Intradermal Delivery of TLR Agonists in a Human Explant Skin Model: Preferential Activation of Migratory Dendritic Cells by Polyribosinic-Polyribocytidylic Acid and Peptidoglycans

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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 and dermal DCs 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 TLR2 and -3 agonists peptidoglycan (PGN) and polyribosinic-polyribocytidylic acid (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 polarization. The enhanced priming capacity of Poly I:C–stimulated DCs was associated with a strong upregulation of appropriate costimulatory molecules, including CD70, whereas that of PGN-stimulated DCs 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/nucleotide-binding oligomerization domain 2 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. The Journal of Immunology, 2013, 190: 3338–3345.

Vacccines aimed at the induction of neutralizing Abs 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).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CBA, cytokine bead array; Ct, threshold cycle; DC, dendritic cell; DDC, dermal dendritic cell; HPS, human pooled serum; HPV, human papillomavirus; LC, Langerhans cell; MPLA, monophosphoryl lipid A; NOD, nucleotide-binding oligomerization domain; PD-L1, programmed cell death ligand-1; PGN, peptidoglycan; Poly I.C, polyribosinic-polyribocytidylic acid; PRR, pattern recognition receptor; TLR-L, TLR ligand.

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the cell membrane to bind bacterial structural molecules such as peptidoglycans (PGN) and LPS (TLR1, -2, -4, -5, and -6) or intracellular in endosomal compartments to bind viral or bacterial RNA or DNA (TLR3, -7, -8, and -9). Subsequent signaling through MyD88 or Toll/IL-1R domain-containing adapter inducing IFN-β leads to activation of the NF-κB and MAPK pathways, resulting in AP activation and proinflammatory 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 nucleotide-binding oligomerization domain (NOD)-like receptors (NOD1 and NOD2) or RIG-I-like helicases (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 PAM3CYSK40.3HCl (PAM3CSK4, TLR1/2/6), PGN (TLR2/1/NO2D2-L), PGL-3, and costimulatory molecules and their capacity to prime allogeneic T cells and induce Th1/2/17 responses. We report that Poly I:C and PAM3CYSK40.3HCl (PAM3CSK4, TLR1/2-L) may serve as Toll/IL-1R domain-containing adapter (MyD88 or Toll/IL-1R domain-containing adapter inducing IFN-β) leads to activation of the NF-κB and MAPK pathways, resulting in AP activation and proinflammatory 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 nucleotide-binding oligomerization domain (NOD)-like receptors (NOD1 and NOD2) or RIG-I-like helicases (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 PAM3CYSK40.3HCl (PAM3CSK4, TLR1/2-L), PGN (TLR2-L/NO2D2-L), PGL-3, and costimulatory 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. Whereas for Poly I:C this appeared to be due to upregulation of costimulatory molecules, PGN was a powerful inducer of proinflammatory cytokine release, thereby promoting Th1 induction. From these observations, we conclude that TLR2/ NO2D2 and TLR3 ligands are attractive candidate adjuvants for intradermally delivered therapeutic vaccines.

Materials 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 24 h of surgery. Cytokines or TLR-Ls (endotoxin contaminations not >1 EU/mg, excepting MPLA and LPS) were dissolved in serum-free medium. DCs were all found to express CD86, CD83, and CD54 (ICAM-1) and GM-CSF, IL-1β, TNF-α, IL-6, IL-12p40, IL-10, IL-17, and IFN-γ (12). To induce DC maturation and the induction and skewing of T cells (10, 11). Moreover, alternative intracellular microbial PRRs, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NOD1 and NOD2) or RIG-I-like helicases (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 PAM3CYSK40.3HCl (PAM3CSK4, TLR1/2-L), PGN (TLR2-L/NO2D2-L), PGL-3, and costimulatory 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. Whereas for Poly I:C this appeared to be due to upregulation of costimulatory molecules, PGN was a powerful inducer of proinflammatory cytokine release, thereby promoting Th1 induction. From these observations, we conclude that TLR2/ NO2D2 and TLR3 ligands are attractive candidate adjuvants for intradermally delivered therapeutic vaccines.

Microarray analysis of TLR transcriptional profiles

Epidermal and dermal fractions were prepared from dermato-sliced human skin (3 mm) as described previously (13). After CD14-guided MACS (Miltenyi Biotec, 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 100 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) 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, and 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 (Invitrogen). cDNA was synthesized using a reverse transcription kit (Promega) 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) as previously described (15). Briefly, 4 μl Power SYBR Green master mix (Applied Biosystems) was mixed with 2 μl solution containing 5 mmol/μl bisulfate-oligonucleotides and 2 μl cDNA solution (1/100 of the cDNA synthesis product). The threshold cycle (Ct) value is defined as the number of PCR cycles in which 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 García-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^{\text{Ct}[\text{GAPDH}]} – \text{Ct}[\text{target}].

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

Results
Effects of TLR-Ls 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). An overview of the phenotype of the three major discernable subsets is presented in Table I, and in Supplemental Fig. 2, the employed gating strategy is presented. Of note, LC and CD1a+ DDCs are mature subsets with a high T cell stimulatory potential, whereas the CD14+ DDCs are immature cells with macrophage-like features and a poor capacity for T cell stimulation (Table I) (12). Beside these three major subsets, also smaller CD1a+CD14+ and CD1a-CD14- populations (percentages varying per donor and ranging between 5 and 25%) were discernable (Supplemental Fig. 2). CD1a+ CD14+ DDCs were macrophage-like cells that phenotypically resembled the CD14+ subset, whereas the double-negative population appeared to be a DDC subset of intermediate maturation, based on expression of CD83 and costimulatory markers (data not shown). As these minor migratory DDC subsets were not modulated in number or phenotype by any of the tested TLR agonists (data not shown), migration rates in this study were analyzed for the three major subsets only (Fig. 1). 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, whereas it inhibited migration of the CD14+ immature subsets (12). In this study, we confirm these observations (Fig. 1), and in all subsequent experiments, GM-CSF plus IL-4 was included as a positive control. TLR agonists were intradermally injected at subtoxic active doses (see Materials and Methods) that observed for medium controls (Fig. 1). Of note, unlike GM-CSF plus IL-4, none of the tested TLR-Ls was able to significantly downregulate the frequencies of migrated immature CD14+ DDC.

<table>
<thead>
<tr>
<th>DC Subset</th>
<th>Marker Profile*</th>
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<tbody>
<tr>
<td>LC</td>
<td>CD1ahi, langerin(^a), E-cadherin(^a), DC-SIGN(^a), CD11c(^a), CD1c(^a), CD163(^a), CD83(^a), CD80(^a), PD-L1(^a)</td>
</tr>
<tr>
<td>CD1a+ DDC</td>
<td>CD1a+hi, langerin(^a), E-cadherin(^a), DC-SIGN(^a), CD11c(^a), CD1c(^a), CD163(^a), CD83(^a), CD80(^a), PD-L1(^a)</td>
</tr>
<tr>
<td>CD14+ DDC</td>
<td>CD1ahi, langerin(^a), E-cadherin(^a), DC-SIGN(^a) (30%)(^a), CD11c(^a), CD1c(^a), CD163(^a) (15%)(^a), CD83(^a), CD80(^a), PD-L1(^a) (20%)</td>
</tr>
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*Based on multicolor FACS analyses as described by de Gruijl et al. (12) and J.J. Lindenberg, D. Oosterhoff, C.C. Sombroek, S.M. Lougheed, E. Hooijberg, A.G.M. Stam, S.J.A.M. Santegoets, H.J. Tijssen, J. Buter, H.M. Pinedo, A.J.M. van den Eertwegh, R.J. Schepen, H.J.P.M. Koene, R. van de ven, and T.D. de Gruijl (submitted for publication), marker expression was considered negative (−) when percentage positive cells did not exceed 10%. 

\(^{a}\)Upregulated by intradermal GM-CSF and IL-4 injection prior to explant culture.

To assess the influence of the TLR-L on the local cytokine balance, explant-conditioned media were collected 48 h 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), whereas low but detectable levels of TNF-α were induced by Poly I:C (Fig. 3). In addition, variable but consistently elevated levels of the proinflammatory 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 DCs to induce the proliferation of allogeneic T cells (Fig. 4A). Interestingly, DCs migrated from GM-CSF plus 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 TLRs. 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 IFN-γ and TNF-α (Fig. 4B). In addition, PGN was able to induce the release of variable levels of IL-17A by the primed T cells (Fig. 4B, bottom panel).

PRR transcriptional profiles in LCs, DDCs, and keratinocytes
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 TLR transcripts as compared with C-type lectin

Table I. DC subsets and their phenotype

TLR-MEDIATED ACTIVATION OF DERMAL DC
receptor transcripts selectively expressed by LCs (langerin, CD207) or by DDCs (macrophage mannose receptor, 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 analyzed. 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 quantitative RT-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 upregulation of TLR1–3 and -5 transcription, confirming functional expression of TLR3 in 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.

**p-ERK Phosflow measurements in the emigrated DC subsets**

Finally, as an indication of the relative activation of the different migratory DC subsets under the influence of the intradermally delivered TLR-L for which demonstrable effects were observed, p-ERK or p–NF-κB, both previously shown to act downstream in the signaling pathways of all identified TLR, were considered for detection by Phosflow in combination with staining for CD1a and CD14. As phosphorylation events are usually rapid and typically occur within 30 min, we first determined if p-ERK or p–NF-κB-p70 was still measurable in monocyte-derived DCs 48 h after Poly I:C or LPS stimulation and found increased p-ERK expression to indeed be detectable at that time (data not shown). We therefore assessed p-ERK expression in the 48 h–migrated DC subsets after intradermal delivery of GM-CSF plus IL-4, or the denoted TLR agonists. Flow cytometric histograms of the expression of the indicated activation markers on all of 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 ± SEM of three to six experiments are shown. Asterisks denote significant differences versus medium controls, *p < 0.05.
studied DC subsets; in some experimental groups, these shifts reached significance (Fig. 6B). An overview of results from three independent experiments (Fig. 6B) shows that basal p-ERK levels in the LC and CD1a+ DDC subsets were considerably higher than in the CD14+ DDC subset, in keeping with the more activated state of these CD1a-expressing subsets (Table I). Although PGN and Poly I:C (like GM-CSF plus IL-4) induced moderately elevated p-ERK levels in all three subsets, the p-ERK–increasing effect of LPS appeared to be restricted to the LC subset (Fig. 6B).

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 adjuvants deposited in the skin can interact with a complex mix of cells, among 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. Of note, in a previous and separate set of experiments we had already shown that intradermal delivery of the TLR9 agonist PF-3512676 (a CpG-B oligonucleotide formerly known as CpG 7909) did not result in phenotypic or functional activation of human skin explant emigrated DCs (A. Baars, T.D. de Gruijl, S.M. Lougheed, C.C. Sombroek, S.M. van Ham, R.J. Scheper, H.M. Pinedo, and A.J.M. van den Eertwegh, unpublished observations). This general lack of skin-derived DC activation upon TLR-L treatment is in striking contrast to our own observation for monocyte-derived DCs (Supplemental Fig. 1). Strong activation by TLR-Ls was also reported for isolated monocyte-
Freshly isolated LCs and CD1a⁺ DDCs were analyzed by genome-wide transcriptional profiling. Shown are the transcript levels for the indicated PRR and C-type lectin receptor genes (means from three 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 quantitative RT-PCR (qPCR). 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 (100 ng) and again tested for TLR transcript expression as described in (B). Means ± SEM 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 PRR and C-type lectin receptor genes (means from three 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 quantitative RT-PCR (qPCR). 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 (100 ng) and again tested for TLR transcript expression as described in (B). Means ± SEM are shown.

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. (18) recently reported that selective overexpression of miR-146a in LCs interfered with TLR2-mediated signaling and caused their nonresponsiveness to PGN. 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 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; 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 plus 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. (8) for LCs and DDCs. 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⁺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. (10), who reported high TLR2 and -3 expression as well as expression of RIG-I and MDA5 in foreskin, cervical, and vaginal keratinocytes. 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 and MDA5 or RIG-I (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. TLR3 and MDA5/RIG-I 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, 21). Generalized increases in p-ERK expression in both LC and CD14⁺ DDC as well as in CD1a⁺ DDC may argue in favor of the latter option. Interestingly, out of a panel of TLR-L–based adjuvants s.c. injected with a DEC250-targeted HIV-gag protein vaccine in mice, Poly I:C was identified as the superior adjuvant (22). 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 (22, 23). Conceivably, a similar mechanism may have been at work in our skin-explant model. Recently, selective induction of the CD8⁺ T cell–activating costimulatory molecule CD70 was reported on LCs but not on DDCs in response to viral signals (24). Indeed, intradermal injection of Poly I:C (a synthetic viral pathogen-associated molecular pattern 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 CD1a⁺ DDCs (12, 19).

PGN appeared to achieve type 1 DC maturation through triggering the release of proinflammatory cytokines, either through TLR2/NOD2 binding in CD1a⁺ DDCs or, as previously described, in keratinocytes (25). Stimulation of T cells resulted in Th1 polarization reflected by significantly elevated TNF-α and IFN-γ release (Fig. 4B). This confirms findings by others, showing Th1/ Th17 induction by PGN through a TLR2/NOD2-dependent
mechanism (26). Of note, the TLR2/TLR1 agonist PAM3CSK4 did not induce any DC maturation, in line with findings from a mouse study in vivo (22), thus confirming the likely involvement of the intracellular PRR NOD2 in these PGN-induced effects. Based on in vivo findings from the Spörri and Reis e Sousa groups (27, 28), direct APC activation by TLR agonists, rather than indirect activation through proinflammatory mediators released by accessory cells, ensured proper differentiation and long-term memory of Th1 and CD8+ effector T cells. 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.

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 proinflammatory cytokine release (most notably IL-1β and IL-6; see also Fig. 3), leading to the observed DC activation. Of note, the observed unique ability of LPS among the other tested TLR-L to increase the frequency of migrating LC corroborates recent findings by Schneider et al. (29) and is in line with the LPS-induced increase in p-ERK levels exclusively in LCs (Fig. 6B). Remarkably, we observed no stimulatory effects for the TLR7/8 agonist R848 (resiquimod), despite the fact that it emerged as an

![Image](https://example.com/image.png)

**FIGURE 6.** TLR-L–induced p-ERK expression among human skin-emigrated DC subsets. GM-CSF plus IL-4, PGN, Poly I:C, or LPS were intradermally injected into human skin, explants were subsequently taken and cultured for 48 h, after which explants were discarded, and emigrated DCs were harvested and stained for CD1a and CD14 in conjunction with intracellular p-ERK. (A) Representative results for Poly I:C show increased p-ERK expression levels upon Poly I:C delivery (closed histograms) relative to medium (open histograms) in all three CD1a+ LC, CD1a+ DDC, and CD14+ DDC subsets. (B) Results for all tested conditions from three independent experiments. Data are presented in mean fluorescence (MF) indices (i.e., mean fluorescence intensity [MFI] p-ERK mAb divided by mean fluorescence intensity no mAb). Asterisks denote significant differences versus medium controls, *p < 0.05.
effective vaccine adjuvant, second only to Poly I:C from an in vivo study in humans (22). 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 in LCs (8). The effects of both Poly I:C and PGN. Indeed, as Poly I:C and PGN of skin DCs was hampered by unavailability of autologous PBMC and low numbers of migrated DCs. Nevertheless, priming and skewing of allogeneic T cells clearly demonstrated favorable effects of both Poly I:C and PGN. 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 proinflammatory cytokine release and Th1 polarization), possible synergism through the combined administration of both 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 and/or MD25 and TLR2/NO2D, are attractive candidate adjuvants for intradermally delivered therapeutic vaccines targeting cutaneous DCs and should be further explored as such. Assessment of the effects of the TLR-L on the Ag-specific T cell–priming ability of skin DCs was hampered by unavailability of autologous PBMC and low numbers of migrated DCs. Nevertheless, priming and skewing of allogeneic T cells clearly demonstrated favorable effects of both Poly I:C and PGN. 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 proinflammatory cytokine release and Th1 polarization), possible synergism through the combined administration of both 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.

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Disclosures
The authors have no financial conflicts of interest.

References

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).
Supplementary figure 2. Gating strategy for migratory skin dendritic cell (DC) subsets. DCs migrated from human skin explants at day 2 of culture were first gated on high Forward and Side Scatter properties (not shown) and subsequently on the basis of CD1a and CD14 expression. Frequencies among the migrated DCs of Langerhans cells (LC, CD1a<sup>+</sup>CD14<sup>-</sup>), CD1a<sup>+</sup>CD14<sup>-</sup> (CD1a<sup>+</sup>) and CD1a<sup>+</sup>CD14<sup>-</sup> (CD14<sup>-</sup>) dermal DCs (DDC) were quantified.