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Macrophage- and Neutrophil-Derived TNF-α Instructs Skin Langerhans Cells To Prime Antiviral Immune Responses

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Dendritic cells are major APCs that can efficiently prime immune responses. However, the roles of skin-resident Langerhans cells (LCs) in eliciting immune responses have not been fully understood. In this study, we demonstrate for the first time, to our knowledge, that LCs in cynomolgus macaque skin are capable of inducing antiviral-specific immune responses in vivo. Targeting HIV-Gag or influenza hemagglutinin Ags to skin LCs using recombobant fusion proteins of anti-Langerin Ab and Ags resulted in the induction of the viral Ag-specific responses. We further demonstrated that such Ag-specific immune responses elicited by skin LCs were greatly enhanced by TLR ligands, polyriboinosinic polyribocytidylic acid, and R848. These enhancements were not due to the direct actions of TLR ligands on LCs, but mainly dependent on TNF-α secreted from macrophages and neutrophils recruited to local tissues. Skin LC activation and migration out of the epidermis are associated with macrophage and neutrophil infiltration into the tissues. More importantly, blocking TNF-α abrogated the activation and migration of skin LCs. This study highlights that the cross-talk between innate immune cells in local tissues is an important component for the establishment of adaptive immunity. Understanding the importance of local immune networks will help us to design new and effective vaccines against microbial pathogens.

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The skin is an important site for vaccine delivery. Anti-smallpox vaccine, the most successful vaccine ever in humans, is administered by skin scarification or puncture, and the bacille Calmette-Guérin vaccine and new commercial anti-influenza vaccines are injected intradermally (1). In steady state, human skin contains many cells specializing in immune surveillance, including CD1a and langerin-expressing Langerhans cells (LCs) in the epidermis and CD1a+CD14+ dendritic cells (DCs) and macrophages in the dermis (2). CD141high DCs originating from the blood also have enhanced cross-presenting capabilities (3). These various cell subsets in the skin sample Ags in their environment by diverse mechanisms. They are activated by danger signals, leading to their migration to the draining lymph nodes to interact with Ag-specific T and B lymphocytes, resulting in the priming and induction of adaptive immune responses. Diverse specific immune response profiles may be generated by different DC subsets or may result from DC plasticity upon Ag encounter and activation signals. These properties could be exploited in new generations of vaccines designed to enhance, redirect, and fine-tune the desired immune response (4–6). We recently reported, in non-human primates (NHPs), which have an immune system organized similarly to humans, that targeting the same Ag to different C-type lectin receptors expressed by skin DCs results in different Th cells secreting IL-10 or IFN-γ (7). However, despite the accumulation of substantial amounts of knowledge from in vitro and mouse models over the last 10 y, little is known about the mechanisms of vaccine interaction with primate DCs in vivo, in the skin microenvironment. The translation of this new concept into the rational design of human vaccines will require detailed characterization of complex cascades of events at the injection site and in peripheral tissues following the injection of vaccine Ags and adjuvants.

NHP and human DCs have very similar distributions and functions (8–10). In vitro, LCs loaded with Ags, through incubation with soluble peptides or infection with viral vectors, prime naïve T cells efficiently. In humans, LCs extracted from the epidermis efficiently induce MLRs and trigger the production of Th1 and Th2 cytokines by CD4+ T cells (11, 12). CD14+ dermal DCs prime T cells less efficiently than LCs, providing a strong rationale for using fusions of a vaccine Ag to anti-langerin Abs to target LCs in vivo. TLR ligands (TLR-Ls) have been reported to be essential for the efficacy of DC-targeted vaccines and are certainly major players in immune response polarization. TLRs bind pathogen-associated mo-

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Abbreviations used in this article: DC, dendritic cell; HA, hemagglutinin; i.d., intradermal; LC, Langerhans cell; NHP, nonhuman primate; PMN, polymorphonuclear neutrophil; poly(LC), polyribosinosinic polyribocytidylic acid; TLR-L, TLR ligand.

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lecular patterns, such as dsRNA, ssRNA, and microbial DNA. Such interaction with specific ligands induces the activation of TLR-expressing cells in the skin, such as DCs, macrophages, and inflammatory cells (13, 14). Synthetic TLR agonists have therefore been designed for use as vaccine adjuvants. Their use intensifies Th1- and Th2-oriented immune responses (15–17). However, as for skin DCs, little is known about the cellular and molecular events occurring at the site of TLR-L injection. We show in this study that langerin-targeted vaccines can efficiently prime Ag-specific immune responses in NHP that could be enhanced by a synthetic dsRNA, polyriboinosinic polycytidylic acid [poly(I:C)], which is a TLR3 agonist. We also deciphered the in situ mechanisms underlying the activation and migration of LCs triggered by poly(I:C) or resiquimod (R848), which acts as a ligand of TLR7/8.

Materials and Methods

Animals

Adult male cynomolgus macaques (Macaca fascicularis) imported from Mauritius and weighing 4–8 kg were housed in Commissariat à l’Énergie Atomique facilities (accreditation RLG, B 92-032-02) and handled (investigator accreditation RLG, B 92-073; FM, C 92-241) in accordance with European guidelines for NHP care (EU Directive N 2010/63/EU). Before the start of the study, the animals were tested and found to be seronegative for several pathogens (SIV, simian T lymphotropic virus, filovirus, hepatitis B virus, herpes B, and measles). Animals were sedated with ketamine hydrochloride (10–20 mg/kg; Rhone-Mérieux, Lyon, France) during handling. Animals were euthanized by sedation with ketamine, followed by i.v. injection of a lethal dose of sodium pentobarbital. The regional animal care and use committee (Comité Régional d’Ethique Ile de France Sud, reference 12-013) and the Baylor Research Institute animal care and use committee (reference A10-015) have reviewed and approved this study.

Reagents

R848 and high molecular mass poly(I:C) were purchased from InvivoGen (San Diego, CA). R848 (resiquimod) is an imidazoquinoline compound binding TLR7 and TLR9. High molecular mass poly(I:C) is a synthetic analog of dsRNA, with a mean size of 1.5–8 kb, that binds TLR3. Anti-DC receptor and control Ab Gag (p24) and influenza virus hemagglutinin (HA) (strain A/PR/8/34) fusion proteins were prepared as described in Flamar et al. (18), except that the anti-CD207 mAb fusions used variable regions derived from the in-house anti-human langerin hybridoma (15B10, Genbank accession number KF021227), or the human anti-CD207 mAb coupled to HIV-gag protein, its control isotype IgG4, and the Gag protein were provided by the Barshop Institute for Immunology Research (Dallas, TX). Fluorescent labeling of these fusion proteins and the anti-HLA-DR mAb (clone L243; Ozyme, St. Quentin en Yvelines, France) was performed using, respectively, Fluorochrome 682 (F682) and 490 (F490) kits (Interchim, Montluçon, France). A total of 10 μg IgG4-Gag-F682 mAb, anti-Lang-Gag-F682 mAb, or Gag-F682 protein was injected i.d. with 10 μg anti-HLA-DR-F490 mAb in 100 μl PBS solution in adult NHP under anesthesia. Biopsies at the injection sites were moved 2 h after in vivo i.d. injection of fluorescent mAbs. Each skin biopsy was placed in a 6-well plate (MatTek, Ashland, MA) in contact with RPMI 1640 containing 100 μg/ml penicillin/streptomycin/neomycin and 5% FCS to analyze dermis and epidermis. Fluorescent images were captured through a Plan Fluor 20× DIC objective (NA: 0.45) on a Nikon A1 confocal laser-scanning microscope system attached to an inverted ECLIPSE Ti (Nikon, Tokyo, Japan) held at 37°C under a 5% CO2 atmosphere.

Immunizations

Groups of three to six cynomolgus macaques underwent inoculation in weeks 0, 6, and 15, with 1 ml (10 i.d. injections of 100 μl) indicated vaccine preparation. Each preparation contained 62.5 μg HIV Gag protein, correspondi to 250 μg total protein when associated with Abs in fusion proteins. Poly(I:C) was added at a final concentration of 125 μg/ml where indicated. Sera were collected from vaccinated animals for the titration of Gab-specific Abs with the Gag-specific IgG Ab ELISA, as described (18). Immunization groups were designed according to MHC genotypes of animals (Supplemental Fig. 1). Groups of four NHPs primed with influenza A/PR/8/34 virus underwent inoculation, in weeks 11 and 17, with adjuvanted anti-langerin-HA vaccine [containing 62.5 μg HA protein, corresponding to 250 μg total protein when associated with Abs in fusion proteins, supplemented with 125 μg poly(I:C)] or with Vaxigrip. Sera were collected for titration of hemagglutination inhibition, as described (18).

Surface plasmon resonance assay

Surface plasmon resonance assay-binding measurements were performed on SensiQ Pioneer (SensiQ Technologies, Oklahoma City, OK) using sera samples from NHPs immunized with the anti-CD207-Gag conjugate (Lang-Gag group) or the anti–CD207–Gag conjugate mixed with poly(I:C) (Lang-Gag + PIC group). Anti-Lang-Gag and anti-Lang mAbs were immobilized (30 μg/ml in pH 5.0 10 mM sodium acetate) on individual flow cells using amine-coupling chemistry on a COOH sensor chip at 25°C. Channel 2 was used to capture anti-Lang–Gag, and channel 3 was used for anti-Lang mAbs. Channel 2 was left as a reference to subtract nonspecific binding. Pooled immunized sera samples from NHPs immunized with the anti–CD207–Gag conjugate (Lang-Gag + PIC group) were injected over the surfaces for 10 min. Surfaces were regenerated by injecting two short pulses (30 s) of 50 mM phosphoric acid (pH 2.0). Specific responses for each sera sample were obtained by subtracting 4°C and then for 2 h at 37°C, to facilitate separation of epidermal and dermal sheets. The epidermis was then incubated with 0.25% trypsin (Eurobio, Courtaboeuf, France) for 20 min at room temperature. The dermis was incubated with 2 mg/ml type D collagenase (Roche Diagnostic, Meylan, France) in RPMI 1640 at 37°C for 1 h, with shaking. Each resulting cell suspension was passed through a filter with 100-μm pores before use.

Flow cytometry analysis

Cell mortality was assessed with a blue fluorescent reactive dye from the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen Life Technology, Paisley, U.K.). FeRs and other nonspecific binding sites were blocked by incubation with a 5% solution of pooled macaque sera. The details on Abs used are listed in Table I. Fluorochrome-free Abs were detected with either PE-labeled goat-antimouse secondary Ab (Jackson, Newmarket, U.K.) (for anti-CD207 mAb) or a secondary Ab coupled to an Alexa Fluor fluorochrome, with the Zenon kit (Invitrogen Life Technology). Acquisition was performed on an LSRFortessa or an LSRII cytometer (BD Biosciences, Le Pont de Claix, France). The data obtained were analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunohistoﬂuorescence staining

Fresh skin biopsy specimens were flash frozen in a liquid nitrogen bath. Sections were cut, stained with the Abs listed above conjugated with Alexa Fluor fluorochromes, and mounted in DAPI-containing mounting medium (Invitrogen Life Technology).

In vivo immunohistoﬂuorescence staining

The human anti-CD207 mAb coupled to HIV-gag protein, its control isotype IgG4-gag, and the Gag protein were provided by the Barshop Institute for Immunology Research (Dallas, TX). Fluorescent labeling of these fusion proteins and the anti-HLA-DR mAb (clone L243; Ozyme, St. Quentin en Yvelines, France) was performed using, respectively, Fluorochrome 682 (F682) and 490 (F490) kits (Interchim, Montluçon, France). A total of 10 μg IgG4-Gag-F682 mAb, anti-Lang-Gag-F682 mAb, or Gag-F682 protein was injected i.d. with 10 μg anti-HLA-DR-F490 mAb in 100 μl PBS solution in adult NHP under anesthesia. Biopsies at the injection sites were moved 2 h after in vivo i.d. injection of fluorescent mAbs. Each skin biopsy was placed in a 6-well plate (MatTek, Ashland, MA) in contact with RPMI 1640 containing 100 μg/ml penicillin/streptomycin/neomycin and 5% FCS to analyze dermis and epidermis. Fluorescent images were captured through a Plan Fluor 20× DIC objective (NA: 0.45) on a Nikon A1 confocal laser-scanning microscope system attached to an inverted ECLIPSE Ti (Nikon, Tokyo, Japan) held at 37°C under a 5% CO2 atmosphere.

Intradermal injections and biopsies

We injected 200 μg R848 or poly(I:C) in 100 μl PBS, or PBS alone, by intradermal (i.d.) route into the backs of the animals, via a 29-gauge needle. Punch biopsies (8 mm in diameter) were performed on anesthetized animals 1, 3, or 8 d after injections. We added 1 ng etanercept where indicated. In all cases, the final volume injected i.d. was 100 μl per injection.

Skin APC extraction

Cells were extracted from fresh skin biopsy specimens with modified versions of published protocols (19, 20). Briefly, the s.c. fat was removed and the specimen was then incubated with 4 mg/ml bacterial protease dispase grade II (Roche Diagnostic, Meylan, France) in PBS for 12–16 h at

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Flow cytometry analysis of the surface expression of CD80, CD83, and CD86. After incubation for 16 h, LC activation/maturation status was assessed by and CD209 was analyzed. Most of the HLA-DR expression of CD207, CD11b, CD11c, CD14, CD163, and CD209 was determined with the Milliplex MAP NHP Immunoassay kit (Millipore, Billericay, MA). LCs were stimulated by dispensing 10^6 cells per well with 2 ml complete medium supplemented with the following human cytokines at 24-well culture plates, by incubating 10^6 granulocytes per well with 2 ml complete medium plus 1 ml supernatant from the granulocyte and monocyte stimulation experiments described above, with or without 200 μg/ml macaque serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, with or without 10 μg/ml R848 or polycl[1,C] for 16 h before harvesting the supernatant. Concentrations of GM-CSF, IFN-γ, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, IL-18, MIP-1α, MIP-1β, and TNF-α in the supernatants were determined with the Milliplex MAP NHP Immunoassay kit (Millipore, Billericay, MA). LCs were stimulated by dispensing 10^6 cells resulting from enzymatic disruption of the epidermis into a well containing 2 ml complete medium with or without 10 μg/ml R848 or polycl[1,C] for 16 h in a 24-well plate. Alternatively, epidermal cells were incubated in complete medium supplemented with the following human cytokines at a concentration of 20 ng/ml: TNF-α, IL-1β, IL-6, IL-8, MIP-1α, or MIP-1β. After incubation for 16 h, LC activation/mutation status was assessed by flow cytometry analysis of the surface expression of CD80, CD83, and CD86.

Statistical analysis

Data are expressed as the mean ± SD, unless specified in figure legends. Statistical analyses were performed with Prism 5.0 (GraphPad Software, La Jolla, CA) software, using the appropriate nonparametric test: Wilcoxon–Mann-Whitney unpaired, or Spearman’s correlation tests.

**Results**

A langerin-targeting vaccine primes an Ag-specific antiviral response in NHP

We have shown that LCs can prime Ag-specific immune responses in vitro models and humanized mice (12, 18, 22). We assessed the concept of a vaccine targeting langerin (CD207)-expressing cells, by generating anti-langerin Abs fused to HIV Gag or influenza H1N1 HA viral Ags, which we tested in cynomolgus macaques. NHPs are a more relevant model than mice for the testing of human vaccines. We have previously demonstrated that NHP and human DC subsets have similar tissue distributions and bear analogous differentiation and activation markers, including C-type lectin receptors (9). Diverse DC populations can be identified in the macaque skin, as follows: LCs, which express langerin (CD207), are found exclusively in the epidermis, whereas DCs expressing DC-SIGN (CD209) are localized in the dermis (Fig. 1A). CD209 may also stain activated dermal macrophages. LCs also express high levels of CD1a and form a network in the epidermis with 300–1000 cells/cm^2 (Fig. 1A). At steady state, they are characterized as CD45^–HLA-DR^{high}CD11c^CD11b^CD14^{high}CD207^{high} (Table 1) and account for 0.71% ± 0.23% (n = 12) of all epidermal cells (Fig. 1B). NHP LCs do not express CD14 and CD163 on their membranes and display only low levels of CD209 expression. In the dermis, CD45^–HLA-DR^{–} cells include macrophages and DCs, but no CD207^{+} cells (Fig. 1C). Dermal macrophages are CD11c^{low}CD163^{+} and account for 1.48% ± 0.70% (n = 18) of all cells at steady state. Similar findings have been reported for human skin (2). Dermal DCs (CD11c^{–}CD163^{–}) comprise at least two distinct populations expressing intermediate levels of CD1a (0.60% ± 0.39%, n = 12) or CD14 (2.20% ± 1.45%, n = 12). As previously reported (23), only a small pro-

**FIGURE 1.** Identification of DCs, macrophages, and neutrophils in NHP skin at steady state. (A) Skin sections, perpendicular to the surface (upper image), were stained with DAPI (blue), anti-CD207 (red), and anti-CD209 (green) mAbs. Epidermis sections, parallel to the skin surface (lower image), were stained with DAPI (blue) and anti-CD1a (red) mAb. Scale bars, 20 μm. (B) Suspensions of cells from the epidermis were analyzed by flow cytometry. LCs (HLA-DR^{–}CD1a^{+}) accounted for 0.71% ± 0.23% of the living cells, and their expression of CD207, CD11b, CD11c, CD14, CD163, and CD209 was analyzed. Most of the HLA-DR^{–}CD1a^{–} cells were keratinocytes (cytokeratin^+). Iso-type-matched staining overlays are shown in solid gray curves. (C) Suspensions of cells from the dermis were analyzed by flow cytometry. PMNs were identified as CD45^–HLA-DR^{–}CD66^{+} cells. Macrophages were identified as CD45^–HLA-DR^{–}CD11c^{–}CD163^{–} cells. Dermal DCs were identified as CD45^–HLA-DR^{–}CD11c^{–}CD163^{–} cells, and included CD14^{+} DCs (2.20% ± 1.45% of dermis cells) and CD1a^{+} DCs (0.60% ± 0.70% of dermis cells). At steady state, PMNs and macrophages accounted for 0.88% ± 0.45% and 1.48% ± 0.70% of dermis cells, respectively.
portion (0.88% ± 0.45%, n = 13) of CD45^+HLA-DR^- dermal cells are polymorphonuclear cells expressing CD66abce (Fig. 1C). The anti-langerin mAb that we have developed to target human epidermal LCs also recognized the LCs of cynomolgus macaques (Fig. 1A, 1B). This anti-CD207 mAb, fused to HIV-Gag, was used to immunize NHPs. We first demonstrated that this fusion protein specifically targeted the epidermal LCs. As early as 2 h following i.d. injection of the fluorescent-labeled fusion protein, the Ag colocalized with almost all HLA-DR^+ cells of the epidermis (Fig. 2A). There was no Ag associated with other types of epidermal cells. By contrast, none of the IgG4-Gag and Gag proteins injected i.d. as controls appeared to be associated with epidermal cells expressing HLA-DR, demonstrating that only the anti-langerin-Gag fusion protein could specifically target LCs with very high efficacy. In the dermis, only part of the anti-langerin-Gag and IgG4-Gag immunogens appeared to colocalize with HLA-DR--expressing cells, whereas no such staining could be evidenced when Gag alone was injected. Three i.d. injections of the anti-langerin-Gag vaccine (250 μg each), at weeks 0, 6, and 15, were sufficient to induce a significant anti-Gag Ab response in NHPs (Fig. 2B). Remarkably, the induction of this response did not require the use of an adjuvant. By contrast, animals injected with HIV-Gag protein alone or with the IgG4-Gag isotype control, not require the use of an adjuvant. By contrast, animals injected with HIV-Gag protein alone or with the IgG4-Gag isotype control, did not induce a significant Ab response in mice (24) and NHPs (25). We confirmed that poly(I:C) also acted as an adjuvant for our anti-langerin-Gag and anti-langerin-HA conjugates. Three i.d. injections of the anti-langerin-Gag vaccine in the presence of poly(I:C), compared with the vaccine without adjuvant, enhanced Gag-specific Ab response in macaques, although this was not statistically significant (p = 0.069, Fig. 2C). We then assayed recall responses analogous to those induced by vaccines in human adults exposed to seasonal influenza infections, by injecting the anti-langerin-HA fusion protein into NHPs primed with influenza A/PR/8/34 virus. Two injections (weeks 11 and 17) of the adjuvanted anti-langerin-HA vaccine induced high titers of protective Abs, as demonstrated by measurements of hemagglutination inhibition in the serum (Table I). These levels are significantly higher (p = 0.028) than those obtained for control animals primed with A/PR/8/34 and boosted twice with Vaxigrip (Fig. 2D). The Ab responses against the targeting component of the vaccine were measured (Fig. 2E). Interestingly, these responses remained 2- to 3-fold lower than the Gag-specific responses after the third vaccine injection.

The increased responses obtained with the use of poly(I:C) suggested a synergy between the effect of the adjuvant and the targeting of LCs. Information about early cellular and molecular changes at the site of poly(I:C) injection associated with the enhanced immunogenicity of the vaccine should provide hints for the rational design of future strategies. We studied, in particular, local interactions of R848, a TLR7/8 agonist, or poly(I:C) with LCs. The i.d. injection of 200 μg R848 or poly(I:C) induced skin inflammation, which was not seen with an identical volume (100 μl) of PBS used as a control. Staining of skin sections with H&E 72 h after injection with R848 revealed perivascular, neutrophilic dermatitis-associated epidermal spongiosis and exocytosis of leukocytes (Fig. 3A). The i.d. injection of 200 μg poly(I:C) induced similar, but less marked changes.

The density of LCs in the epidermis changed considerably after R848 or poly(I:C) i.d. injection (Fig. 3B). R848 induced an initial small increase in LC number (1.19% ± 0.32%, at 24 h), which was not statistically significant. However, the numbers of langerin- and CD1a-positive cells were significantly lower 72 h after injection (p = 0.0156, Fig. 3C). We excluded the possibility that the disappearance of LCs reflected the downregulation of CD207 only, because CD1a expression was not affected by TLR-L injection (Fig. 3D). Although the macroscopic signs of inflammation at the injection site

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**Table I. Details of Abs used for flow cytometry**

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The i.d. injection of TLR ligands favors LC migration and activation

LCs require costimulatory signals for maturation and migration and for optimal Ag processing and presentation to T and B lymphocytes. We therefore considered the use of synthetic TLR-Ls as immune stimulant to enhance Ag-specific responses in vaccinated NHPs. The targeting of Ags to DCs through DEC205 has been shown to be strongly dependent on the simultaneous administration of TLR3-L in mice (24) and NHPs (25). The increased responses obtained with the use of poly(I:C) suggested a synergy between the effect of the adjuvant and the targeting of LCs.
returned to normal 8 d after R848 injection, LC density remained lower compared with PBS injection site (0.38% ± 0.16% and 0.99% ± 0.60%, respectively; Supplemental Fig. 2). By contrast, the number of CD209+ dermal cells was similar or slightly higher (although not significant). However, this marker cannot discriminate between possible migration of dermal DCs out of the skin and recruitment of macrophages.

Similar changes were observed after the i.d. injection of 200 µg poly(I:C). At 72 h postinjection, there were significantly fewer epidermal LCs (p = 0.0313) than in control skin treated with PBS (Fig. 3C). This smaller number of LCs was associated with lower levels of CD207 expression on the membrane (Fig. 3D), usually linked to LC activation. We confirmed that injections of R848 and poly(I:C) enhanced the expression of LC activation and maturation markers (CD80, CD83, and CD86). The changes observed were more marked 24 h than 72 h after injection (i.e., for the LCs that had not left the epidermis; Fig. 3E). Thus, i.d. injection of TLR7/8-L or TLR3-L clearly induced LC maturation and activation and their migration out of the epidermis.

The migration of LCs is associated with the local recruitment of polymorphonuclear cells and macrophages

HLA-DR+CD66+ polymorphonuclear cells were identified as mostly polymorphonuclear neutrophils (PMNs) on the basis of their phenotype (HLA-DR-CD66+FceR1-CD123+; Supplemental Fig. 3) and histological features in the skin (Fig. 3A). The i.d. injection of 200 µg R848 or poly(I:C) was associated with a massive local recruitment of PMNs and macrophages (Fig. 4). Indeed, the frequency of PMNs increased to 8.18% ± 6.92% at 24 h and 8.52% ± 12.93% at 72 h after R848 injection, and significantly higher than

![Image](https://example.com/image1.png)

**FIGURE 2.** Immunogenicity of Ag targeting to the LC-specific receptor CD207. (A) The targeting of LCs by the anti–CD207-Gag conjugate (Lang-Gag) was compared with the isotype control conjugate (IgG4-Gag) and Gag alone (Gag). Fluorescent labeled proteins (red) were injected i.d. together with anti–HLA-DR mAb (green). Injection sites were surgically removed 2 h after in vivo injection to analyze the fluorescence signals, in the dermis and the epidermis, with a confocal laser-scanning microscope system. Scale bars, 20 µm. (B) Gag-specific Abs were titrated in sera from NHPs immunized with the anti–CD207-Gag conjugate (Lang-Gag), Gag alone (Gag), or the isotype control conjugate (IgG4-Gag). (C) The area under the curves from weeks 0 to 18 (AUC week 0–18) of Gag-specific Ab responses was compared for NHP groups receiving Gag protein alone (Gag), IgG4 isotype control-Gag conjugate (IgG4-Gag), anti–CD207-Gag conjugate (Lang-Gag), anti–CD207-Gag conjugate mixed with poly(I:C) (Lang-Gag + PIC). *p < 0.05, **p < 0.01. (D) Hemagglutination inhibition was measured in the serum of NHPs primed with influenza A/PR/8/34 virus and boosted with the anti–CD207-HA conjugate plus poly(I:C) (Lang-HA + PIC) or with Vaxigrip. (E) Gag-specific Ab responses (anti-Gag Ab) were compared with the Ab responses against the targeting component of the vaccine (anti-Ig Ab) in sera from NHPs immunized with the anti–CD207-Gag conjugate (Lang-Gag) or the anti–CD207-Gag conjugate mixed with poly(I:C) (Lang-Gag + PIC). Surface plasmon resonance assay was used to measure resonance units (RU) obtained with sera collected before the first vaccine injection (week −4), after the second injection (week 8), and after the third injection (week 17). Data are represented as the means ± SEM of groups of three to six animals. Vertical dotted lines indicate the injections (B and D).
after PBS injection ($p = 0.0156$ and $p = 0.0156$, respectively). No significant PMN infiltration was observed in PBS-injected skin, as shown by comparison with steady-state skin ($p = 0.6885$ and $p = 0.4246$ at 24 and 72 h, respectively). Poly(I:C) injection resulted in similar changes with respect to PBS injection ($4.19\% \pm 2.08\%$, $p = 0.0313$, at 24 h and $5.02\% \pm 2.89\%$, $p = 0.0313$, at 72 h). Macrophages also infiltrated the skin after injection. PBS alone induced a moderate but significant increase in the proportion of macrophages in the dermis with respect to untreated skin ($3.15\% \pm 1.36\%$, $p = 0.0020$, at 24 h and 3.77\% $\pm 2.49\%$, $p = 0.0253$, at 72 h). However, R848 injection strongly enhanced macrophage infiltration, as shown by comparison with PBS injection at 24 h ($8.41\% \pm 2.77\%$, $p = 0.0156$) and 72 h ($10.78\% \pm 5.69\%$, $p = 0.0313$). Similarly, poly(I:C) resulted in higher levels of macrophage infiltration at 24 h ($9.30\% \pm 5.73\%$, $p = 0.0156$) and 72 h ($8.62\% \pm 3.55\%$, $p = 0.0313$) than PBS injection (Fig. 4B). The decrease in LC density in the epidermis at 72 h was correlated with the recruitment of PMNs to the dermis 24 h after R848 ($p = 0.0002$) and poly(I:C) ($p = 0.0067$) injections. Furthermore, the recruitment of these inflammatory cells was associated with an enhanced expression of CD80, CD83, and CD86 surface markers 24 and 72 h after TLR-L injection. The relative expression levels of the activation/maturation markers after TLR agonist injection were compared with those after PBS injection. Data are expressed as the means $\pm$ SD of groups of four to eight animals. PIC, poly(I:C); *$p < 0.05$, **$p < 0.01$. 

**FIGURE 3.** LC responses to the i.d. injection of TLR-Ls. (A) PBS, R848, or poly(I:C) was injected i.d. into NHPs, and a skin biopsy was carried out 72 h later. Skin sections were stained with H&E. Scale bars, 50 $\mu$m. (B) Skin sites were biopsied 72 h after PBS, R848, or poly(I:C) injection, and frozen sections were stained with DAPI, anti-CD207, anti-CD1a, and anti-CD209 mAbs. Dotted lines indicate the frontiers between the epidermis (left) and dermis (right). Scale bars, 20 $\mu$m. (C) Cells were extracted from the epidermis, and LCs were identified on the basis of HLA-DR and CD1a expression. Skin biopsies from sites injected with R848 or poly(I:C) were compared with autologous sites injected with PBS. (D) The expression of CD207 and CD1a at the cell surface was analyzed in HLA-DR$^+$ CD1a$^+$ epidermis cells at 24 and 72 h postinjection, and the results obtained were compared between injection sites treated with PBS, R848, or poly(I:C). One representative experiment of eight for PBS, seven for R848, and five for poly(I:C) is shown. (E) The activation/maturation of LCs (HLA-DR$^+$ CD1a$^+$) in the epidermis was determined by assessing the expression levels of CD80, CD83, and CD86 surface markers 24 and 72 h after TLR-L injection. The relative expression levels of the activation/maturation markers after TLR agonist injection were compared with those after PBS injection. Data are expressed as the means $\pm$ SD of groups of four to eight animals. PIC, poly(I:C); *$p < 0.05$, **$p < 0.01$. 

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become involved in the inflammatory cascade triggering LC migration out of the skin. Indeed, R848-associated LC activation and migration appear paradoxical, as several previous studies have shown that these cells do not express TLR7 and TLR8 (26) or that they express these receptors only at very low levels (27). In addition, the direct incubation of epidermal cells extracted from NHP skin (containing keratinocytes and 0.5–1% LCs) with 10 μg/ml R848 did not increase the expression of LC activation/maturation markers (Fig. 5A). By contrast, supernatant from freshly isolated blood PMNs and monocytes/macrophages exposed to 10 μg/ml R848 induced significant levels of LC activation. These data support the hypothesis that both types of inflammatory cells may produce soluble factors responsible for LC activation and migration. Poly(I:C) did not directly activate NHP skin LCs ex vivo, but supernatants from monocytes stimulated with 10 μg/ml poly(I:C) did. However, supernatants from poly(I:C)-exposed PMNs did not appear to have a significant effect on LC activation, although these cells have been reported to express TLR3 intracellularly (26). This discrepancy could be dependent on the state of maturation at the time of treatment with poly(I:C), which did not allow full signaling for induction of activation markers in immature cells.

**FIGURE 4.** Modification of the dermal density of PMNs and macrophages induced by i.d. injection of TLR-Ls. (A) The frequency of PMNs (HLA-DR–CD66+) in dermal cell suspensions was analyzed for skin biopsy specimens collected 24 and 72 h after i.d. injection of PBS, R848, or poly(I:C). Biopsy specimens of R848- or poly(I:C)-injected skin were compared with autologous specimens of PBS-injected skin. (B) The frequency of macrophages (HLA-DR+ CD163+) in dermal cell suspensions was analyzed. Biopsy specimens of R848- or poly(I:C)-injected skin were compared with autologous specimens of PBS-injected skin. (C) The density of LCs in the epidermis 72 h after injection was plotted (upper panels) as a function of PMN (left column) or macrophage (right column) dermal frequency at 24 h. Similar plots were generated for the relative expression level of activation/maturation molecules on LCs 24 h after injection. PIC, poly(I:C); *p < 0.05.

**Tnf-α produced by PMNs and monocytes/macrophages activates LCs**

We analyzed the cytokines produced by granulocytes and monocytes exposed in vitro to R848 or poly(I:C). TNF-α, IL-1β, IL-6, IL-8, IL-12, MIP-1α, and MIP-1β were the most abundant factors in supernatants of R848-exposed PMNs and R848- or poly(I:C)-exposed monocytes (Fig. 5B). The lack of LC activation by supernatants from poly(I:C)-incubated PMNs was confirmed by the absence of cytokines into the corresponding supernatants. We then incubated epidermal cells with recombinant forms of each of these cytokines. Only stimulation with TNF-α resulted in the acquisition of a mature/activated profile by LCs ex vivo. Incubation with IL-1β, IL-6, IL-8, MIP-1α, or MIP-1β had only a moderate impact, if any, on LC activation (Fig. 5C). Moreover, the addition of etanercept (a soluble TNF-α receptor fused to an IgG1 fragment) to supernatants from R848-exposed PMNs and R848- or poly(I:C)-exposed monocytes/macrophages resulted in the complete abolition of LC activation. Altogether, these results indicate that TNF-α is an essential factor in the supernatants for LC activation and maturation. They also strongly suggest that TNF-α secreted by locally recruited PMNs and macrophages may be the
key factor in the inflammatory cascade activating LCs after i.d. injection of R848 or poly(I:C). We would therefore expect R848 and poly(I:C) to have similar effects in vivo, mediated by the secretion of TNF-α, because both PMNs and macrophages are recruited. We therefore injected R848 or poly(I:C) mixed with the indicated recombinant cytokines. Supernatants of TLR-L–exposed PMNs or monocytes were supplemented with etanercept (anti–TNF-α), and their levels of LC activation/maturation were then compared. Results are represented as the means ± SD of 3–30 experiments. ns, nonsignificant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

The data reported in this work highlight the relevance of targeting DCs to improve vaccine efficacy. To our knowledge, we provide the first demonstration, in a primate species highly relevant for the evaluation of human vaccines, that direct targeting to LCs is a very efficient strategy for increasing the immunogenicity of viral Ags. Significant specific Ab responses were obtained without the need for an adjuvant, but higher titers were obtained when the Ag fused to the anti-langerin Ab was cojected with poly(I:C).

The effects of direct Ag targeting to LCs suggest that this DC population plays a specific and active role in triggering the humoral response. Other studies have focused on the use of anti-lectin Abs to target DCs. The incubation of human DCs with anti–DCIR-fused Ag (28) leads to in vitro CD8+ T cell cross-priming and the secretion of Th1 cytokines. The addition of TLR7/8-L (CL075) enhanced cross-presentation and cross-priming. In mice (29), skin DCs targeted with a fusion protein consisting of anti–DEC-205 Ab effectively presented Ag to CD4+ and CD8+ T cells in vitro. An Ag fused to anti-CD207 Ab has been shown to induce IFN-γ–producing CD4+ and CD8+ T lymphocytes in vivo, in mice without coadministered adjuvant. However, directing Ag specifically to mouse LCs via CD207 Ab, rather than both resident mouse CD207+ DC populations, evoked Ag-specific CD4+, but not CD8+, T cell expansion (31). Influenza Ag targeted to mouse CD207+ DCs raised Ag-specific Ab responses without coadministered adjuvant (18), as did OVA targeted to mouse DCs via Clec9A (32). However, the efficiency of DC-targeted vaccine strategies in the absence of synthetic TLR-Ls coinjected as an immunostimulant had yet to be assessed in NHPs. A pioneering in vitro study of human LCs (12) suggested that LCs were involved in triggering the CTL response rather than in the humoral response. However, LCs may not be in the same state in vivo and may be influenced differently by the microenvironment. Furthermore, when LCs are targeted in vivo, the intracellular signal delivered via the membrane receptor may strongly influence the resulting immune response, as suggested for mouse LCs (29) and NHP DCs (7). Maximal specific responses were obtained after three injections of the recombinant vaccine. Although these repeated injections induced moderate responses against the targeting component of the vaccine, we cannot exclude that they might
LCs do not produce TLR7 or TLR8 mRNA (27) and do not respond directly to TLR7/8-Ls (26). It is therefore likely that other cell populations are locally activated by R848 and produce signals that then activate LCs. We observed a correlation between LC migration and the local recruitment of PMNs and macrophages, suggesting that these cells act as “go-betweens.” Moreover, skin LCs are activated by the supernatants of PMNs exposed to R848 or monocytes exposed to R848 or poly(LC), confirming the role of these inflammatory cells in the adjuvant effect of these two TLR-Ls.

This role of PMNs is consistent with their expression of TLR8 and TLR7 (36) and their recruitment and activation by R848 in humans (13, 37) and in NHPs (38). The coincidence of a decrease in LC density in the epidermis and the local recruitment of granulocytes has already been observed in response to other stimuli, during aminolevulinic acid–photodynamic therapy for example (39). Direct interplay between LCs and granulocytes has also been suggested by previous studies. Indeed, mast cell–deficient mice (Kit<sup>Wsh/Wsh</sup>) were shown to display lower levels of LC emigration from the epidermis following R837 local application than control mice (40). In the antitumor vaccine mouse model based on CD95L-overexpressing ex vivo generated DCs, massive PMN infiltration was observed at the injection site (41), and such infiltration was shown to be required for tumor regression. In vitro, PMNs could induce the activation of monocyte-derived DCs (42) by both soluble factors and cell-to-cell contact. These findings, combined with our data, provide strong support for the hypothesis that granulocytes are involved in the generation of vaccine-induced adaptive immune responses, through the delivery of activation signals to DCs (including LCs) at the immunization site. This implies that PMN activation is beneficial during the vaccination process and that adjuvants could be selected on the basis of their effect on PMNs.

The effect of poly(I:C) probably involves more complex interactions. Flacher et al. (26) reported that LCs were activated by poly(I:C), although this was not confirmed in our study or in previous works (43). Differences in TLR-L doses and in the origin and purity of the cell populations used may account for these discrepancies. Keratinocytes and inflammatory cells, such as macrophages that express TLR3 to significant levels, may play a predominant role in the adjuvant effect observed with poly(I:C) (43). Further evidence for the predominant role of macrophages is provided by the demonstration that poly(I:C) can activate macrophages in different ways, including TLR3-independent Mac-1 dependent activation of LCs leads to their migration (47). Alternatively, TLR-L injections may stimulate TNF-α production by keratinocytes (48). However, keratinocytes do not express TLR7 or TLR8 mRNA (27) and do not respond directly to TLR7/8-Ls (26). It is therefore likely that other cell populations are locally activated by R848 and produce signals that then activate LCs. We observed a correlation between LC migration and the local recruitment of PMNs and macrophages, suggesting that these cells act as “go-betweens.” Moreover, skin LCs are activated by the supernatants of PMNs exposed to R848 or monocytes exposed to R848 or poly(LC), confirming the role of these inflammatory cells in the adjuvant effect of these two TLR-Ls.

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The TNF-α produced by PMNs and macrophages seems to be a major factor in LC activation. We and others (46) have shown that the TNF-α–dependent activation of LCs leads to their migration. However, it may also activate dermal cells, such as fibroblasts, which, in turn, secrete CCL2 and CCL5, favoring LC migration (47). Alternatively, TLR-L injections may stimulate TNF-α production by keratinocytes (48). However, keratinocytes do not express TLR7/8 (49–51) and do not secrete cytokines when exposed to TLR7/8 ligands in vitro (52). Keratinocytes express TLR3 (43, 52), but they may nevertheless not be the main source of TNF-α in the inflammatory processes reported in this work, because we did not observe LC activation when total epidermal cells, consisting mostly of keratinocytes, were exposed to R848 or poly(I:C) ex vivo. Dermal DCs may also participate in the production of TNF-α, after i.d. injection of TLR-L. Indeed, the stimulation of these cells by poly(I:C) has been demonstrated to lead to the production of TNF-α (3). Nevertheless, the low frequency of the dermal DCs and the moderate amount of TNF-α decrease the efficacy of the boosts. Therefore, future development of this targeting strategy for preclinical and clinical studies would need to optimize the vector sequence to avoid unsuitable responses.
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produced suggest a minor contribution of these cells in the activation of LCs in comparison with PMN and macrophages in inflammatory conditions.

In conclusion, we demonstrate in this study that R848 and poly(I:C) activate LCs in vivo mostly indirectly, by activating innate immune cells (PMNs and macrophages). The secretion of TNF-α, a cytokine already known to potentiate vaccine-induced specific responses (53–55), plays a predominant role in this process. Finally, we also provide evidence that the specific targeting of Ags to LCs can increase vaccine efficacy. Such an approach, in combination with carefully designed adjuvant strategies, could be used to increase vaccine potency and safety, while minimizing the doses of vaccines and adjuvants required.

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Disclosures

J.B., G.Z., and S.O. are named inventors on a patent application from the Baylor Research Institute: Vaccines directed to Langerhans cells, United States patent application publication 20110081343 A1. J.B. and G.Z. are named inventors on a patent application from the Baylor Research Institute: HIV vaccine based on targeting maximized Gag and Nef to dendritic cells, United States patent application publication 20100135994A1. The other authors have no financial conflicts of interest.

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