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CHAPTER 6

Intradermal delivery of TLR agonists in a human explant skin model: preferential activation of migratory Dendritic Cells by Poly I:C and Peptidoglycans

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Submitted

ABSTRACT

TLR agonists are attractive candidate adjuvants for therapeutic cancer vaccines as they can induce a balanced humoral and T-cell mediated immune response. With a dense network of dendritic cells (DCs) and draining lymphatics, the skin provides an ideal portal for vaccine delivery. Beside direct DC activation, TLR agonists may also induce DC activation through triggering the release of inflammatory mediators by accessory cells in the skin microenvironment. Therefore, a human skin explant model was used to explore the *in vivo* potential of intradermally delivered TLR agonists to stimulate Langerhans Cells (LCs) and dermal DCs (DDCs) in their natural complex tissue environment. The skin-emigrated DCs were phenotyped and analyzed for T-cell stimulatory capacity. We report that, of six tested TLR-agonists, the TLR-2 and -3 agonists PGN and Poly I:C were uniquely able to enhance the T-cell priming ability of skin-emigrated DCs, which in the case of PGN was accompanied by Th1/Th17 polarization. The enhanced priming capacity of Poly I:C stimulated DC was associated with a strong upregulation of appropriate costimulatory molecules, including CD70, while that of PGN-stimulated DC was associated with the release of a broad array of proinflammatory cytokines. Transcriptional profiling further supported the notion that the PGN- and Poly I:C-induced effects were mediated through binding to TLR2/NOD2 and TLR3/MDA5 respectively. These data warrant further exploration of PGN and Poly I:C, alone or in combination, as DC-targeted adjuvants for intradermal cancer vaccines.

INTRODUCTION

Vaccines aimed at the induction of neutralizing antibodies are widely used with great success to prevent microbial infections. Therapeutic vaccination is being developed for treatment of chronic infections and cancer, and aims to generate protective T-cell immunity. Although some clinical successes have been reported, particularly in the field of cancer vaccination, much is yet to be gained in terms of efficacy (1,2).

In vivo studies have shown that the vaccine delivery route determines the instruction of T cells by locoregional antigen-presenting cells (APCs), thereby affecting the homing capacity of primed T cells (reviewed by Kupper and Fuhlbrigge (3)). Langerhans cells (LCs) and dermal dendritic cells (DDCs) reside in the epidermis and dermis, respectively. Both subsets are well equipped to sense pathogenic threats in their microenvironment, to take up antigen and to migrate to the draining lymph nodes (LN) where they prime and program T cells. Hence, neoplastic diseases that present within the skin (*e.g.* melanoma and human papilloma virus (HPV)-induced tumors) may benefit most from intradermal vaccination, as in the LN the migrated LCs or DDCs will imprint the T cells to express a pattern of homing and chemokine receptors that facilitates migration to the skin. Immunization with poxviruses is a clear example of a remarkably successful induction of strong and long-lasting antibody and T-cell responses by vaccination via the skin (4). Also, intradermal vaccination with synthetic long peptides (SLPs) derived from HPV type 16 oncoproteins resulted in the induction of both $CD4^+$ and $CD8^+$ T cells specific for HPV that were able to migrate to the skin (5).

The choice of adjuvant will influence the type of immune response induced by therapeutic vaccines. More and more, it is realized that for effective anti-tumor immunity a balanced humoral and cell-mediated response with long-term T-cell memory is obligatory. Based on pre-clinical mouse models, human *in vitro* experiments and early clinical experience, compounds targeting toll like receptors (TLRs) are promising adjuvant candidates in this regard (6,7). TLRs are trans-membrane pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs). TLRs can be expressed at the cell membrane, to bind bacterial structural molecules such as peptidoglycans (PGN) and lipopolysaccharides (LPS) (TLR1,2,4,5,6), or intracellular in endosomal compartments, to bind viral or bacterial RNA or DNA (TLR 3,7,8,9). Subsequent signaling through MyD88 or TRIF leads to activation of the NF-κB and MAPK pathways resulting in APC activation and pro-inflammatory cytokine and chemokine release. Human epidermal LCs have been reported to express only virus-recognizing TLRs, to keep them from inadvertently being activated by commensal bacteria. In contrast DDCs express TLRs recognizing both bacteria and viruses (8). So far, the ability of TLR agonists to stimulate cutaneous DCs has mostly been tested *in vitro* (9). However, other skin-resident cells such as keratinocytes, melanocytes, endothelial cells and fibroblasts also express TLRs and can release cytokines that contribute to DC maturation and the induction and skewing of T cells (10,11). Moreover, alternative intracellular microbial PRRs, such as NOD-like receptors (NLR, NOD1 and NOD2) or RIG-I-like Helicases (RLH, MDA5 and RIG-I), can be expressed by DCs and accessory cells, and share ligands with TLRs (7). Therefore, a human skin explant model was used to explore the *in vivo* potential of TLR agonists to stimulate LCs and DDCs in their natural complex tissue environment (12). By intradermal delivery of TLR-ligands (TLR-Ls), intradermal vaccination with TLR-based adjuvants was thus mimicked *ex vivo*. The compounds tested included PAM_3CYSK_4.3HCl (PAM3CSK4, TLR1/2-L), PGN (TLR2-L/NOD2-L), LPS and its detoxified

active constituent monophosphoryl lipid A (MPLA, TLR4-L), Polyribosinic:polyribocytidic acid (Poly I:C, TLR3-L), and R848 (Resiquimod, TLR7/8-L), all of which are either used or considered as vaccine adjuvants (7). So-called crawl-out DCs, migrating from the skin explants, were analyzed for their expression of maturation and co-stimulatory molecules and their capacity to prime allogeneic T cells and induce Th1/2/17 responses. We report that Poly I:C and PGN are uniquely able to enhance the T-cell priming ability of skin-emigrated DCs. While for Poly I:C this appeared to be due to up-regulation of co-stimulatory molecules, PGN was a powerful inducer of pro-inflammatory cytokine release, thereby promoting Th1 induction. From these observations, we conclude that TLR2/NOD2 and TLR3 ligands are attractive candidate adjuvants for intradermally delivered therapeutic vaccines.

MATERIAL AND METHODS

Preparation and culture of skin explants. Healthy human skin was obtained after informed consent from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (Amsterdam, The Netherlands) or the Tergooi hospital (Hilversum, The Netherlands), following hospital guidelines, within 24h of surgery. Cytokines or TLR-Ls (endotoxin contaminations not exceeding 1 EU/mg, excepting MPLA and LPS) were dissolved in serum-free medium (IMDM) and injected intradermally into skin: GM-CSF (100 ng/biopsy, Berlex Laboratories Inc. Montville, NJ), IL-4 (10 ng/biopsy, R&D), PGN (10 μg/biopsy, InvivoGen), Poly I:C (20 μg/biopsy, InvivoGen), R848 (10 μg/biopsy, InvivoGen), LPS (250 ng/biopsy, Sigma Aldrich), MPLA (250 ng/biopsy, InvivoGen), PAM3CSK4 (10 μg/biopsy, InvivoGen). These amounts were based either on optimal maturational effects or on maximal sub-toxic doses found upon titration and were all found active on monocyte-derived DCs *in vitro* (see SFig.1). Of note, the TLR5 agonist flagellin was found to be too toxic in active concentration ranges (based on findings for monocyte-derived DCs) and was therefore not further included in these studies. Using a 500 μl microfine syringe (Becton Dickinson), 20 μl was injected into skin so that an urtica formed. A biopsy was immediately taken of the injection site with a 6 mm biopsy punch (Microtec) and floated in 1 well of a 48 well plate, which contained 1 ml IMDM supplemented with 5% Human Pooled Serum (HPS, Sanquin Blood Supply, Amsterdam, The Netherlands), 100 IU/ml sodium penicillin (Yamanouchi Pharma), 100 μg/ml streptomycin sulfate (Radiumfarma-Fisiopharma), and 2 mM L-glutamine (Invitrogen Life Technologies). Per experimental condition, 10-30 biopsies were taken. Biopsies and medium were placed in an incubator (37^{0}C, 5% CO_2) for 2 days, after which biopsies were discarded and migrated cells and conditioned media were harvested. Conditioned media from 48h-cultured full-thickness skin explants were analyzed for cytokine content with the inflammatory cytokine bead array (CBA) kit from BD Biosciences (San Jose, CA), and 1:2 diluted for CCL5 content with a DuoSet development system (R&D sytems, Minneapolis, MN), both according to the manufacturers' instructions.

Phenotypical analysis. Phenotypical analyses were performed by flow cytometry. Cells were washed in PBS supplemented with 1 % BSA and 0.02% NaN_3 and incubated for 30 min. at room temperature in the presence of appropriate dilutions of FITC or PE fluorochrome-conjugated specific mAbs to CD14, CD1a, B7-H1 (PD- L1), CD80, CD86, CD70, HLA-DR (BD, San Jose, CA), or CD83 (Beckman Coulter Immunotech), or corresponding isotype-matched control mAbs (BD, San Jose, CA). The cells were subsequently analyzed, using a FACSCalibur and Cellquest-Pro

FACS analysis software (Becton Dickinson, San Jose, CA). As described, DC were gated by their high Forward and Side Scatter properties (12).

Mixed Leukocyte Reaction (MLR). Migrated DCs were resuspended at $1x10^5$/ml in IMDM with 10% HPS, penicillin, streptomycin, and glutamine and 100 µl was plated in triplicate in a 96-well round bottom sterile culture plate. Peripheral blood lymphocytes (PBLs) were isolated from allogeneic healthy donors after plastic adherence to deplete monocytes and labelled with 3 µM CFSE (Invitrogen) for 10 minutes at 37°C. After washing, the labelled PBL were resuspended at $1x10^6$ /ml in IMDM with 10% HPS, penicillin, streptomycin, and glutamine and 100 µl was added to wells containing migrated DCs, and to 3 wells containing only medium. Control wells of unlabelled PBLs were also included. On days 3, 6, 8 and 10 cell culture samples were taken and analysed for proliferation by flow cytometry. On day 6, supernatants were collected and subsequently analyzed for cytokines secreted by the T cells using a Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Microarray analysis of TLR transcriptional profiles. Epidermal and dermal fractions were prepared from dermatome-sliced human skin (3mm) as described previously(13). After CD1a-guided MACS (Miltenyi, Bergisch Gladbach, Germany), RNA, isolated from LCs and DDCs from three different donors, was dissolved in TRIzol reagent (Invitrogen Life Technologies) and stored at –20°C. After chloroform extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol, lyophilized, and dissolved in 10 µl distilled water. Fragmentation, hybridization, and scanning of the Human Genome U133 Plus 2.0 arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA) and as described (13). The arrays were scanned with a GeneArray scanner (Affymetrix). Data analysis was performed with GeneSpring 7.1 software (Agilent Technologies). Gene expression levels of TLR, NOD2, RIG-I, or MDA5, present on the Human Genome U133 Plus 2.0 Arrays, are presented as mean signal values of triplicate DDC or LC samples (signal intensity range from 0- 22,000). Samples with an absent detection call in one to three of the replicate samples were set at 0 (i.e. no reliably detected expression).

Real-time quantitative RT-PCR. Cells were lysed and mRNA was specifically isolated by capture of poly(A)-RNA in streptavidin-coated tubes using an mRNA capture kit (Roche, USA). cDNA was synthesized using a reverse transcription system kit (Promega, USA) following the manufacturer's guidelines. cDNA was diluted 1:2 in nuclease-free water upon synthesis and stored at -20°C until analysis. Specific primers for human TLR1–10 were designed as described (14). Real-time PCR was performed using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems, USA) as previously described (15). Briefly, 4 µl of the Power SYBR Green master mix (Applied Biosystems) were mixed with 2 µl of a solution containing 5 nmol/µl of both oligonucleotides and 2 µl of a cDNA solution (1/100 of the cDNA synthesis product). The cycle threshold (Ct) value is defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value (fixed at 0.045 relative fluorescence units). GAPDH was selected as the endogenous reference gene from a set of 10 functionally unrelated housekeeping genes according to Garcia-Vallejo *et al*. (15). For each sample, the relative abundance of target mRNA was calculated from the obtained Ct values for both target and endogenous reference gene GAPDH by applying the following formula: relative mRNA expression =2[Ct(GAPDH) -Ct(target)].

Statistical analyses. DC subset frequencies, marker expression levels, cytokine release levels, and proliferated T cell fractions were compared between conditions using the 2-sided repeated measures one-way ANOVA test with post-hoc Tukey multiple comparison analysis. Prism 4.0 statistical software (GraphPad Software Inc., La Jolla, CA) was used. Differences were considered significant when $P<0.05$.

RESULTS

Effects of TLR-L on subset distribution among the skin-emigrated DC. We previously identified several DC subsets among DCs that had migrated from human skin explants, based on multicolor flow cytometry ((12) and Lindenberg, *submitted*). An overview of the phenotype of the four major discernable subsets is presented in Table I. Of note, LC and CD1a$^+$ DDC are mature subsets with a high T-cell stimulatory potential, whereas the CD1a$^+$CD14$^+$ and CD14$^+$ DDC subsets are immature cells with macrophage-like features and a poor capacity for T-cell stimulation (Table I, (12)). We previously reported that intradermal injection of GM-CSF and IL-4 prior to skin explant culture, led to predominant migration of the mature CD1a$^+$ subsets, while inhibiting migration of the CD14$^+$ immature subsets (12). Here we confirm these observations (Fig.1) and in all subsequent experiments GM-CSF+IL-4 was included as a positive control. TLR agonists were intradermally injected at subtoxic active doses (see Material and Methods) that induced phenotypic and functional activation in vitro of highly pure monocyte-derived DC (SFig.1). None of the intradermally delivered TLR-Ls significantly increased (or decreased) the absolute number of migrated DCs per explant at 48h after start of culture (range of mean absolute numbers: 1800-4000; data not shown). LPS injection into the dermis prior to explant culture significantly increased the frequency of migrating LC, while injection of either Poly I:C or LPS increased the proportion of migrated CD1a$^+$ DDC over that observed for medium controls (Fig.1). Of note, unlike GM-CSF+IL-4, none of the tested TLR-Ls was able to significantly down-regulate the frequencies of migrated immature CD14$^+$ DDC.

TLR-L effects on DC maturation, inflammatory cytokine release and T cell induction. Whereas intradermal delivery of most of the tested TLR-Ls had no effect on phenotypic maturation of the migrated cells, Poly I:C consistently upregulated the expression of CD80, CD83, CD86, and HLA-DR, as well as of the coinhibitory molecule PD-L1 (B7-H1) (Fig.2A). Similarly, LPS induced

Table I. DC subsets and their phenotype.

DC subset	Marker profile*
LC	CD1a^{hi}, Langerin$^+$, E-Cadherin$^+$, CD11c^{int}, CD1c$^+$, CD83$^{+,}$ CD80$^+$, PD-L1$^+$
CD1a+ DDC	DC-SIGN$^+$, CD11c^{hi}, CD1c$^+$, CD83$^+$, CD80$^+$, PD-L1$^+$
CD14+ DDC	DC-SIGN$^+$(30%)†, CD11c^{hi}, CD1c$^+$, CD163$^+$(15%)‡, CD83$^-$, CD80$^-$, PD-L1$^+$(20%)‡
CD1a+CD14+ DDC	DC-SIGN$^+$(20%)†, CD11c^{hi}, CD1c$^+$, CD163$^+$(35%)‡, CD83$^-$, CD80$^+$(50%), PD-L1$^+$(30%)‡

* Based on multicolor FACS analyses as described by de Gruijl *et al*. (12) and Lindenberg *et al*., *submitted,* marker expression was considered negative (-) when percentage positive cells did not exceed 10%; hi=high fluorescence intensity levels, int=intermediate fluorescence intensity levels.
†Up-regulated by intradermal GM-CSF and IL-4 injection prior to explant culture
‡Up-regulated by intradermal IL-10 injection prior to explant culture

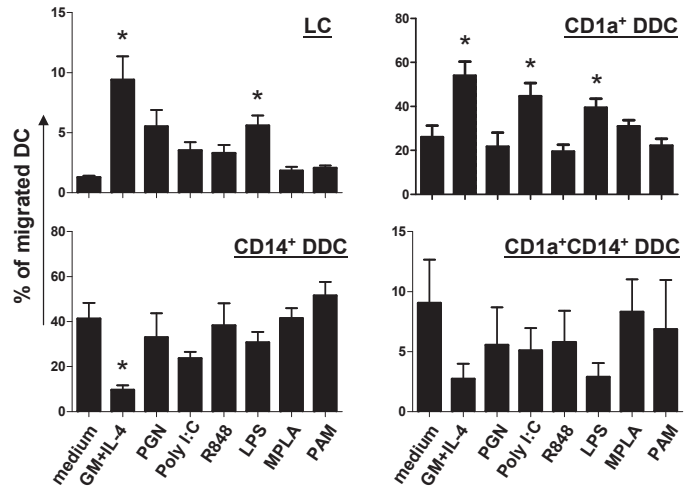

Figure 1. Subset distribution among skin emigrated DCs. Based on CD14 and C1a expression, four major subsets were discerned among skin-emigrated DCs (gated by high forward and side scatter properties) and their frequency determined for each of the test conditions. Means ± s.e.m. are shown based on 6 independent experiments. Asterisks denote significant differences vs. medium controls.

a significant upregulation of the percentages of DCs expressing CD83 and PD-L1, but the expression of CD80 and CD70 was enhanced by Poly I:C only (Fig.2B).

To assess the influence of the TLR-L on the local cytokine balance, explant-conditioned media were collected 48h after the start of culture, and tested for their inflammatory cytokine and chemokine content. As demonstrated in Fig.3, PGN by far proved to be a superior inducer of inflammatory cytokine release with high levels of IL-6, IL-8, and IL-1β, but also of the suppressive cytokine IL-10. IL-10 was produced upon administration of most TLR-Ls, with the notable exception of Poly I:C (Fig. 3). IL-12p70 was undetectable in the explant-conditioned media (not shown), while low but detectable levels of TNFα were induced by Poly I:C (Fig.3). In addition, variable but consistently elevated levels of the pro-inflammatory chemokine CCL5 were observed after intradermal delivery of Poly I:C (Fig.3).

Poly I:C and PGN were the only tested TLR-Ls that significantly enhanced the capacity of the skin-emigrated DC to induce the proliferation of allogeneic T cells (Fig.4A). Interestingly, DC migrated from GM-CSF+IL-4-injected skin explants, induced low but selective IL-4 release by the allogeneic T cells (Fig.4B, top panel) but this was not observed when DC were activated through their TLR. Whereas multiple TLR-Ls induced the release of IL-6, only PGN was able to induce the release of high levels of the Th1 cytokines IFNγ and TNFα (Fig.4B). In addition, PGN was uniquely able to induce the release of variable but consistently elevated levels of IL-17A by the primed T cells (Fig.4B, bottom panel).

PRR transcriptional profiles in LC, DDC and keratinocytes are conform the responsiveness to TLR-L. To assess PRR expression in the skin environment, transcriptional analysis of TLRs was performed in isolated LCs, CD1a$^+$ DDCs and keratinocytes from healthy human skin. As demonstrated by microarray data (Fig.5A), both LCs and DDCs generally expressed low levels of

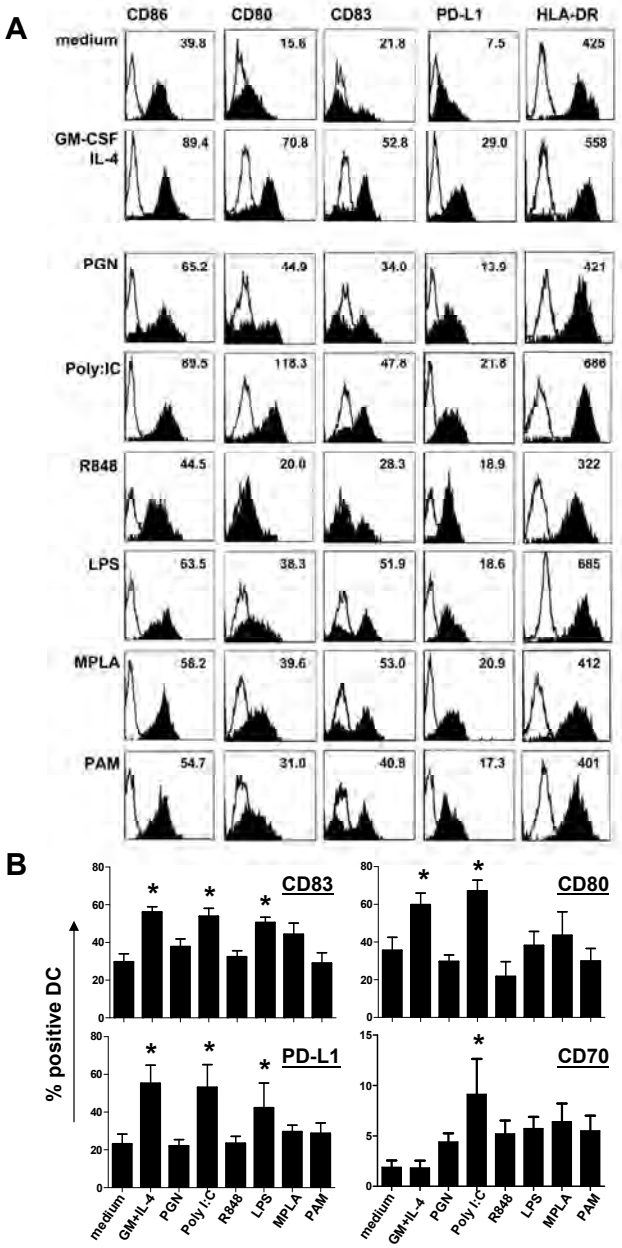

Figure 2. Expression of activation markers on skin-emigrated DCs. A) Expression of activation markers on the total population of skin-emigrated DCs at 48 hr after injection (and start of explant culture) of medium, GM-CSF+IL-4, or the denoted TLR agonists. Flowcytometric histograms of the expression of the indicated activation markers on all the cells within the live gate of one representative experiment. Fluorescence intensities are listed. Open histograms: IgG isotype controls; closed histograms: activation marker expression. B) Percentages of DCs that express the indicated activation markers as described in (A). Means ± s.e.m. of 3-6 experiments are shown. Asterisks denote significant differences vs. medium controls.

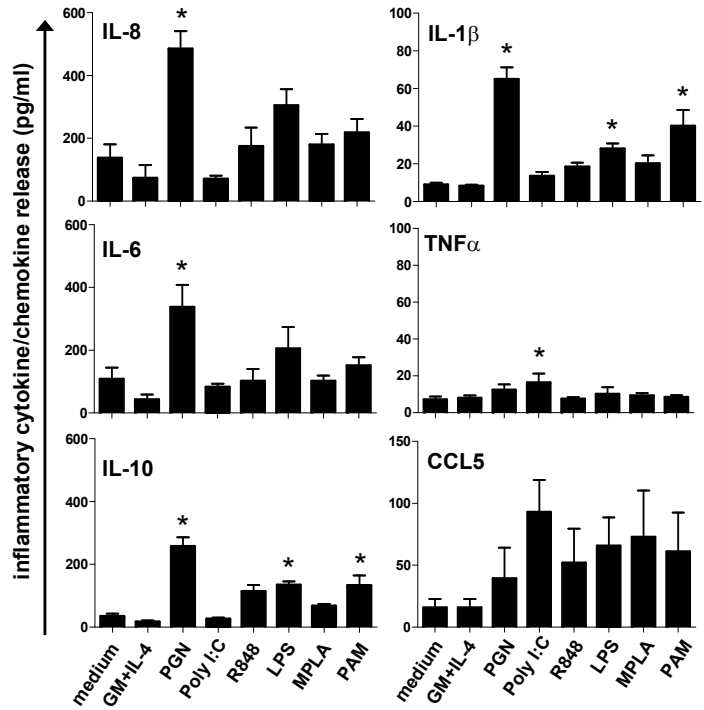

Figure 3. Inflammatory cytokine release from skin explants upon TLR-L injection. 48 hr after injection of TLR-Ls or controls and start of culture, skin explants were discarded and the medium was analyzed for the levels of the listed inflammatory cytokines by CBA or of CCL5 by ELISA. Means ± s.e.m. of 5 experiments are shown. Asterisks denote significant differences vs. medium controls.

TLR transcripts as compared to C-type Lectin Receptor (CLR) transcripts selectively expressed by LCs (Langerin, CD207) or by DDCs (Macrophage Mannose Receptor [MMR], CD206). Only TLR2 mRNA levels in DDC were relatively high. As PGN can also bind to NOD2 and Poly I:C to RIG-I/MDA5, expression of these receptors was also analysed. Relatively high expression levels were observed for both NOD2 and MDA5 in LCs as well as DDCs (Fig.5A). Epidermis-derived keratinocytes were tested by qRT-PCR for the expression of TLR transcripts. As shown in Fig.5B, resting keratinocytes expressed mostly TLR2, -3, and -5. Stimulation of keratinocytes with the TLR3-L Poly I:C led to up-regulation of TLR1-3, and -5 transcription, confirming functional expression of TLR3 on keratinocytes (Fig.5C). Thus, the observed DC activation by PGN and Poly I:C in the human explant cultures was in line with the detected expression of TLR2/NOD2 transcripts in DDCs and of MDA5 and TLR3 transcripts in LCs/DDCs and keratinocytes.

DISCUSSION

Immunization through the skin leads to the induction of T-cell mediated and humoral immunity (16). TLR agonists have been proposed as prime adjuvant candidates for therapeutic vaccines but most of our knowledge on TLR agonists and their effects on skin-mediated immunization

stems either from murine *in vivo* studies or from *in vitro* studies with isolated human primary LCs or DDCs, and more often from their monocyte-derived counterparts. However, vaccines and adjuvant deposited in the skin can interact with a complex mix of cells, amongst others DCs and keratinocytes. Therefore, we sought to assess the overall effect of these interactions in the context of intact human skin and intradermally injected a panel of TLR-Ls into human skin explants to study their effects on DC migration, activation, and subsequent T-cell priming in an organotypic culture model. Our studies point out Poly I:C and PGN as prime candidate adjuvants for clinical translation to skin-based cancer vaccines.

Of a panel of six tested TLR agonists, only the TLR3-L Poly I:C consistently induced phenotypic activation of the skin-emigrated DC population. This general lack of skin-derived DC activation upon TLR-L treatment is in striking contrast to our own observation for Monocyte-derived DCs (SFig.1). Strong activation by TLR-Ls was also reported for isolated monocyte-derived LCs (8,17). Of note, also overall migration rates of the skin APCs were not affected by intradermal injection of any of the tested TLR agonists. This general lack of reactivity in primary LCs and DDCs in the context of their tissue microenvironment may in part be explained by their low TLR transcript levels (Fig.5A), but may also be caused by specific local suppression. For instance, Jurkin *et al*. recently reported that selective overexpression of miR-146a in LCs interfered with TLR2-mediated signaling and caused their non-responsiveness to PGN (18). Alternatively, TLR triggering in DCs or skin-resident cells such as keratinocytes, melanocytes or fibroblasts, may have caused the release of suppressive IL-10 to

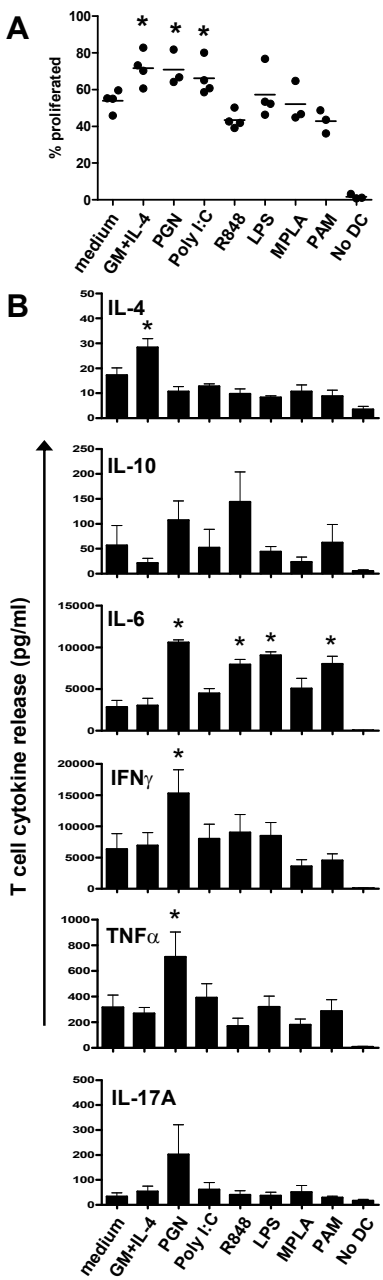

Figure 4. Allogeneic T cell priming and differentiation induction by skin-emigrated DCs. Emigrated DCs were washed and cultured with allogeneic CFSE-labeled PBLs for 6 days. The cells were analyzed by flow cytometry for proliferation by dilution of CFSE intensity (in % proliferated cells within the live lymphocyte gate). Means from 4 experiments are shown. B) Supernatants were collected from the mixed leukocyte cultures at day 6 and analyzed for Th cytokines by CBA. Means ± s.e.m. from 4 experiments are shown.

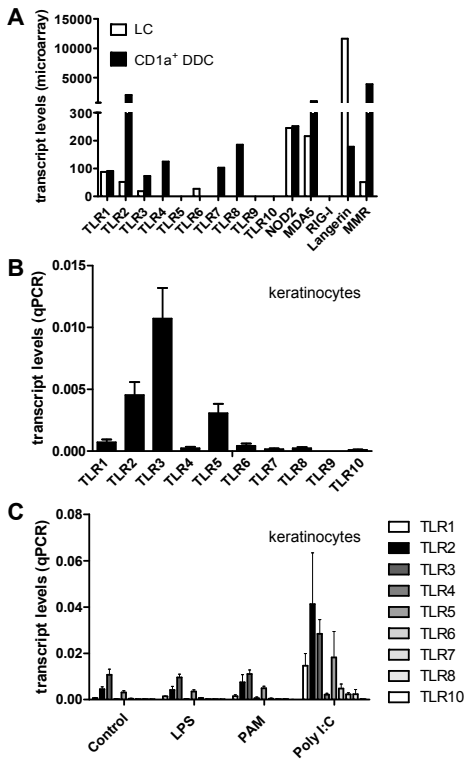

Figure 5. TLR mRNA expression in cell subsets of the skin. A) Freshly isolated LCs and CD1a$^+$ DDCs were analyzed by genome-wide transcriptional profiling. Shown are the transcript levels for the indicated Pattern recognition Receptor (PRR) and C-type Lectin receptor (CLR) genes (means from 3 separate donor samples; if one was negative the expression was set to 0 to ensure *bona fide* expression). Open bars: LC transcripts; closed bars: DDC transcripts. B) Skin-derived primary keratinocytes were profiled for TLR transcript expression by qRT-PCR. Transcript levels for TLR1-10 are indicated relative to GAPDH as a reference gene. C) Skin-derived keratinocytes were stimulated by the indicated TLR agonists (100ug) and again tested for TLR transcript expression as described under (B). Means ± s.e.m. are shown.

such an extent that it interfered with DC activation. In favor of the latter option, the only TLR agonist that did not induce elevated IL-10 release from the skin explants (i.e. Poly I:C, see Fig.3), was also the only agonist to consistently induce DC maturation. This elevated IL-10 release may also explain why intradermal injection of TLR-Ls, in contrast to GM-CSF+IL-4, did not reduce the migration frequency of CD14$^+$ DDCs (Fig.1B), as we previously described IL-10 to skew migratory DCs in skin from a mature to this immature CD14$^+$ phenotype (12,19).

Transcriptional profiling of cell types isolated from dissociated healthy human skin revealed TLR expression in LCs and CD1a$^+$ DDCs to be generally low, but other intracellular PRRs like MDA5 and NOD2 were also expressed. Keratinocytes expressed TLR2 and TLR3 and treatment of these cells with Poly I:C resulted in increased TLR expression, demonstrating functionality of the expressed TLR3. Roughly, our findings reflect the TLR expression profile previously described by van der Aar *et al.* for LCs and DDCs (8). Inconsistencies may be explained by differences in the employed methodologies or by differences in the isolated DC populations (notably CD1a$^+$ DDCs in our study versus all CD11c^{hi}HLA-DR$^+$ DDCs in their study). Also, the high TLR2 and -3 mRNA expression levels observed by us in isolated keratinocytes are in line with data from Karim *et al.* who reported high TLR2 and -3 expression as well as expression of RIG-I and MDA5 in foreskin, cervical, and vaginal keratinocytes (10). Importantly, these PRR expression profiles altogether support the observed stimulatory effects of PGN and Poly I:C on the skin-emigrated DCs, with PGN likely binding and activating through TLR2/NOD2 and Poly I:C through TLR3/MDA5 (7).

Intradermally delivered Poly I:C induced both DC maturation and an increased migration frequency of the mature CD1a$^+$ DDC subset, resulting in an overall significantly increased T-cell stimulatory ability of the skin-emigrated DCs in an MLR with allogeneic T cells. It is not clear if the effect of Poly I:C on DCs is solely caused by direct activation through TLR3/MDA5, as CD1a$^+$ DDCs express low levels of TLR3 but higher levels of MDA5, or indirectly. Both TLR3 and MDA5 are also functionally expressed by keratinocytes and as such may have triggered the release of DC-activating cytokines that subsequently can induce DC maturation (20). Interestingly, out of a panel of TLR-L based adjuvants subcutaneously injected with a DEC250-targeted HIV-gag protein vaccine in mice, Poly I:C was identified as the superior adjuvant (21). Its observed *in vivo* adjuvanticity, effecting efficient Th1 activation, depended on the release of type-1 IFN by stromal cells and DCs. Indeed, both keratinocytes and fibroblasts may be the source of Poly I:C-induced type-1 IFN (10, 21,22). Conceivably a similar mechanism may have been at work in our skin explant model. Recently, selective induction of the CD8$^+$ T cell activating co-stimulatory molecule CD70 was reported on LCs but not on DDCs in response to viral signals (23). Indeed, intradermal injection of Poly I:C (a synthetic viral PAMP mimic) resulted in a subset of skin-emigrated DCs *de novo* expressing CD70 (Fig.2B). Although Langerin double staining was not performed and their LC identity could thus not be unequivocally confirmed, the small percentages of CD70$^+$ cells correspond with the low frequencies of LCs among the skin-emigrated DCs (Fig.1B). As expected, CD70 was not highly up-regulated on Monocyte-derived DCs upon TLR triggering, in keeping with the reported exclusive up-regulation of CD70 on LCs (23).

PGN appeared to achieve a type-1 DC maturation through triggering the release of pro-inflammatory cytokines, either through TLR2/NOD2 binding in CD1a$^+$ DDCs or in keratinocytes (25).. Stimulation of T cells resulted in a Th1 polarization reflected by significantly elevated TNFα and IFNγ release, as well as in Th17 polarization, reflected by the release of IL-17 (Fig.4B). This confirms findings by others, showing Th1/Th17 induction by PGN through a TLR2/NOD2-dependent mechanism (24). Of note, the TLR2/TLR1 agonist PAM3CSK4 did not induce any DC maturation, in line with findings from a mouse study *in vivo* (21), thus confirming the likely involvement of the intracellular PRR NOD2 in these PGN-induced effects.

In addition, minor DC-activating effects were observed for LPS. Increased LC and CD1a$^+$ DDC migration frequencies and significantly enhanced CD83 and PD-L1 expression levels were observed. Although low TLR4 expression in LCs, DDCs and keratinocytes was observed, reported TLR4 expression in dermal fibroblasts (11) may have facilitated pro-inflammatory cytokine release (most notably IL-1β and IL-6, see also Fig.3), leading to the observed DC activation.

Remarkably, we observed no stimulatory effects for the TLR7/8 agonist R848 (Resiquimod), despite the fact that it emerged as an effective vaccine adjuvant –second only to Poly I:C from an *in vivo* study in humans (21). R848 was applied epicutaneously, like the closely related TLR7 agonist Imiquimod, which has previously been recognized as a possibly effective vaccine adjuvant (7). Conceivably, topical application more effectively addresses low-level TLR7/8 expressed in LCs (8). Moreover, topical application of Imiquimod has previously been described to lead to the recruitment of both myeloid and plasmacytoid DCs (26). Co-administration of systemic FLT3-L further mobilized DC precursors and enhanced the DC-stimulatory effects of Imiquimod (27). In line with these observations, we found R848 (like all the other TLR-Ls tested) to induce CCL5 release from the skin explants (although at highly variable levels, see Fig.3),

consistent with possible *in vivo* recruitment of DCs or their precursors from the blood. Clearly, the skin explant model employed in the current study is not suitable to address this issue.

In conclusion, Poly I:C and PGN, respectively binding TLR3/MDA5 and TLR2/NOD2, are attractive candidate adjuvants for intradermally delivered therapeutic vaccines targeting cutaneous DCs, and should be further explored as such. Based on *in vivo* findings from the Spörri and Reis e Sousa groups, direct APC activation by TLR agonists, rather than indirect activation through pro-inflammatory mediators released by accessory cells, ensures proper differentiation and long-term memory of Th1 and $CD8^+$ effector T-cells (28,29). In view of the relatively high expression levels of TLR2 and NOD2 in the mature and T-cell stimulatory $CD1a^+$ DDC subset and its proven ability to induce Th1 differentiation, inclusion of PGN in any TLR-targeted adjuvant formulation seems an attractive option in this regard. Indeed, as Poly I:C and PGN achieve their T-cell stimulatory effects through different mechanisms (Poly I:C through phenotypic DC activation and PGN through pro-inflammatory cytokine release and Th1/Th17 polarization), possible synergism through the combined administration of both deserves particular attention in future studies.

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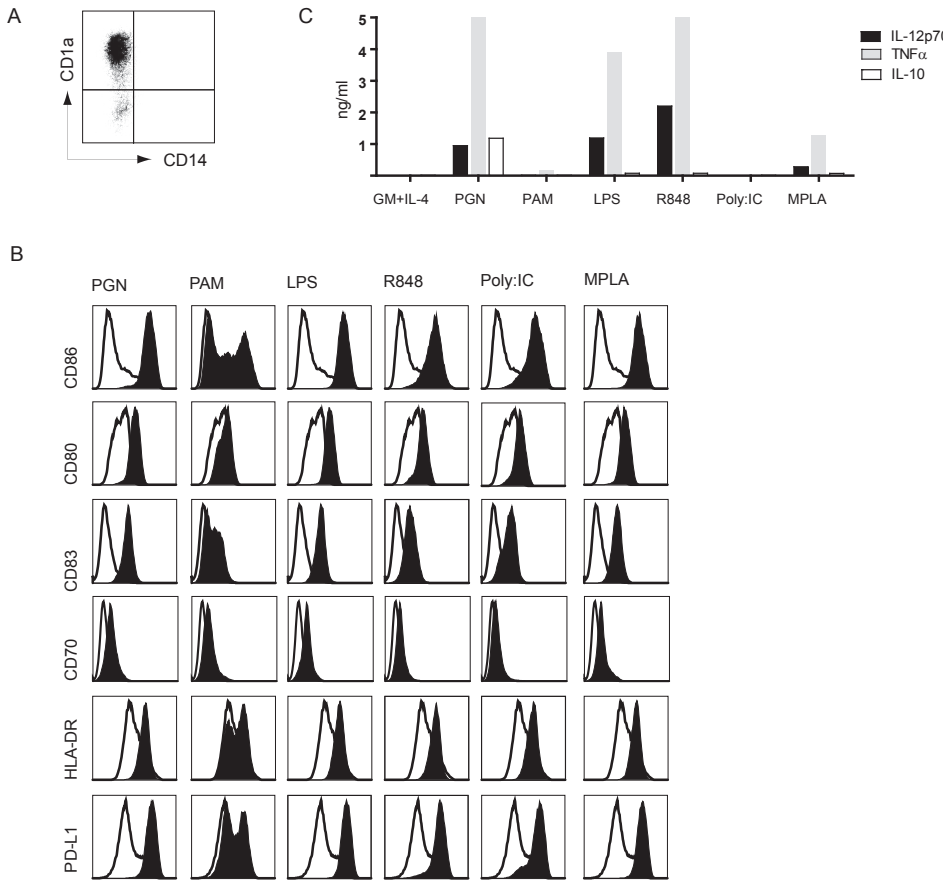
SUPPLEMENTAL FIGURES



Supplementary figure 1. TLR-ligands activate human monocyte derived dendritic cells. A) CD14+ isolated monocytes (>95% pure) were differentiated with IL-4 and GM-CSF and analyzed at day 6 of culture by flowcytometry. B) mo-DC were stimulated with indicated TLR-agonists for 48 hr and analyzed by flowcytometry for expression of co stimulatory molecules Open histograms: immature/unactivated DC (GM+IL 4). Example of 2 5 different donors tested. C) Cytokine production of B analyzed by CBA (one representative experiment).