separated through an OptiPrep density gradient (Accurate Chemical & Scientific Corp., Westbury, NY) (Pena-Cruz *et al.*, 2001). iLCs were enriched at 1.057 and 1.068 gml^{-1} densities, with purity of 75% as judged by CD1a expression, and viability was >95%. The primary contaminating cells were keratinocytes. Purified keratinocytes were obtained from the 1.090-g ml^{-1} density fraction. mLCs and DDCs were obtained by incubating the epidermis or dermis, respectively, for 48–72 hours in RPMI-1640 (Mediatech, Herndon, VA) with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells that had migrated out of the epidermis were further purified by density gradient as above. mLCs were enriched at 1.057 g ml^{-1} and purity was >90% and viability >95%. DDCs were enriched at 1.057 gml^{-1} and purity was >90% as judged by CD11c expression and viability >95%.

Confocal microscopy

iLCs were placed on 12-mm-diameter glass coverslips previously treated with polylysine (Sigma, St Louis, MO). The attached cells were fixed in 3.9% paraformaldehyde, permeabilized using 0.05% saponin (Sigma, St Louis, MO), and stained using CD1a (OKT6-FITC) and PD-1 (EH12-PE) for 1 hour (Diaz-Griffero *et al.*, 2002). Preparations were mounted using the Prolong Antifade kit (Invitrogen, Los Angeles, CA). Images were obtained using a Bio-Rad Radiance 2000 laser-scanning confocal microscope with Nikon × 60 1.4 numerical aperture optics.

Immunohistochemistry

Human skin was fixed in formalin, paraffin-embedded, and sectioned at 5 μm thickness. For single-color immunohistochemistry, deparaffinized sections were incubated with mAbs against CD207 (Immunotech, Marseille, France) or PD-1 (EH12) (Dorfman *et al.*, 2006), and then with biotinylated goat anti-mouse IgG followed by treatment with streptavidin–peroxidase complex (Vector Laboratories, Burlingame, CA) and counter-stained with Giemsa. For double staining, frozen sections were fixed with acetone and methanol (1:1). After washing with PBS and incubation in 3% BSA, tissue was incubated with PD-1 mAb (EH12) overnight at 4 °C. After washing with PBS, sections were incubated with goat anti-mouse IgG conjugated with Texas Red (Invitrogen, Eugene, OR) for 1 hour at room temperature, washed twice with PBS, and incubated with CD1a (OKT6-FITC) for 2 hours at 4 °C. After washing, the slide was mounted with Vectashield with 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). A Nikon Eclipse E800 microsope and a MetaVue imaging system were used for fluorescence microscopy (Molecular Devices, Madison, WI).

Flow cytometry

iLCs and mLCs were analyzed for cell-surface protein expression using the following mAbs: CD1a, OKT6-FITC (Ortho-mune, Raritan, NJ): Langerin, CD207-PE, and CD86-PE (Immunotech, Marseille, France), CD80-PE, CD80-V450, CD83-PE, and CD86-APC (BD Biosciences, San Diego, CA). mAbs against PD-1 (EH12, IgG1) (Dorfman *et al.*, 2006), PD-L1 (29E.2A3, IgG2b), and PD-L2 (24F.10C12, IgG2a) have been described by Brown *et al.* (2003). MOPC31C (IgG1), MPC11 (IgG2b), and C1.18.4 (IgG2a) were used as isotype controls (Bio-X-Cell, West Lebanon, NH). Phycoerythrin (PE)-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b were used as secondary antibodies (Southern Biotech, Birmingham, AL). Flow cytometry was performed using BD FACSCanto II and analyzed using the BD

FACSDiva software (Becton Dickinson, San Jose, CA); and on a Cytomics FC500 (Beckman Coulter, Fullerton, CA) and using the FlowJo software (Tree Star, Ashland, OR).

Cytokine assay

Cell culture supernatants were collected at 24 hours and assayed by multiplex ELISA (Aushon Biosystems, Billerica, MA).

Preparation of coated beads and stimulation of LCs

Approximately 10×10^6 sulfate latex beads (5 μm diameter) (Invitrogen, Eugene, OR) in 1ml PBS were incubated with 10 μg ml^{-1} PD-L1-Ig or PD-L2-Ig fusion protein (murine IgG2a Fc) (Latchman *et al.*, 2001), or the mIgG2a mAb, for 90 minutes at 37°C. The endotoxin levels of all proteins were less than 2 EU mg^{-1}. Fetal bovine serum was then added to 1% and beads were washed. iLCs in a round-bottomed, 96-well (1×10^5 but in one experiment, 1.7×10^5) were incubated with coated beads (5×10^5/well) in the presence or absence of 400 ng ml^{-1} of the TLR2 agonist, Pam3Cys (EMC Microcollections, Tübingen, Germany). Supernatants were harvested after 24 hours and assayed for cytokine production.

RT–PCR

Total RNA was extracted from iLCs, mLCs, and human keratinocytes using Trizol (Invitrogen, Carlsbad, CA). cDNA was prepared from 100 ng of total RNA according to the manufacturer's protocol (Applied Biosystems, Foster City, CA) and one-tenth of the reaction mixture was used for PCR using Taqman reagents (Applied Biosystems, Foster City, CA). PCR consisted of the following steps: initiation at 95 °C for 10 minutes, then 40 cycles of 95 °C, 15 seconds, and 60 °C, 1 minute. The primer/probe sets for qRT–PCR were from Applied Biosystems and were the following: PD-1 (PDCD1 Hs00169472_m1), PD-L1 (CD274 Hs00204257_m1), PD-L2 (PDCD1LG2 Hs00228839_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1). PCR was performed using the Taqman Universal PCR Master Mix and the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and relative PD-L1 and PD-L2 expression was calculated using the formula $2^{-\Delta CT}$ (Kobayashi *et al.*, 2007).

For semi-quantitative PCR, PCR products of PD-1 and GAPDH were separated by electrophoresis on agarose gel (Cambrex, Rockland, ME) and ethidium bromide-stained DNA bands were photographed under UV light. Jurkat hPD-1, a cell line transfected with human PD-1 cDNA, was used as a PD-1-positive control (Latchman *et al.*, 2001).

Mixed leukocyte reaction

Blood from human normal donors was obtained with DFCI IRB approval and centrifuged on Ficoll–Hypaque. CD4 T cells were purified by negative selection according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). To induce PD-1 expression, 10×10^6 CD4 T cells were stimulated in media with 1 μg ml^{-1} CD28 mAb (37407; R&D Systems, Minneapolis, MN) in a 10 cm tissue culture plate coated with 1 μg ml^{-1} CD3 mAb (OKT3; eBioscience, San Diego, CA). T cells were harvested after 24 hours, washed, and 1×10^5 CD4 T cells were incubated with 1×10^2 allogeneic mLCs per well, and proliferation measured by $[^3H]$-thymidine incorporation (0.5 μCi $well^{-1}$; New England Nuclear, Boston, MA) for the last 16 hours of a 6-day culture. Where indicated, mLCs were incubated with 20 μg ml^{-1} PD-L1, PD-L2, or isotype control mAb(s) for 1 hour and washed once before culture with T cells. One group of T cells was incubated with PD-1 mAb for 1 hour and washed once before culture with mLCs. Wells were done in quadruplicate.

Statistical analysis

Statistical analysis was performed using Excel (Microsoft, Redmond, WA). Paired experimental groups were compared using a two-tailed Student's *t*-test.

Abbreviations

APCs	antigen-presenting cells
DCs	dendritic cells
DDCs	dermal DCs
i	immature
LCs	Langerhans cells
m	migratory
MIP	macrophage inflammatory protein
PBS	phosphate-buffered saline
PD-1	Programmed Death-1
PD-L1 and PD-L2	PD-1 ligands
PE	phycoerythrin
TLR	Toll-like receptor

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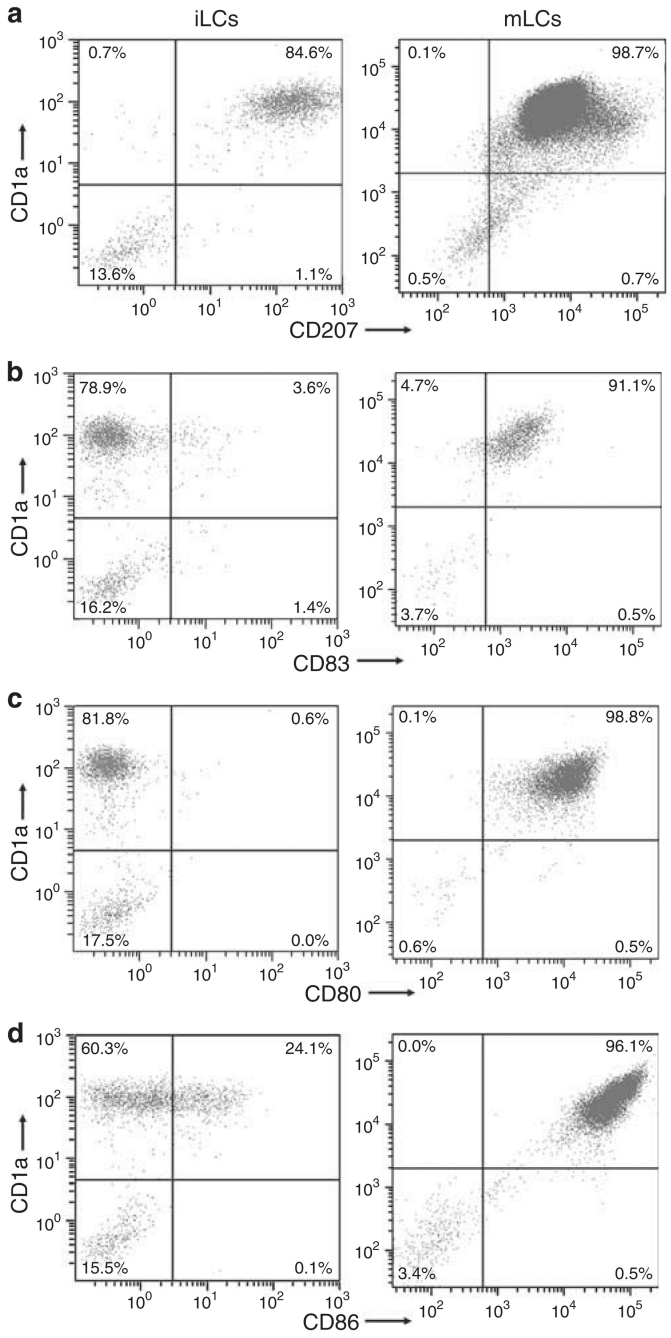

Figure 1. Phenotypic differences between iLCs and mLCs

iLCs and mLCs were prepared as described under Materials and Methods and phenotyped using CD1a-FITC and the indicated PE-conjugated mAbs by flow cytometry: CD207 (**a**); CD83 (**b**); CD80 (**c**);and CD86 (**d**). Similar results were seen in three independent experiments.

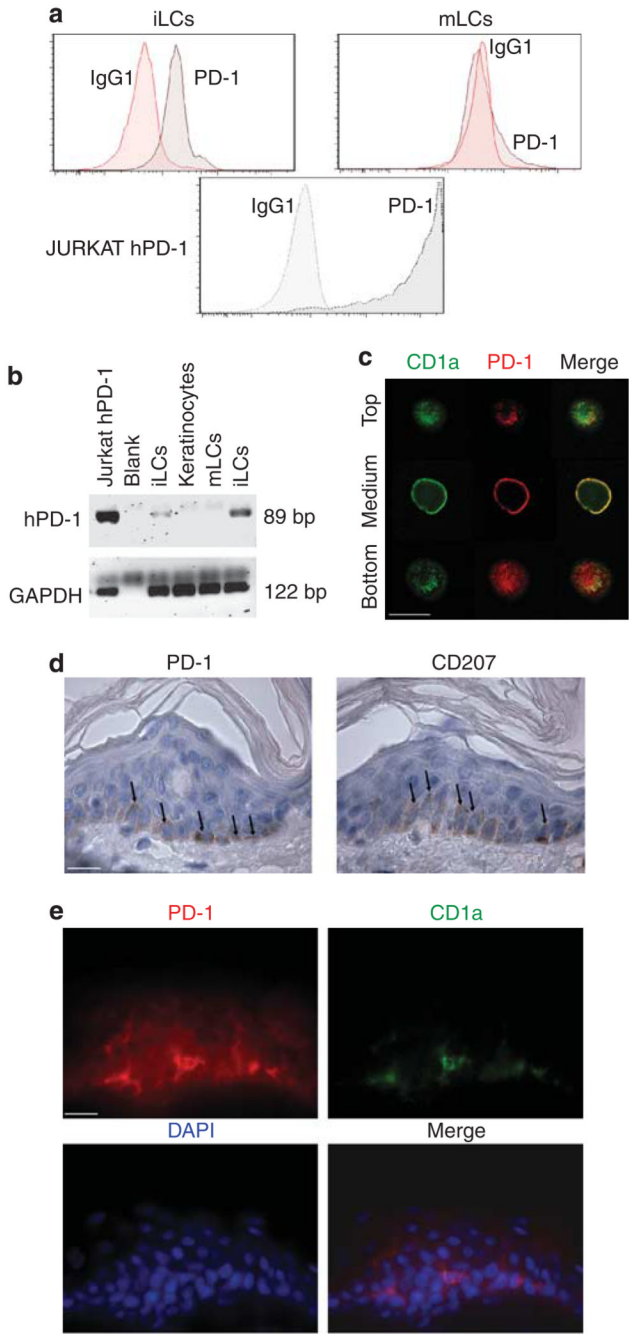

Figure 2. PD-1 expression on LCs

(**a**) Purified iLCs and mLCs were phenotyped by flow cytometry using the PD-1-PE mAb, gating on CD1a-FITC$^+$ cells. Jurkat cells transfected with PD-1 were included as positive control. The results are representative of three independent experiments. (**b**) RNA was prepared from the indicated cells and examined for PD-1 mRNA expression by RT–PCR and gel electrophoresis of PCR products. (**c**) iLCs were stained with CD1a-FITC and PD-1-PE and expression was localized by confocal microscopy. (**d**) Sequential histological sections of normal human skin were stained using PD-1 and CD207 mAbs. (**e**) A frozen section of human skin was sequentially stained for PD-1 and CD1a as described under

Materials and Methods. 4′-6-Diamidino-2-phenylindole was used to visualize nuclei and the three images were merged to judge co-expression. Bar = 20 μm (**c**–**e**).

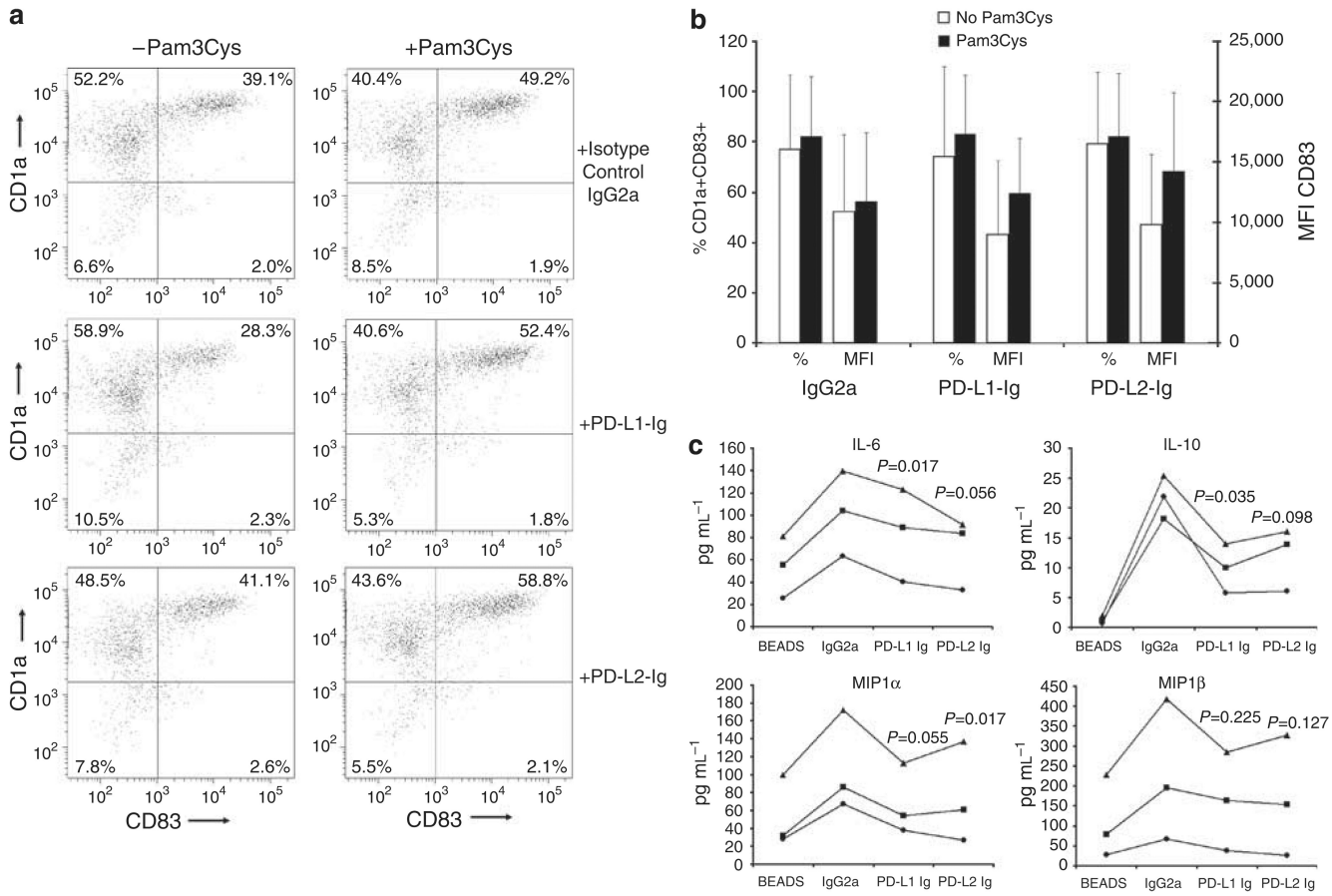

Figure 3. PD-1 engagement reduces TLR2-induced cytokine production by iLCs but does not affect expression of CD83

iLCs were incubated with the TLR2 agonist, Pam3Cys, and beads were coated with control IgG2a, PD-L1-Ig, or PD-L2-Ig. (**a**) After 24 hours, CD83 expression was evaluated by flow cytometry; (**b**) the percentage and mean fluorescence intensity (MFI) of CD83 expression (mean ± SD of three experiments) is shown. The open bars represent cells treated with the indicated Ig fusion proteins and the filled bars represent cells treated with Ig fusion protein plus Pam3Cys. (**c**) Supernatants were harvested at 24 hours and assayed for IL-6 and MIP-1α levels. Results from three experiments are shown with the statistical significance, *P*-values, shown on each graph.

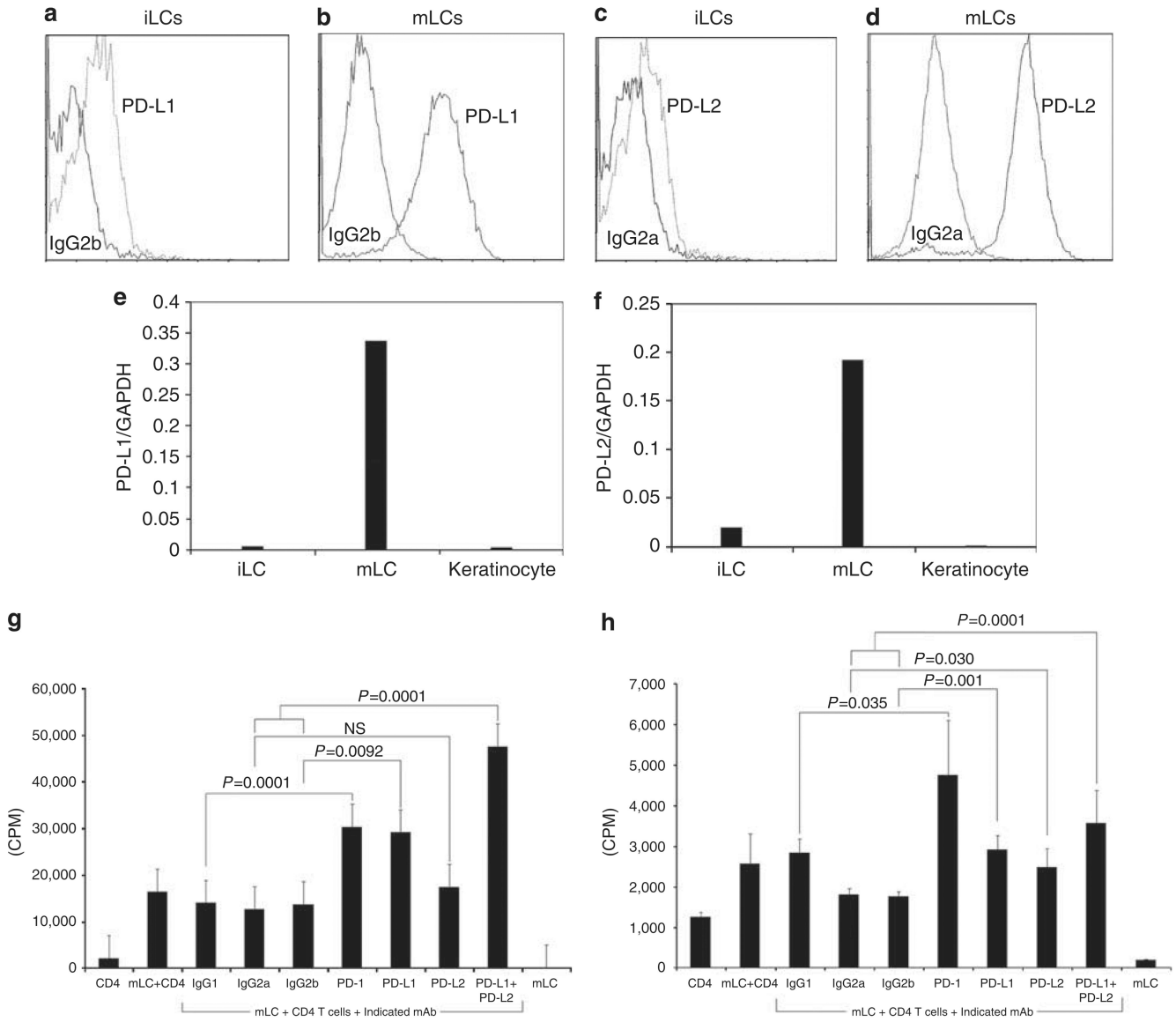

Figure 4. PD-1 ligands are upregulated after migration of LCs and blockade of the PD-1 pathway upregulates CD4 T-cell alloresponses to mLCs

(**a**–**d**) iLCs and mLCs were stained using PD-L1 or PD-L2 mAbs and analyzed by flow cytometry. RNA from iLCs, mLCs, and keratinocytes was analyzed for (**e**) PD-L1 and (**f**) PD-L2 mRNA expression by qRT–PCR. (**g**, **h**) mLCs were incubated with 20 μg ml^{-1} of the indicated mAb(s) for 1 hour and washed once. One group of CD4 T cells was incubated with PD-1 mAb for 1 hour and washed once. 1×10^2 mLCs and 1×10^5 previously activated allogeneic CD4 T cells were cultured together and proliferation was measured by [^{3}H]-thymidine incorporation for the last 16 hours of a (**g**) 6-day culture or (**h**) 40-hour culture. Statistical analysis was performed using Student's *t*-test. The experiments in panels **a**–**d** and **g** were repeated 3–5 times and the experiment in panel **h** was repeated once.

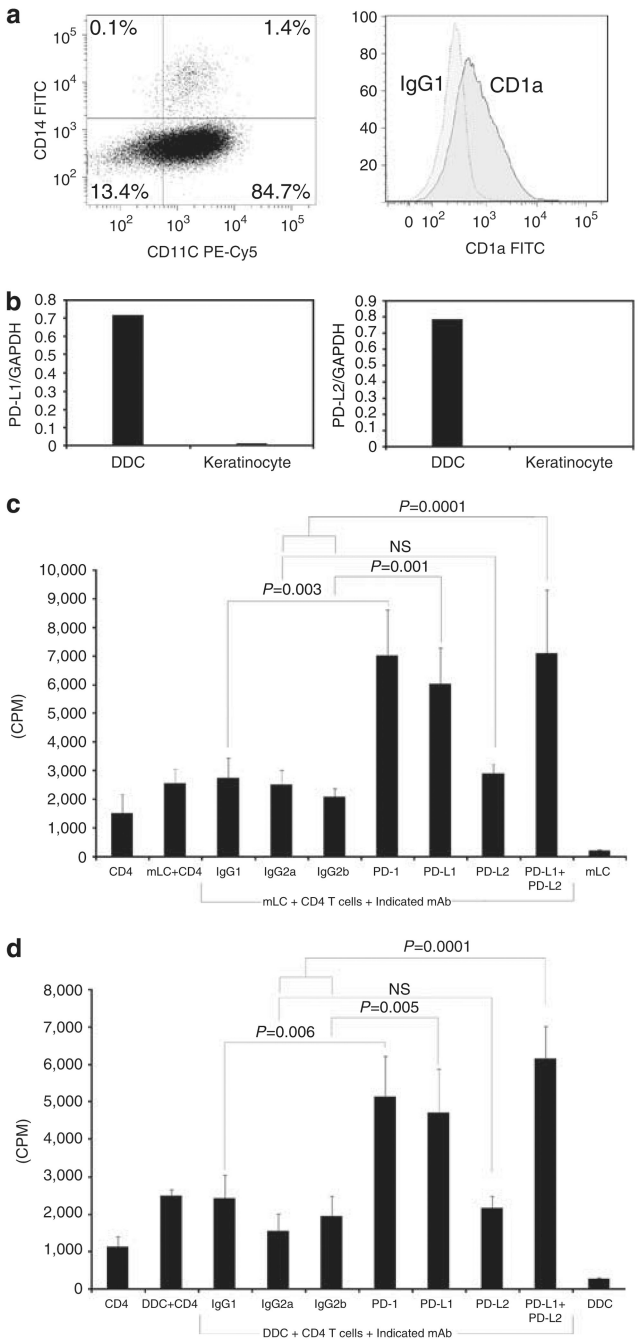

Figure 5. Blockade of the PD-1 pathway enhances alloresponses to both mLCs and DDCs

(**a**) DDCs were analyzed using CD11c, CD14, and CD1a mAbs, and flow cytometry. (**b**) Expression of PD-L1 and PD-L2 mRNAs in DDCs and keratinocytes was determined by qRT–PCR. (**c**, **d**) mLCs and DDCs from the same donor were treated with the indicated mAbs and incubated with previously activated CD4 T cells as described in Figure 4 and proliferation was measured by [^{3}H]-thymidine incorporation for the last 16 hours of a 6-day culture.

Table 1

Expression of co-stimulatory cell-surface proteins by iLCs after 24 hours of PD-1 engagement and stimulation with or without TLR2 agonist

	IgG2a beads				PD-L1-Ig beads				PD-L2-Ig beads			
	+Pam3Cys		−Pam3Cys		+Pam3Cys		−Pam3Cys		+Pam3Cys		−Pam3Cys	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
CD80	79.1	733	68.1	751	71.0	764	71.4	653	70.3	770	65.1	598
CD83	92.1	7,030	91.5	9,665	93.6	10,226	89.9	6,463	92.2	9,680	86.9	5,181
CD86	94.6	30,617	93.5	54,618	94.9	43,657	92.5	31,414	94.0	37,792	94.1	30,617

Abbreviations: iLC, immature Langerhans cell; LC, Langerhans cell; MFI, mean fluorescence intensity; PD-1, Programmed Death-1; PD-L1 and PD-L2, PD-1 ligands; TLR, Toll-like receptor.