Plasmacytoid Dendritic Cells Infiltrate the Skin in Positive Tuberculin Skin Test Indurations

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Plasmacytoid dendritic cells (pDCs) are rarely present in normal skin but have been shown to infiltrate lesions of infections or autoimmune disorders. Here, we report that several DC subsets including CD123$^+$ BDCA-2/CD303$^+$ pDCs accumulate in the dermis in indurations induced by the tuberculin skin test (TST), used to screen immune sensitization by *Mycobacterium tuberculosis*. Although the purified protein derivate (PPD) used in the TST did not itself induce pDC recruitment or IFN-α production, the positive skin reactions showed high expression of the IFN-α-inducible protein MxA. In contrast, the local immune response to PPD was associated with substantial cell death and high expression of the cationic antimicrobial peptide LL37, which together can provide a means for pDC activation and IFN-α production. *In vitro*, pDCs showed low uptake of PPD compared with CD11c$^+$ and BDCA-3/CD141$^+$ myeloid DC subsets. Furthermore, supernatants from pDCs activated with LL37–DNA complexes reduced the high PPD uptake in myeloid DCs, as well as decreased their capacity to activate T-cell proliferation. Infiltrating pDCs in the TST reaction site may thus have a regulatory effect upon the antigen processing and presentation functions of surrounding potent myeloid DC subsets to limit potentially detrimental and excessive immune stimulation.

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INTRODUCTION

Tuberculin skin tests (TSTs) are performed by intradermal injection of purified protein derivate (PPD), produced and sterilized from multiple strains of *Mycobacterium tuberculosis* (Magnusson and Bentzon, 1958), and are clinically used to evaluate immune sensitization by *M. tuberculosis* or the Bacillus Calmette-Guérin vaccine. In sensitized individuals, a delayed-type hypersensitivity reaction is induced at the injection site. The sequence of events leading to the local immune reaction associated with a positive TST is incompletely defined but is likely initiated by PPD uptake of dendritic cells (DCs) in the skin for subsequent antigen presentation and stimulation of *M. tuberculosis*-specific memory T cells (Poulter *et al.*, 1982; Scheynius *et al.*, 1982). DCs are known to orchestrate immune reactions occurring at the interface of innate and adaptive immunity (Banchereau *et al.*, 2000). There are several distinct subsets of DCs described, based on lineage origin and anatomical location. DCs resident in the skin are almost exclusively of the myeloid lineage. Recently, a distinctly different subset, plasmacytoid DCs (pDCs), normally absent in skin, were shown to migrate to inflamed skin lesions of autoimmune reactions such as psoriasis (Nestle *et al.*, 2005; Albanesi *et al.*, 2010) and lupus erythematosus (Farkas *et al.*, 2001; Meller *et al.*, 2005), as well as to virally induced lesions of herpes simplex (Donaghy *et al.*, 2009; Peng *et al.*, 2009) and varicella (Gerlini *et al.*, 2006; Huch *et al.*, 2010). pDCs are thought to have a central role in the response to viruses through their capacity to produce high amounts of IFN-α, which is primarily induced by microbial nucleic acid signaling via Toll-like receptors 7 and 9 (Gilliet *et al.*, 2008). In addition, pDCs can produce IFN-α in response to human (self) DNA or RNA if complexed to the antimicrobial peptide LL37 (Lande *et al.*, 2007; Ganguly *et al.*, 2009). This complex formation enables endocytosis of self-DNA and -RNA and subsequent Toll-like receptor signaling in the endosome. Thus, pDC may also regulate immune responses via IFN-α in the absence of pathogen exposure.

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Abbreviations: hDNA, human DNA; HMGB-1, high-mobility group B 1; LDH, lactate dehydrogenase; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; PPD, purified protein derivate; TST, tuberculin skin test

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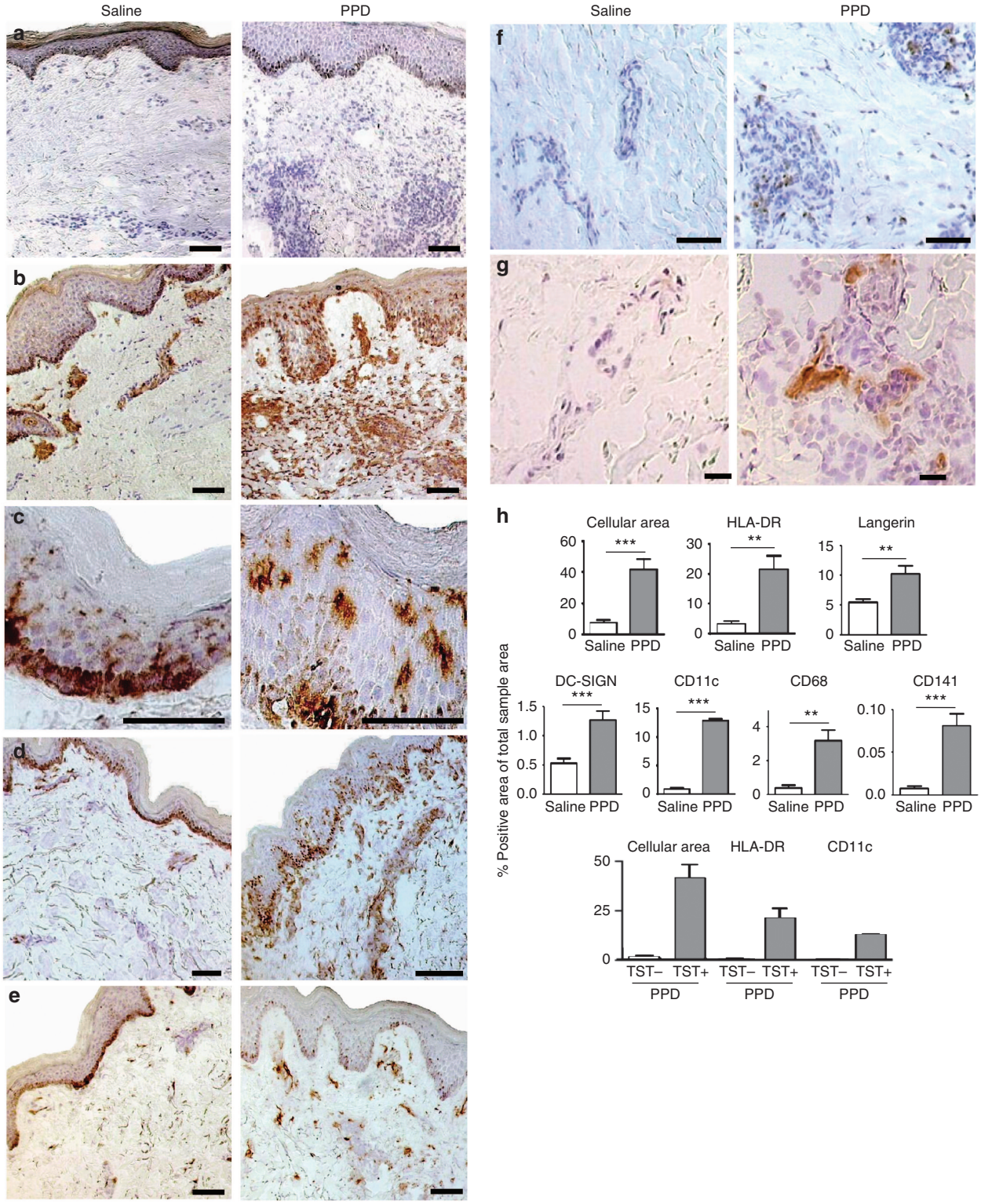


Figure 1. PPD-injected sites show increased cellularity and infiltration of dendritic cells (DCs). Cryosections of donor-matched skin punch biopsy samples of the tuberculin skin test (TST) site were taken 48 hours post injection. Representative images are shown of saline-injected versus purified protein derivate (PPD)-injected sites from TST-positive donors. Increased cellularity (**a**) as measured by cell nuclei counterstained blue with hematoxylin, and increased expression (brown) of HLA-DR (**b**), Langerin (**c**), DC-SIGN (**d**), CD11c (**e**), CD68 (**f**), and CD141/BDCA-3 (**g**) at the TST site. All scale bars = 25 μm. Note that the dark basal membrane represents melanin-rich cells and not positive staining. (**h**) Bar graphs show mean ± SEM. Data were collected from image analysis of dermis only, except for Langerin, where epidermis only was analyzed. DC-SIGN, dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin.

In this study, we investigated the presence and relative roles of distinct DC subsets in the skin in the TST reaction. In biopsy specimens from healthy individuals displaying a positive TST, we found a large infiltration of several DC subsets expressing markers associated with myeloid DCs (mDCs) and pDCs. This was accompanied by upregulation of the antimicrobial peptide LL37 and an increased expression of multiple markers for cell death, suggesting the release of self nucleic acids. No infiltration of cells was found after PPD injection in individuals with a negative TST, nor did PPD exposure activate DCs *in vitro*, suggesting that it is the milieu in the positive TST reaction that leads to recruitment of DCs. Supernatants from LL37–DNA-activated pDCs *in vitro* reduced the otherwise high uptake of PPD by mDCs, induced their maturation, as well as altered the activation of T-cell proliferation and cytokine production profile. Infiltrating pDCs may therefore show a regulatory function to control the level of antigen presentation and immune activation in the TST reaction.

RESULTS

DCs infiltrate the TST site

Cryosections of skin punch biopsy specimens taken at 48 hours after injection of PPD, or saline, in healthy donors with a positive TST reaction were analyzed by immunohistochemistry and quantitative image analysis. Inflammation was observed in all positive TST sites as evidenced by a significant increase in cellularity quantified by the area of hematoxylin-stained nuclei in the tissue (Figure 1a and h, $P=0.0008$, $n=7$). The efficiency of DCs to present PPD antigen to $CD4^+$ and $CD8^+$ T cells, which are known to infiltrate the positive TST reaction (Poulter *et al.*, 1982; Scheynius *et al.*, 1982; Platt *et al.*, 1983), likely depends on both recruitment of DCs to the skin and their antigen-processing capacity. To evaluate the degree of DC influx to the TST site, we first assessed the level of MHC class II (HLA-DR) expression. The percentage of $HLA\text{-}DR^+$ area out of the total area of the dermis was found to be significantly higher in the TST reaction than in the donor-matched saline-injected control sites (Figure 1b and h, $P=0.0019$, $n=9$). In the controls, the majority of $HLA\text{-}DR^+$ cells were typically present in clusters in the dermis. In contrast, the $HLA\text{-}DR^+$ cells in the TST reaction were scattered throughout both the dermis and epidermis, although predominantly in the pericapillary areas. To further characterize these cells, a series of well-defined markers were used. The C-type lectin receptor Langerin, specific for Langerhans cells, was constitutively present in the epidermis (Valladeau *et al.*, 2003) and significantly upregulated in the TST reaction (Figure 1c and h, $P=0.0061$, $n=8$). Dendritic cell-specific intracellular adhesion 3 grabbing was constitutively expressed in dermis and significantly increased in the TST reaction (Figure 1d and h, $P=0.0006$, $n=8$). Furthermore, CD11c (Figure 1e and h, $P\leqslant0.0001$, $n=8$) and CD68 (Figure 1f and h, $P=0.0013$, $n=10$), expressed by DCs of the myeloid lineage, as well as macrophages, were almost exclusively present in the dermis and markedly increased in the TST sites as compared with controls. CD141 (BDCA-3), recently reported to define a distinct myeloid DC subset (Jongbloed *et al.*, 2010), was absent or expressed at very low levels in the controls, but was detected in all TST biopsy specimens (Figure 1g and h, $P=0.0009$, $n=10$). In accordance with the fact that PPD is designed to not induce immune activation in individuals lacking preexisting immunity, there was no infiltration after injection of individuals displaying a negative TST as evidenced by the absence of increased cellularity or expression of HLA-DR and CD11c (Figure 1h). From these results, we concluded that multiple mDCs of the myeloid lineage infiltrated the positive TST indurations.

pDCs are recruited to the positive TST site

As mentioned earlier, pDCs do not usually reside in the skin. This was confirmed by rare or absent CD123 expression in the saline controls. In contrast, $CD123^+$ pDCs were present in all positive TST biopsy samples ($P=0.0043$, $n=10$, Figure 2a and c). The expression of CD123 was confined to the dermis and mainly found in clusters in which distinct individual cells with high CD123 expression were discernible. In addition, there was occasionally low-intensity CD123 staining in streak formations, presumably representing endothelial cells as described (Hirbod *et al.*, 2009). Another pDC marker, CD303 (BDCA-2), was also significantly upregulated in the TST sites ($P\leqslant0.0001$, $n=9$, Figure 2b and d), and was highly co-expressed with CD123 (Figure 2f). However, CD303 was less prevalent, consistent with its documented downregulation on activated pDCs (Figdor *et al.*, 2002). No increase in CD123 or CD303 expression was detected, indicating an absence of pDC recruitment, in biopsy samples from individuals with a negative TST (Figure 2e).

High expression of the IFN-α-inducible protein MxA and cell-death markers in the TST reaction

Despite the infiltration of pDCs in response to the positive TST, IFN-α was not detected (data not shown). In contrast, staining of MxA, a GTPase specifically induced by IFN-α/-β exposure (Simon *et al.*, 1991; Fah *et al.*, 1995), was readily detected after PPD injection, whereas it was undetectable in the saline controls (Figure 3a and d, $P\leqslant0.0001$, $n=9$). As the presence of MxA strongly implies production of IFN-α/-β, the time of biopsy sample collection, or production levels may explain the lack of IFN-α detection. MxA was present not only in the dermis, in close proximity to $CD123^+$ $CD303^+$ pDCs, but also in the epidermis. It is plausible that pDC-produced IFN-α/-β can diffuse through the tissue inducing upregulation of MxA. Alternatively, cells in the epidermis distinct from pDCs may have produced IFN-α. IFN-α production by pDCs in psoriatic lesions, a non-pathogen-containing, yet inflammatory, milieu, was recently demonstrated to be induced by self-DNA or -RNA from dying cells in complex with the antimicrobial peptide LL37 (Lande *et al.*, 2007; Ganguly *et al.*, 2009). Analyses of the expression levels of LL37 at the TST sites showed a strong upregulation in dermis, compared with a low constitutive expression in the controls (Figure 3b and d, $P\leqslant0.0001$, $n=10$). With regard to cell death, the levels of active Caspase-3, primarily indicating

apoptosis (Duan *et al.*, 2003), were highly upregulated in the positive TST site (Figure 3c and d, $P=0.0002$, $n=9$). PPD injection *per se* induced no or a very small increase in MxA, LL37, or Caspase-3 expression, as shown in donors with negative TST reactions (Figure 3d). High intracellular and extracellular presence of high-mobility group protein B-1 (Figure 3e) and lactate dehydrogenase (Figure 3f) in the TST sites indicated leakage of these proteins due to loss of cell membrane integrity associated with necrosis in particular (Scaffidi *et al.*, 2002; Duan *et al.*, 2003; Yun *et al.*, 2008; Morishima *et al.*, 2010). This cell leakage implies a simultaneous release of self nucleic acids from cells undergoing necrosis or secondary apoptosis in the TST reaction, which could thus be available for complex formation with the high levels of LL37.

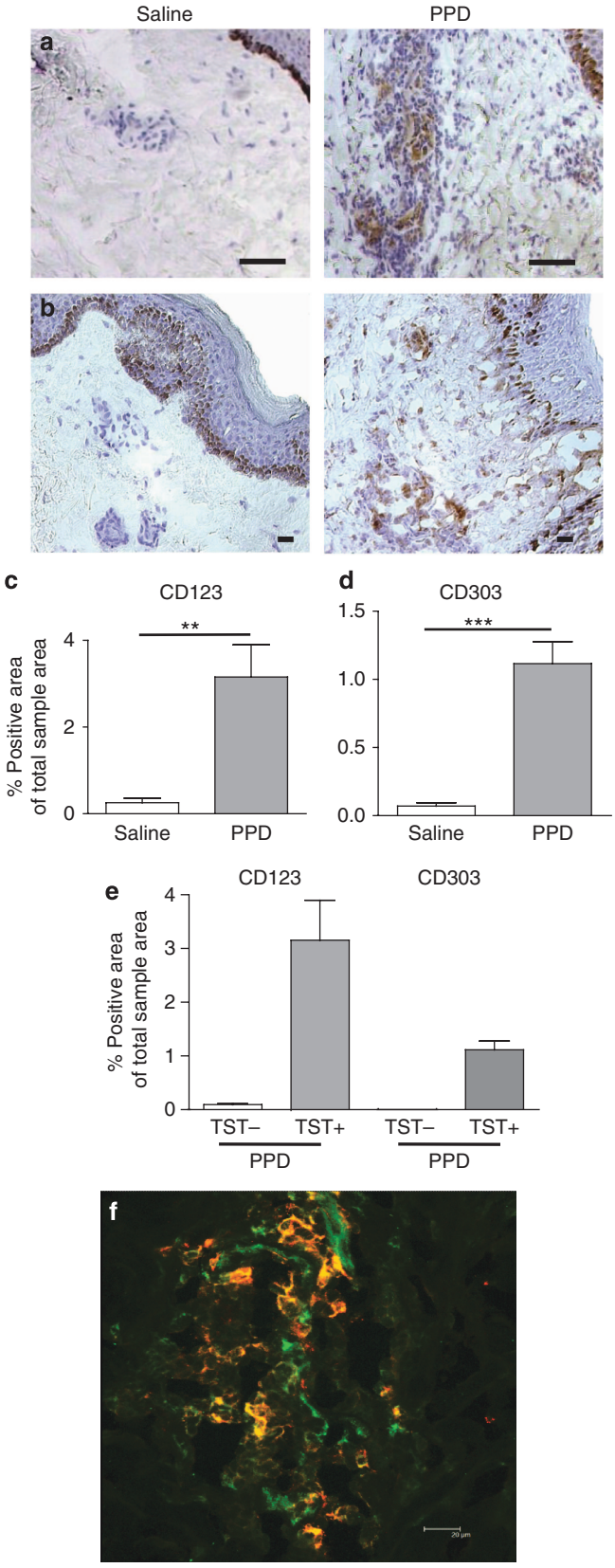


Figure 2. Recruitment of CD123$^+$CD303$^+$ pDCs to the TST site. Plasmacytoid dendritic cells (pDCs) were detected by expression of both CD123 (**a**, **c**) and CD303 (BDCA-2) (**b**, **d**), and were significantly increased at the purified protein derivate (PPD) injection site of tuberculin skin test (TST)-positive individuals. The expression was restricted to the dermis. Bars = 25 μm. Note that the dark basal membrane represents melanin-rich cells and not positive staining. All graphs show mean ± SEM. (**e**) pDCs were not recruited to the site of PPD injection in TST-negative individuals. (**f**) Representative immunofluorescent costaining (which appears yellow) of CD123 (green) and CD303 (red) to confirm the presence of pDCs at the TST site.

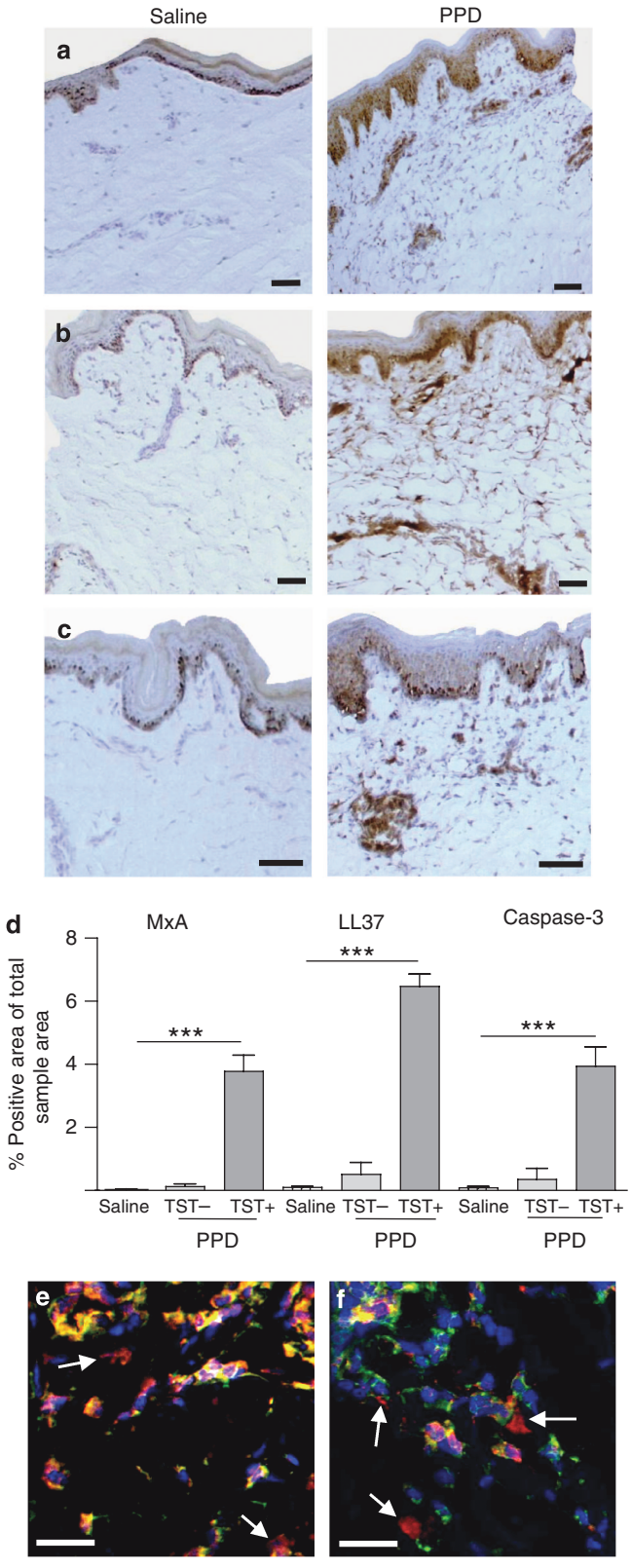
Differential capacity for PPD uptake in DC subsets

To investigate DC functions pertaining to uptake and processing of PPD for antigen presentation, and whether a milieu of LL37–DNA complexes influenced this, we conducted experiments *in vitro* using isolated DC subsets. pDCs are generally considered to be inefficient at antigen presentation compared with other DC subsets, which may partially relate to lesser capacity to internalize antigens (Villadangos and Young, 2008). To this end, CD123$^+$ pDCs and CD11c$^+$ mDCs were pulsed with fluorescently labeled PPD for 2 hours. The internalization of PPD was assessed by comparing PPD binding at 4 °C with active uptake at 37 °C by flow cytometry, as well as by verifying intracellular punctuate signals by microscopy (Figure 4a). pDCs showed significantly lower uptake of PPD compared with both conventional mDCs ($P=0.0008$) and cross-presenting CD141$^+$ DCs (Figure 4b and c, $P=0.02$, $n=3$).

LL37–DNA-stimulated pDCs can regulate mDCs and T-cell function

In line with earlier reports (Lande *et al.*, 2007; Ganguly *et al.*, 2009), we found that IFN-α secretion by pDCs exposed to complexes of LL37 and human DNA was enhanced compared with the low levels found in response to either LL37 or DNA alone (Figure 4d). This was even more pronounced using complexes of LL37 and synthetic CpG ODN. LL37–DNA complexes also induced phenotypic maturation in pDCs as evidenced by upregulation of CD40, CD80, and CD86 (Figure 4e). Exposure of pDCs to PPD alone did not induce IFN-α or maturation. mDCs did not show any detectable IFN-α or maturation in response to either PPD or LL37–DNA (data not shown). In contrast, mDCs matured when exposed to supernatants from LL37–DNA-exposed pDCs (Figure 4f). Further, the high uptake of PPD by mDCs was significantly

reduced when cultured in the presence of pDC supernatants (Figure 4g). This reduced capacity to internalize PPD was unlikely to be due to IFN-α alone, as the addition of recombinant IFN-α at similar levels as in the pDC supernatants did not reproduce this effect. Importantly, LL37–DNA complexes alone did not reduce PPD uptake in mDCs (data not shown). The decreased PPD uptake by mDCs caused by pDC supernatants is unlikely to exclusively relate to the functional transformation associated with maturation, as the effect was observed as early as after 2 hours of incubation with supernatants (data not shown). Finally, mDCs exposed to pDC supernatants showed significantly reduced capacity to stimulate allogeneic $CD4^+$ T-cell proliferation as evidenced by both carboxyfluorescein succinimidyl ester dilution (Figure 5a, representative of seven donors) and thymidine incorporation assays (Figure 5b, $n=4$, $P=0.02$). Again, IFN-α alone did not have the same effect. LL37–DNA-exposed pDCs also directly suppressed T-cell proliferation found in pDC/T-cell cocultures (Figure 5c, $P=0.035$, $n=4$). In addition to reduced proliferation, T cells cocultured with mDCs exposed to pDC supernatant showed altered production of effector cytokines IL-2, tumor necrosis factor-α, and IFN-γ as measured by intracellular cytokine staining (Figure 5d, $P=0.0036$, $n=8$). Further, separate assessment showed that the production of IL-2 was particularly reduced, whereas the levels of tumor necrosis factor-α and IFN-γ remained unchanged (Figure 5e). This concurred with elevated production of IL-6 and IL-10. Thus, pDCs activated by LL37–DNA complexes in the TST site may acquire a function to regulate immune activation by directly or indirectly skewing cytokine production and controlling proliferation of T cells.



DISCUSSION

pDCs have emerged as a highly specialized DC subset that has been shown to possess poorer antigen uptake and presentation capacity compared with other DC subsets, but instead equipped with a high capacity to produce IFN-α/-β (Cella *et al.*, 1999). Thus, their role in controlling viral infections has been particularly explored. However, pDCs have also shown to exert other immunoregulatory properties not involving pathogen responses (Swiecki and Colonna, 2010). Indeed, pDCs were initially described for their role in maintaining tolerance. High levels of pDCs were shown to represent a good prognostic factor for discontinuation of immunosuppressive treatment after liver and stem cell transplantation, and may hence be important to prevent transplant rejection or graft-versus-host disease, respectively, (Mazariegos *et al.*, 2003; Rajasekar *et al.*, 2008). To this end, pDCs were shown to mediate oral tolerance to food antigens

Figure 3. Presence of potential stimuli for IFN-α production at the TST site. (**a**, **d**) High expression of the IFN-α-inducible protein MxA in the tuberculin skin test (TST) site, but not in the saline site, was detected. (**b**, **d**) High levels of LL37 were present in the dermis of the TST sites. In addition, Caspase-3, associated with cell death, was also increased (**c**, **d**). These increases were not seen in TST-negative individuals. Representative images of (**e**) high-mobility group protein B-1 (HMGB-1) (red) and (**f**) lactate dehydrogenase (LD) (red) together with HLA-ABC (green) and 4′,6-diamidino-2-phenylindole (blue). (**e**, **f**) Arrows denote extracellularly located HMGB-1 and LD as a possible consequence of leakage out of necrotic cells. Bars = 25 μm. All graphs show mean ± SEM.

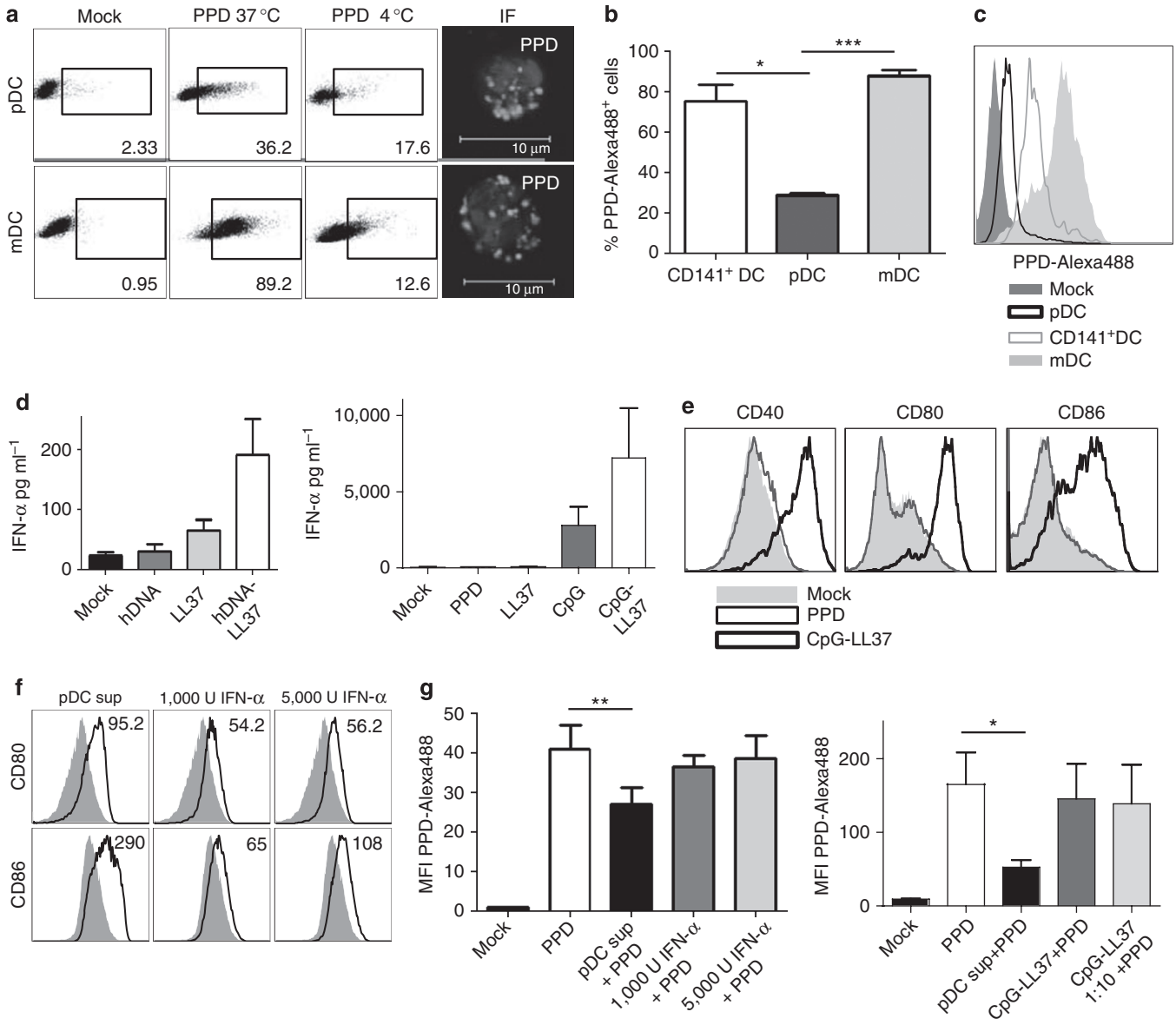


Figure 4. LL37/DNA-activated plasmacytoid dendritic cells (pDCs) affect myeloid dendritic cells (mDCs) phenotype and purified protein derivate (PPD) uptake. (**a**) Gates indicate percentages of CD11c$^+$ mDCs and CD123$^+$ pDCs with binding (4 °C) and/or uptake (37 °C) of PPD-Alexa488. Single-plane confocal images show intracellular PPD-Alexa488 in dendritic cells (DCs) pulsed at 37 °C. (**b**) Bar graph shows mean ± SEM of mean fluorescence intensity values (MFIs) of PPD-Alexa488 uptake, $n = 3$. (**c**) Representative histogram of the PPD uptake. (**d**) IFN-α production of pDCs exposed to complexes of LL37 and human or CpG DNA. (**e**) Phenotypic maturation of pDCs exposed to LL37–DNA. (**f**) Phenotypic maturation in mDCs exposed overnight to supernatants from stimulated pDCs or IFN-α. Solid gray lines represent unexposed mDCs. Numbers indicate MFI. (**g**) mDCs pulsed with PPD-Alexa488 for 2 hours after overnight stimulation as indicated. IF, immunofluorescence.

by suppressing CD4$^+$ and CD8$^+$ T-cell-specific delayed-type hypersensitivity responses (Goubier *et al.*, 2008). The TST reaction examined in this study also represents delayed-type hypersensitivity. A role for DCs in the development of positive TST reactions was early implied (Scheynius *et al.*, 1982; Beck, 1991; Sarrazin *et al.*, 2009). Our data indicate that there is a rapid and rather robust infiltration of several DC subsets, including pDCs in the positive TST reaction. As this was not found in negative TST, the inflammation induced locally by infiltrating, presumably PPD-specific, T cells likely regulates the pDC recruitment. Although pDCs are not normally located in the skin, they are recruited to allergic contact hypersensitivity reactions (Bangert *et al.*, 2003), as well as to the autoimmune reactions associated with lupus erythematosus (Farkas *et al.*, 2001; Meller *et al.*, 2005) and psoriasis (Nestle *et al.*, 2005; Albanesi *et al.*, 2010). In the latter, infiltrated pDCs were shown to produce IFN-α. The classical stimuli that induce high levels of IFN-α in pDCs are nucleic acids derived from microbes activating cells via intracellular Toll-like receptor signaling (Stacey *et al.*, 2003; Akira *et al.*,

2006). However, recent reports show that pDCs can react to human RNA or DNA if they are presented as complexes with (the antimicrobial peptide) LL37 (Lande *et al.*, 2007; Ganguly *et al.*, 2009). These studies prompted our further investigation of whether LL37 was upregulated at the TST sites and whether this occurred simultaneously with cell death, leading to the release of nucleic acid material. On the basis of our data showing high levels of LL37 and signs of necrotic tissue, we speculate that LL37–DNA complexes may also be available and activate pDCs that infiltrate the TST sites. In this regard, pDCs could also be activated by complexes formed by self-DNA and the high levels of high-mobility group protein B-1 at the TST site (Tian *et al.*, 2007). Because of the quite distinct differences in the characteristics of DC subsets, they likely possess different and complementary functions to generate, enhance, or suppress immune responses. pDCs showed significantly lower capacity to take up PPD than the mDC subsets. This included the distinct $CD141^+$ mDC subset with superior cross-presenting capacity (Bachem *et al.*, 2010; Jongbloed *et al.*, 2010; Villadangos and Shortman, 2010). Infiltration of $CD141^+$ DCs in response to antigen delivery and inflammation in the skin has not been described previously and it is possible they activate PPD-specific $CD8^+$ T cells in the TST reaction. As pDCs showed much lower uptake of PPD, their primary contribution in the TST reaction may not pertain to antigen processing and presentation to T cells. Instead, they may have an important bystander effect of conditioning surrounding mDC subsets. This bystander effect could consist of a suppressor function to restrain otherwise potentially harmful, excessive immune activation. Although pDCs have been identified to mediate immune responses in skin inflammation in psoriasis (Lande *et al.*, 2007) and wound healing (Gregorio *et al.*, 2010), they may also function to regulate the magnitude of the responses. On this note, pDCs have shown different immune-stimulatory capacities at different differentiation/activation stages (Hadeiba *et al.*, 2008; Schwab *et al.*, 2010; Bjorck *et al.*, 2011). The nature of the antigen likely also has a role in shaping pDC function.

We found that supernatants from pDCs stimulated by LL37–DNA complexes reduced the high capacity of mDCs to take up PPD. This reduction may in part be mediated by IFN-α, although it was evident in our experiments that this it was not the sole factor. Anti-IFN-α/-β antibodies have been shown to block maturation of mDCs (Ganguly *et al.*, 2009), and thus IFN-β could potentially also mediate this effect. Here, mDCs cultured in the presence of pDC supernatants showed reduced capacity to induce effector cytokine production and proliferation of allogeneic $CD4^+$ T cells. Type-I IFN produced by pDCs has been shown to limit T-cell proliferation (Chi *et al.*, 2006), but again the inhibition we observed was not evident with exposure to recombinant IFN-α alone, suggesting that there are alternative suppressive

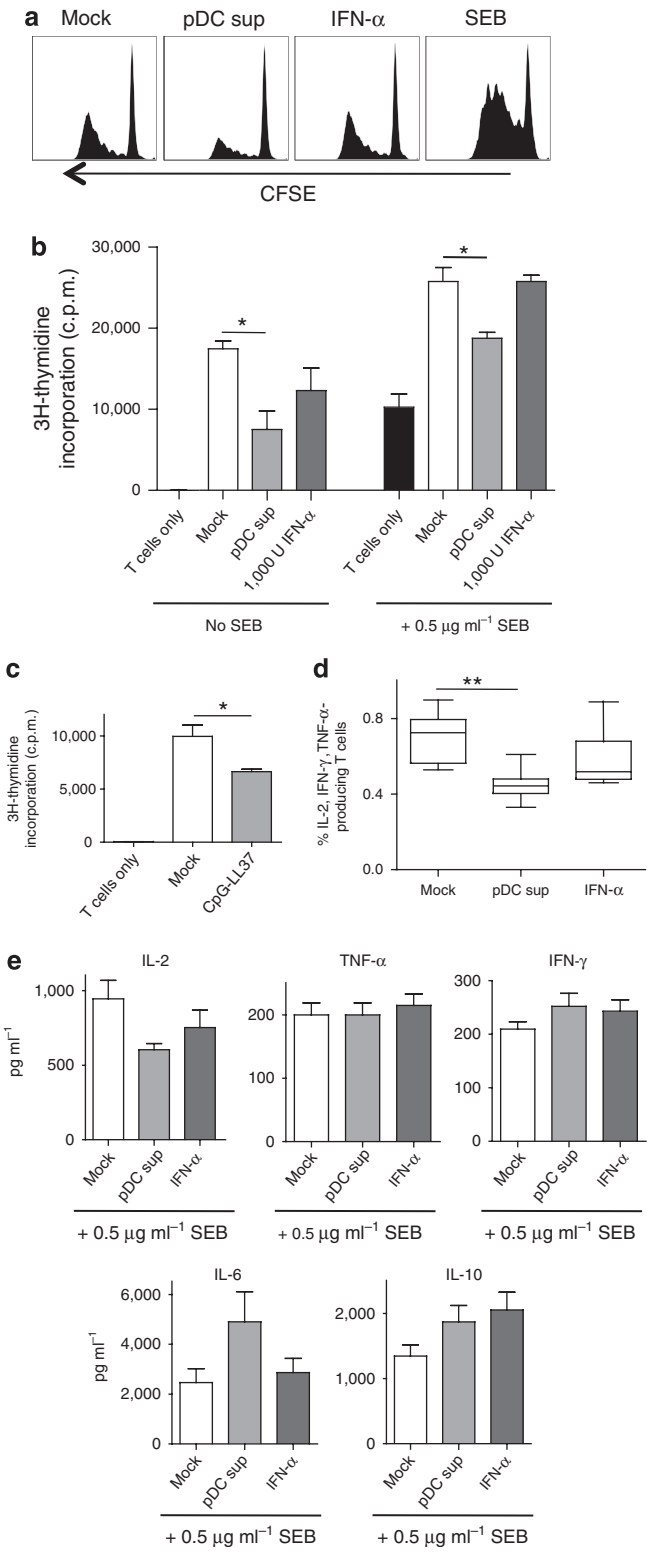


Figure 5. LL37/DNA-activated pDCs alter the T-cell stimulatory capacity of mDCs. T-cell proliferation as measured by (**a**) carboxyfluorescein succinimidyl ester (CFSE) dilution and (**b**) thymidine incorporation in allogeneic $CD4^+$ T cells after 5 days of coculture with myeloid DCs (mDCs) exposed overnight to indicated stimuli. (**c**) T-cell proliferation after 5 days of coculture of T cells and plasmacytoid dendritic cells (pDCs) exposed to indicated stimuli. (**d**) Frequencies of cytokine (IL-2, tumor necrosis factor-(TNF)α, IFN-γ) producing CD4+ T cells measured by intracellular staining after coculture for 16 hours with mDCs exposed overnight to the indicated stimuli and (**e**) levels of IL-2, IL-6, IL-10, TNF-α, and IFN-γ in supernatants from mDC–T-cell cocultures pulsed with staphylococcal enterotoxin B (SEB). c.p.m., counts per minute.

mediators released from the pDCs. Taken together, our data suggest that mDC subsets would take up more PPD delivered at the site of injection than pDCs, and consequently are most likely to perform the majority of the antigen presentation to activate antigen-specific memory T cells. pDCs could still contribute to these functions; however, as mDCs are by far the most frequent DC subsets at the site, even after PPD injection, the uptake and presentation by pDCs may be negligible. Instead, pDCs activated by components from the local inflammation and tissue destruction may partake in controlling the magnitude of immune responses by directly exerting a regulatory effect on T-cell activation or by influencing the functions of mDCs. In our *in vitro* studies, we found that LL37–DNA-activated pDCs can suppress T-cell proliferation and skew the cytokine production. To this end, pDCs have, under other culture conditions, shown to restrain T-cell activation by inducing T-regulatory cells (Moseman *et al.*, 2004; Varani *et al.*, 2007). However, these interactions remain to be shown in the skin *in vivo*. In addition, the kinetics by which the different DC subsets and T cells are recruited after PPD injection could influence the regulation of T-cell activation but are still to be determined. In conclusion, our data further elucidate the emerging multifacetted roles that pDCs have in shaping immune responses. Understanding the different roles of the specific DC subsets infiltrating the skin is critical for defining the responses elicited to pathogens targeting the skin, as well as for the development of new therapies, vaccines, and delivery strategies.

MATERIALS AND METHODS

Collection of human skin punch biopsy specimens

Written informed consent was obtained from all study subjects. The Karolinska Institutet and University of Cape Town Institutional Review Boards approved the experiments, and they were conducted in adherence with the Declaration of Helsinki Principles. Study subjects were recruited in Khayelitsha Township, Cape Town, South Africa. Exclusion criteria are described earlier (Rangaka *et al.*, 2007) and included previously diagnosed tuberculosis, HIV infection, and/or presentation of one of several symptoms, e.g. cough, chest pain, or fever. TST was performed according to international standards and considered positive at $\geqslant$10 mm. The study subjects who displayed a positive TST reaction showed mean 18.6 ± 2.3 mm. Punch biopsy samples were taken as described (Sarrazin *et al.*, 2009) from the PPD injection site and a saline-injected site on the opposite arm at 48 hours and snap-frozen.

Immunohistochemical staining and quantitative image analysis

Cryosections (8 μm) of skin biopsy samples were stained as described (Lore *et al.*, 2002) with anti-HLA-DR, CD11c, CD123, CD8, active Caspase-3 (BD Biosciences, San José, CA), CD68 (Dako, Glostrup, Denmark), CD303 (BDCA-2), dendritic cell-specific intracellular adhesion 3 grabbing, Langerin (R&D Systems, Minneapolis, MN), LL37 (Innovagen, Lund, Sweden), CD141 (BDCA-3) (Miltenyi Biotech, Auburn, CA), MxA (Professor Haller and Kochs, University of Freiburg, Germany), followed by secondary biotinylated antibodies (Abs), i.e., anti-mouse, anti-goat (Dako), or anti-rabbit (Vector Laboratories, Burlingame, CA). The secondary Abs were detected with the peroxidase-based Vectastain Elite ABC kit (Vector Laboratories), and the reaction developed by diaminobenzidine tetrahydrochloride peroxidase substrate kit (Vector Laboratories). Cell nuclei were counterstained with Mayer's Haematoxylin (Histolab Products, Gothenburg, Sweden). The sections were analyzed by a Leica DMR-X microscope (Leica Microsystems GmbH, Wetzlar, Germany) coupled to computerized image analysis (Leica Qwin 5501W, Leica Imaging Systems) as described (Bjork *et al.*, 1996). The epidermis was excluded from the analysis of all markers except for Langerin, where the dermis was excluded. Analyses were confirmed by at least two separate investigators.

Fluorescent costaining

The stainings were performed as above with modifications. Following the incubation with the secondary biotinylated Abs, streptavidin-conjugated Alexa488 or Alexa647 (Molecular Probes, Eugene, OR) was added or, alternatively, Alexa594-conjugated secondary Abs were used. In addition to the cell-specific Abs mentioned above, anti-HLA-ABC (Dako), lactate dehydrogenase (Abcam, Cambridge, UK), high-mobility group protein B-1 (BD Biosciences), and Alexa594-labeled anti-goat (Molecular Probes) antibodies were used. Sections were mounted using SlowFade Gold antifade regent with 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) for nuclear staining.

Isolation of blood pDCs and mDCs

Primary DCs were purified from blood as described (Adams *et al.*, 2009; Douagi *et al.*, 2009; Adams *et al.*, 2011). In short, pDCs and mDCs were isolated from buffy coats treated with monocyte-enrichment cocktail (RosetteSep, Stemcell Technologies, Grenoble, France) or elutriated monocytes by anti-BDCA-4 and anti-CD1c microbeads (Miltenyi Biotech) and AutoMACS separation (Miltenyi Biotech). pDCs and mDCs were cultured in RPMI-1640 with 10% fetal calf serum (Sigma-Aldrich, Schelldorf, Germany) supplemented with IL-3 (1 ng ml^{-1}; R&D Systems) and GM-CSF (2 ng ml^{-1}; PeproTech, Rocky Hill, NJ), respectively.

Stimulation of pDCs with LL37–DNA complexes

Complexes were formed by co-incubation of either 10 μg ml^{-1} human DNA (Biochain Institute, Hayward, CA) or 5 μg ml^{-1} CpG ODN (class B 10103, Coley Pharmaceutical Group GmbH, Düsseldorf, Germany) with 10–50 μg ml^{-1} LL37 (Innovagen, Lund, Sweden) for 30 minutes at room temperature. pDCs were exposed to the complexes at 1×10^6 per ml for 16 hours at 37 °C. Cell-free supernatants were collected and analyzed. Supernatants of pDCs stimulated by CpG–LL37 complexes were used for culture experiments with mDCs.

Analysis of cytokine production and maturation of DCs

IFN-α was measured in supernatants from pDCs by ELISA (*VeriKine*, PBL Interferon Source, Piscataway, NJ). For analysis of phenotypic maturation, isolated DCs were stained with combinations of Abs, including CD1c, CD11c, CD123, CD80, CD83, and CD86 as described (Lore *et al.*, 2005). The cells were collected on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Treestar, San Carlos, CA). Luminex assays (Human Cytokine 10-Plex Panel, Invitrogen), for analysis of cytokines in supernatants from DC and DC/T-cell cultures,

were performed according to the manufacturer's instructions and analyzed by Luminex 200 system (Invitrogen).

Evaluation of PPD uptake capacity

PPD (Statens Serum Institut, Copenhagen, Denmark) was labeled by using an Alexa488 protein labeling kit (Molecular Probes). Isolated pDCs and mDCs were pulsed with $4\,\mu g\,ml^{-1}$ PPD-Alexa488 for 2 hours at 37 or 4 °C and analyzed by flow cytometry. Alternatively, 2×10^6 cells of monocyte-enriched populations (RosetteSep, Stemcell Technologies) were exposed to PPD-Alexa488 for 2 hours and cells expressing CD11c, CD123, or CD141 were analyzed. For confocal microscopy imaging, sorted pDCs and mDCs were transferred to adhesion slides (BioRad Lab, Munich, Germany) as described (Bond *et al.*, 2009). The slides were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories) for nuclear staining.

Functional assays of pDC effects on mDCs

IFN-α at 1,000–5,000 U ml^{-1} or supernatants from pDCs at 1:1 were added to cultures of mDCs. mDCs were incubated for 24 hours, washed, exposed to PPD-Alexa488 for 2 hours, and analyzed for PPD uptake and CD80/CD86 expression. For analysis of T-cell stimulatory capacity, pDCs or mDCs were cocultured with unlabeled or carboxyfluorescein succinimidyl ester-labeled, purified allogeneic $CD4^+$ T cells obtained (as described by Adams *et al.*, 2011) at a ratio of 1:10 for 16 hours for detection of IL-2, tumor necrosis factor-α, and IFN-γ expression by intracellular staining and FACS analysis, or for 5 days for assessment of proliferation, as described (Lore *et al.*, 2005; Adams *et al.*, 2011). Alternatively, proliferation was measured by thymidine incorporation as described (Gujer *et al.*, 2011).

Statistical analyses

Data were analyzed by Student's *t*-test, with GraphPad Prism software (San Diego, CA), and considered significant at $*P \leqslant 0.05$, $**P \leqslant 0.01$, and $***P \leqslant 0.001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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