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In Vivo Signaling through the Neurokinin 1 Receptor Favors Transgene Expression by Langerhans Cells and Promotes the Generation of Th1- and Tc1-Biased Immune Responses

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The proinflammatory capacities of the skin and the presence of high numbers of resident dendritic cells (DCs) constitute an ideal microenvironment for successful immunizations. Regardless of the ability of DCs to respond to local inflammatory signals in an immunostimulatory fashion, the immune functions of skin-resident DCs remain controversial, and epidermal Langerhans cells (LCs) have been referred to recently as anti-inflammatory/protolerogenic APCs. Substance P (SP), released by skin nerve fibers, is a potent proinflammatory neuropeptide that favors development of skin-associated cellular immunity. SP exerts its proinflammatory functions by binding with high affinity to the neurokinin 1 receptor (NK1R). In this study, we tested whether signaling skin cells via the NK1R promotes humoral and cellular immunity during skin genetic immunizations. We used the gene gun to deliver transgenic (tg) Ag to the skin of C57BL/6 mice and the selective NK1R agonist [Sar9Met(O2)11]-SP as a potential proinflammatory Th1-biasing adjuvant. Our strategy expressed tg Ag exclusively in the epidermis and induced a preferential migration of activated LCs to skin-draining lymph nodes. Local administration of the NK1R agonist during skin genetic immunizations increased significantly the expression of tg Ag by a mechanism involving the translocation of NF-κB into the nuclei of cutaneous DCs homing to skin-draining lymph nodes. Importantly, our immunization approach resulted in Th1 and T cytotoxic (CTL)-1 bias of effector T cells that supported cellular and Ab-mediated immune responses. We demonstrate that signaling skin cells via the NK1R provides the adjuvant effect which favors the immunostimulatory functions of LCs. The Journal of Immunology, 2007, 178: 7006–7017.

Efficient vaccine approaches for tumors and infectious diseases are designed to induce CD8+ T cytotoxic (CTL)-1 (Tc1)1 lymphocytes that are able to eliminate intracellular pathogens and tumor cells and CD4+ Th1 lymphocytes that support cellular immunity and CTL memory (1–6).

The most efficient immunization methods to generate Th1 and Tc1 immunity rely on the direct administration of Ag to the skin, a highly immunogenic organ populated by dendritic cells (DCs), including epidermal Langerhans cells (LCs), dermal DCs (DDCs), and a pool of DC precursors (7–9). It is currently believed that, as professional APCs, skin-resident DCs are extremely efficient at 1) providing the adjuvant effect which favors the immunostimulatory functions of LCs.

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Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from The Jackson Laboratory and used between the ages of 6 and 12 wk. Mice were housed under specific pathogen-free conditions and treated according to the University of Pittsburgh’s institutional animal care guidelines.

Genetic immunization

A He-powered (300 μJ) GG was used to deliver naked plasmid DNA (pDNA) encapsulated into 1-μm gold particles (Bio-Rad) to the skin of B6 mice, as described previously (25, 27). The following plasmids encoding reporter proteins or the model Ag chicken OVA under control of the human IE-CMV promoter were used in this study. We used the synthetic NK1R agonist [Sar{9}Met(O),{11}]-SP (NK1R agonist), a highly stable SP derivative that binds exclusively to the NK1R but not to NK2R or NK3R. We show that, after GG immunization, the expression of tg Ag was limited to the epidermis and sDLNs and that the expression of tg protein was increased significantly by simultaneous administration of the NK1R agonist. Mechanistic studies demonstrated that the NK1R agonist promoted acute inflammation in the skin, which correlated with rapid migration of epidermal LCs and their increased homing to sDLNs. Molecular analysis revealed that the NK1R agonist favored the activation of skin LCs as determined by the high expression of the cell surface I-A{b} molecules and the translocation of NF-κB into the nuclei of GG-transfected LCs homed to sDLNs. Importantly, our immunization approach stimulated efficient Ag-specific humoral, as well as Th1/Th1c-biased, and CTL responses that supported effector cellular immunity as demonstrated in vivo by DTH assays.

Immunofluorescence staining of skin samples and LNs

For assessment of LCs in epidermis, epidermal sheets were fixed in cold acetone (15 min at −20°C), washed in PBS, blocked with 10% normal goat serum in PBS, and incubated with biotin anti-DEC-205 mAb (clone NLDC-145; Cedarlane Laboratories), followed by Cy3-streptavidin (Jackson ImmunoResearch Laboratories). Epidermal sheets were mounted on slides with the dermal side up for microscopic evaluation.

For labeling of frozen tissue samples, samples of skin and LNs were embedded in Tissu-Tek OCT (Miles Laboratories), snap-frozen in prechilled methylbutane (Sigma-Aldrich), and stored at −80°C until use. Cryostat sections (8 μm) were mounted onto slides pretreated with Vectabond (Vector Laboratories), air-dried, and fixed in cold 96% ethanol (10 min). Tissue sections were blocked with 10% normal goat or donkey serum in PBS and then incubated with biotin anti-DEC-205 mAb (clone NLDC-145; Cedarlane Laboratories), followed by Cy3 anti-rabbit IgG and Cy3-streptavidin. For detection of CD11c and langerin in LNs, sections were incubated with biotin anti-CD11c (BD Biosciences) and Alexa Fluor 488 anti-langerin mAb (eBioscience), followed by Cy3-streptavidin. For detection of macrophages in skin, sections were incubated with Alexa Fluor 488 anti-CD4 mAb (Caltag Laboratories) and biotin anti-CD16/32 mAb (eBioscience), followed by Cy3-streptavidin. For detection of T cells in the skin, cross-sections were incubated with Alexa Fluor 488 anti-CD4 mAb (Caltag Laboratories) and biotin anti-CD8a mAb (eBioscience), followed by Cy3-streptavidin. For detection of macrophages in skin, sections were incubated with biotin anti-F4/80 mAb (Caltag Laboratories), followed by Cy3-streptavidin. Nuclei were stained with 4'6'-diamidino-2-phenylindole (DAPI) (Molecular Probes). To identify lymphatic vessels expressing the chemokine CCL21/SLC in LNs, sections of sDLNs were incubated with the lymphatic vessel-specific anti-LYVE-1 mAb and with biotin anti-CD11c/SLC (R&D Systems), followed by Cy3 anti-goat IgG and Cy2-streptavidin (Jackson ImmunoResearch Laboratories).

Flow cytometric analysis of single-cell suspensions

LC-enriched epidermal cell suspensions were blocked with 10% normal donkey serum and incubated (30 min, 4°C) with PE anti-CD11c mAb in combination with goat anti-mouse NK1R polyclonal Ab, recognizing the N terminus epitope of the human and mouse NK1R (Santa Cruz Biotechnolog), followed by FITC-conjugated donkey anti-goat IgG, F(ab')2 (Jackson ImmunoResearch Laboratories). Negative controls included cells incubated with goat serum, followed by FITC anti-goat IgG F(ab')2. LN cells were labeled with combinations of allophycocyanin anti-CD11c, PE anti-I{a}, FITC anti-CD11b mAbs, and Alexa Fluor 488 anti-langerin (CD207) polyclonal Ab. All Abs were purchased from BD Biosciences.
with the exception of langerin (eBioscience). Appropriate fluorochrome-conjugated species and isotype-matched irrelevant mAbs were used as negative controls. After staining, cells were fixed with 2% paraformaldehyde and analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems).

**ELISA and ELISPOT assay**

Analysis of OVA-specific total IgG was performed by ELISA, and the Th1 and Tc1 bias of OVA-specific CD4+ and CD8+ T cells were assessed by ELISPOT assay. For these experiments, mice were immunized on the abdominal skin with pCMV-OVA-TR (one priming plus two boosting doses, 7 days apart) in the presence (or not; control) of the NKIR agonist. For ELISA, 96-well EIA/RIA flat-bottom plates (Costar) were coated with 100 μg/ml OVA in a 0.05 M carbonate-bicarbonate buffer (Sigma-Aldrich) (overnight at 4°C). Then, plates were washed in PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich) and blocked with 3% BSA (w/v) (2 h at room temperature (RT)), and 100 μl of serially diluted serum samples or reference serum (diluted in 10% FBS) was added to the wells and incubated overnight at 4°C. The plates were then rinsed and incubated (1 h at RT) with 0.04 μg/ml biotin goat anti-mouse IgG (Caltag Laboratories) in 10% PBS/BSA. Plates were washed, and 100 μl of an Avidin-HRP (BD Pharmingen) solution diluted in 10% FBS/BSA was added to the wells (30 min at RT). After rinsing, the plates were developed with the tetramethylbenzidine system (Sigma-Aldrich). The reaction was stopped with 0.5 M H2SO4 and read at 450 nm on the Enmax microplate reader (Molecular Devices).

For ELISPOT assays, splenic CD4+ and CD8+ T cells (positively selected by magnetic sorting; Miltenyi Biotec) from B6 mice nonimmunized (control) or GG immunized with pCMV-OVA-TR (in the presence or absence of NKIR agonist) were used as responder cells. As stimulators, we used bone marrow-derived DCs (BMDCs) generated in vitro as described previously (39). Day 6 BMDCs were pulsed (2 h, 37°C) with either 1) 100 ng/ml, the H-2Kk-restricted OVA257-264 peptide (SIINFEKL), 2) 10 μg/ml, the IAg-restricted OVA233-241 peptide (ISQAVHAAHAEINEAGR), or 3) left untreated (control). BMDCs were then gamma irradiated and cocultured with 2 × 105 CD4+ or CD8+ responder T cells, respectively (1:5 stimulator:responder cell ratio) in 96-well ELISPOT plates precoated with anti-IFN-γ or anti-IL-5 mAb (BD Bioscences). ELISPOT plates were cultured for 36 h at 37°C, followed by incubation with biotin anti-IFN-γ or biotin anti-IL-5 mAb, streptavidin-peroxidase, and the substrate 3-amino-9-ethylcarbazole. Spots were quantified with an ImmunoSpot automated counter (Cellular Technology).

**In vivo killing assay**

B6 mice were GG immunized (abdominal skin) with pCMV-OVA-TR (one priming plus one or two boosting doses, 7 days apart) in the presence (or not; control) of the NKIR agonist. Nonimmunized mice were used as controls. Target B6 splenocytes were pulsed with SIINFEKL (0.25 μg/ml, 45 min, 37°C) and labeled with 5 μM CFSE (CFSE-bio). Control (nonpulsed) B6 splenocytes were labeled with 0.5 μM CFSE (CFSE-bio). Five days following the last boosting dose, target cells (CFSE-bio) were added to control cells (CFSE-bio nonpulsed). B6 spleen cells were injected i.v. (2 × 107 cells in 300 μl PBS/mouse; 1:1 target:control cell ratio) into preimmunized and control (untreated) mice. Four hours later, the relative percentage of splenic CFSE-bio and CFSE-low cells was assessed by flow cytometry. The percentage of specific cell lysis (%SCL) was then calculated by using the formula: %SCL = 100 × (1 – (CFSE-low:CFSE-bio of splenocytes from untreated mice)/(CFSE-low:CFSE-bio of splenocytes from immunized mice)) (40).

**DTH assay**

B6 mice were sensitized with the pCMV-OVA-TR delivered with the GG to the abdominal skin (one priming plus two boosting doses, 7 days apart). Sensitization was performed in the presence (or not; control) of the NKIR agonist or the nonpeptide NKIR antagonist L733606 (Torcs Bioscience), both used at an nmo/l/100 μl PBS/dose and administered i.d. locally immediately before immunization. Three days following the last immunization, mice were challenged with the pCMV-OVA-TR delivered with the GG to the dorsal surface of the right ear. Controls included mice sensitized with pCMV-Luc (irrelevant gene) and nonsensitized mice but GG transfected in the ear with pCMV-OVA-TR 24 h before ear measurement. The thickness of the right (challenged) and left ear pinna (control) was measured with a digital caliper (Mitutoyo) at different time points. The severity of the inflammatory response was assessed by the swelling of the challenged ear (right) compared with the thickness of the control ear (left) in sensitized animals and was expressed as percentage increase in ear thickness based on the following formula: (thickness of challenged ear – thickness of control ear/thickness of control ear × 100). In some experiments, mice were injected with the anti-CD154-blocking mAb MR1 (a single 250-μg dose; Bio Express) administered i.p. at the time of immunizations.

**Statistical analysis**

Results from multiple different groups were compared using a one-way ANOVA, followed by Tukey’s multiple comparison post hoc test. Comparison of two groups was performed by a two-tailed Student’s t test. A p value of <0.05 was considered statistically significant.

**Results**

**Immunization using the GG restricts expression of tg Ag to epidermis and sDLNs**

The individual contributions of LCs and DDCs to the outcome of cutaneous immunity remain controversial. Recent studies indicate that DDCs and LN-resident CD8α+ DCs are the main APCs that trigger T cell immunity following skin Ag insult, whereas epidermal LCs play tolerogenic/anti-inflammatory roles (32–37, 41–43). Therefore, we analyzed whether tg Ags encoded by pDNA and GG delivered to the skin (ear) of mice were expressed in the epidermis and/or in the dermis wherein LCs and DDCs reside, respectively. The anatomical localization of the 1-μm gold particles in the skin was investigated immediately after treatment with the GG. This strategy allowed us to analyze the original distribution of the particles in the skin before mobilization of epidermal LCs containing gold particles through the dermal lymphatic vessels. Histological analysis of cross-sections of skin demonstrated the presence of gold particles mainly in the epidermis, with very few beads at the epidermal-dermal junction; however, no particles were found in the dermis (Fig. 1A).

Next, we assessed the expression of the reporter protein EGFP in epidermis and dermis following delivery of pCMV-EGFP by the GG. Twenty-four hours after transfection, the expression of EGFP was detected exclusively in the epidermis (Fig. 1, B–D). This result was further confirmed by quantification of the reporter protein Luc in the whole skin, as well as in separated epidermal and dermal layers of mouse skin transfected by the GG with the pCMV-Luc or with a control plasmid encoding no transgene (pBackbone). High levels of Luc were detected in the cell lysates prepared from whole skin (Fig. 1E), as well as in those obtained from epidermal sheets (Fig. 1F). However, the levels of Luc in the dermis remained low and were similar to those detected in dermis from control mice transfected with pBackbone (Fig. 1F).

Our laboratory and others (24, 26, 44) have shown that treatment of the skin with the GG results in a rapid migration of cutaneous DCs to the sDLNs. As a consequence, reporter proteins encoded by pDNA delivered by the GG were detected in the sDLNs as soon as 24 h after cutaneous immunization (26). However, it is unknown for how long these tg proteins are being expressed in the skin and sDLNs, a relevant aspect for the purpose of genetic immunization. Thus, we delivered pCMV-Luc to abdominal skin of mice, and we analyzed the levels of expression of tg Luc in skin and inguinal (local) sDLN cells up to 7 days following cutaneous immunization by the GG. The levels and length of expression of Luc observed in the skin (Fig. 1G) correlated with the levels and length of Luc expression in the sDLNs (Fig. 1H). The highest expression of Luc in the sDLNs was observed 24 h after immunization and decreased but was still detectable up to 4 days (Fig. 1H). Luc expression in cervical sDLNs situated distant from the area of gene delivery remained low and was similar to background levels observed in sDLNs after transfection of the skin with pBackbone (negative control). Taken together, these results demonstrate that tg Ags delivered to the skin by the GG are expressed up to 3–4 days by cells of the epidermis and by migratory cells that traffic from the epidermis to sDLNs.
LCs and DDCs constitutively express NK1R

Our goal was to test whether the NK1R agonist \([\text{Sar}^9\text{Met(O2)}^{11}]\)-SP enhances the ability of skin DCs to promote the differentiation of Th1 and Tc1 cells following vaccination by the GG; therefore, we analyzed the expression of NK1R in skin cells from untreated mice. By double-immunofluorescence microscopy of cross-sections of skin and using CD11c as a murine DC-specific marker, we demonstrated that the NK1R was constitutively expressed by CD11c-positive epidermal LCs and DDCs, as well as by CD11c-negative cells, including keratinocytes, and some cells located in the dermis (likely, mast cells) (Fig. 2, A–F). Expression of NK1R on the surface of CD11c-positive LCs was further confirmed by flow cytometric analysis of epidermal cell suspensions enriched in LCs (Fig. 2G).

Signaling via NK1R triggers skin acute inflammation and enhances transgene expression

It is our hypothesis that signaling via the NK1R at the time of GG immunization triggers acute inflammation in the skin and therefore provides the appropriate “danger signal” to promote the activation and migration of epidermal LCs transporting tg Ags to the sDLNs. Therefore, we assessed the damage caused to the skin by the NK1R agonist or by the GG in combination (or not; control) with the NK1R agonist administered by local i.d. injection immediately before immunization. The NK1R agonist alone induced a moderate acute inflammatory infiltrate composed mainly of polymorphonuclear granulocytes (PMNs) in the dermis of treated mice (Fig. 3B). Although, fewer PMNs were observed infiltrating the epidermis and in the papillary dermis of mice treated with GG alone compared with untreated skin (Fig. 3, A and C). By contrast, immunization with the GG combined with the NK1R agonist triggered a severe and extensive PMN infiltrate in the epidermis and dermis of mouse skin (Fig. 3D).

Next, we tested whether administration of the NK1R agonist affects the level of expression of tg Ag in skin and sDLNs induced by the GG. To address this question, mice were transfected with pCMV-Luc in the skin (abdomen) with the GG in the presence (or not; control) of the NK1R agonist. Injection of the NK1R agonist in combination with the GG increased significantly the expression of Luc in the skin compared with the skin of mice treated with the GG alone (Fig. 3E), as determined by luminometry in cell lysates obtained from identical sized whole skin samples. Importantly, treatment with the GG and the NK1R agonist resulted in a 6-fold increase of Luc expression in the sDLNs compared with the expression observed in sDLNs obtained from mice treated with the GG alone (Fig. 3F). Luc expression in the sDLNs induced by the GG alone diminished significantly after local administration (i.d.) of the NK1R antagonist L733060. This latter result indicates that the GG exerts, to some extent, an intrinsic NK1R agonistic activity, likely through local release of endogenous SP in the skin.
Signaling via NK1R favors homing of activated LCs and expression of tg proteins in sDLNs

GG transfection of the skin triggers activation and migration of LCs to sDLNs (24, 26, 27). In response to DC activation signals, LCs translocate NF-κB into the nuclei, a phenomenon that triggers LC maturation and enhances the expression of those transgenes controlled by hIE-CMV promoter (39, 45, 46). Therefore, the augmented expression of tg proteins in the sDLNs that follows skin GG transfection with the NK1R agonist may be ascribed to a higher number of activated LCs expressing transgenes and mobilized
to the sDLNs. Thus, we analyzed 1) the mobilization of epidermal LCs to sDLNs, and 2) the expression of tg Luc in sDLNs dependent on NF-/H9260 B translocation by migrating LCs that follows skin GG immunization in the presence of the NK1R agonist.

First, we assessed whether the NK1R agonist decreases the number of LCs resident in the epidermis 24 h after i.d. injection in the presence or absence of GG transfections of the skin (Fig. 4, A–E). The NK1R agonist alone induced a significant decrease in the number of LCs in epidermal sheets compared with control skin samples (p < 0.001) (Fig. 4, A and B). GG transfections alone triggered a highly significant reduction of the density of LCs (>90%, from 1350 ± 90 to 110 ± 30 and LCs/mm² of skin; p < 0.0001) (Fig. 4C). As expected, this pronounced mobilization of epidermal LCs could not be further augmented by GG plus NK1R agonist treatments (Fig. 4, C–E).

Nevertheless, the fact that 90% of LCs diminished from the epidermis following GG treatments does not necessarily imply that all these cells will home efficiently to the sDLNs. Indeed, it has been shown that, after GG or i.d. administration of DC-based vaccines, only a small percentage of cutaneous DCs reach the local sDLNs (44, 47). The NK1R agonist is a proinflammatory neuropptide that, even when administered locally (i.d.), might elicit functional changes in the skin and sDLNs, which may enhance the “danger-induced” chemotaxis of skin LCs, a phenomenon that is highly relevant for the outcome of skin genetic immunizations. We hypothesized that the intense inflammation induced in the skin following GG immunizations in the presence of NK1R agonist might augment the number of lymphatic endothelial cells expressing the chemokines known to attract LCs to the sDLNs. Therefore, we analyzed the presence of lymphatic vessels (identified by the specific lymphatic endothelial cell marker LYVE) expressing the DC chemoattractant CCL21/SLC under our experimental conditions. Twenty-four hours following GG immunizations, we observed an expansion of subcapsular LYVE-1⁺ lymphatic vessels coexpressing CCL21/SLC throughout the whole parenchyma of the sDLNs (Fig. 4F). Importantly, administration of NK1R agonist together with the GG resulted in a significantly increased expansion of lymphatic vessels expressing CCL21/SLC compared with sDLNs isolated from naive mice (Fig. 4F). These results highly suggest that the combination of GG and the NK1R agonist promotes a more efficient homing of skin LCs to sDLNs by increasing the number of lymphatic endothelial cells expressing CCL21/SLC.

To test this hypothesis, we further addressed the homing of LCs to sDLNs under our experimental conditions. First, we analyzed the inflammatory changes induced by the GG and the NK1R agonist in sDLNs. As shown in Fig. 5, 24 h after skin treatment with the GG or the NK1R agonist, we observed sinus hyperplasia limited to the subcapsular and paracortical areas of sDLNs (Fig. 5, B and C). Following the combination of the GG with the NK1R agonist, the sinus hyperplasia replaced a high percentage of the normal histological architecture observed in sDLNs of untreated mice (Fig. 5, A and D). These changes were characterized by infiltration of the subcapsular and paracortical areas of the sDLNs with cells having abundant pale cytoplasm, some of them with indented peripheral nuclei and dendritic membrane processes (Fig. 5, B–D). Importantly, after GG treatment alone or in combination with NK1R agonist, several cells with DC morphology...
FIGURE 5. The NK1R agonist enhances the abilities of the GG to promote sDLN inflammation and homing of activated LCs. A–D. Structure of sDLNs (inguinal) excised from nontreated control mice (A) and from animals treated (24 h prior) with: the NK1R agonist (i.d.) (B), GG (pCMV-Luc) (C), or GG plus NK1R agonist (D). sDLNs from treated mice show sinus hyperplasia characterized by the presence of cells with abundant pale cytoplasm (some with morphological features of DCs indicated by arrows and detailed in the insets) located mainly within the subcortical and paracortical areas (dotted lines). Additionally, 1-μm gold beads were detected in the cytoplasm of DCs 24 h after GG treatments alone or in combination with the NK1R agonist (illustrated in insets of C and D and indicated by asterisks). H&E stain: magnification, ×400; insets, ×1000. E–H, Identification of the population of LCs coexpressing langerin (CD207 (green)) and CD11c (red) (arrows) in the paracortical areas of sDLNs (inguinal) excised from nonimmunized control mice (E) and from animals treated (24 h prior) with NK1R agonist (i.d.) (F), GG (pCMV-Luc) (G), or GG plus NK1R agonist (H). Insets show CD11c+ langerin+ LCs at higher magnification. Immunofluorescence: magnification, ×200; insets, ×1000. I. Quantification by flow cytometry of CD11c+CD11b+MHC-II (IAb)high,langerin+ epidermal LCs and CD11c+CD11b+MHC-II (IAb)high,langerin+ DDCs in the sDLNs (inguinal) of nonimmunized control mice and animals treated (24 h prior) with NK1R agonist (i.d.), GG (pCMV-Luc), or GG plus NK1R agonist. Data are representative of two independent experiments. J. Comparative analysis of tg Luc expression in sDLNs 24 h after GG delivery of pNFκB-Luc or pCMV-Luc in the presence (or not; control) of the NK1R agonist. Means ± 1 SD of the fold increase of RLU compared with background levels are illustrated. Three independent experiments were performed.

contained 1-μm gold beads, a further indication that these cells were mobilized from the skin (Fig. 5, C and D, insets).

A phenotypic analysis performed in sections of sDLNs, 24 and 48 h after treatments, demonstrated that the GG or the NK1R agonist by themselves increased significantly the population of DCs coexpressing CD11c (red) and langerin (CD207 (green)) situated in the subcapsular and in the paracortical areas of sDLNs compared with the population of CD11c and langerin double-positive cells present in the sDLNs of untreated animals (Fig. 5, E–H). A combination of GG and NK1R agonist further increased the number of CD11c+ and langerin double-positive cells in sDLNs compared with NK1R agonist or GG treatments administered alone (Fig. 5H). Few CD11c+ DCs expressing langerin were detected in control inguinal LNs of noninnunized mice or in mesenteric LNs (non-sDLNs) of mice immunized in the abdomen with GG plus the NK1R agonist (data not shown).

To further determine whether treatment with the NK1R agonist facilitates the homing of LCs and/or DDCs to sDLNs, we quantified by flow cytometry the percentage of both migratory skin DC subsets, LCs (CD11c+CD11b+MHC-II (IAb)high,langerin+) and DDCs (CD11c+CD11b+MHC-IIhighlangerin+) present in the sDLNs 24, 48, and 96 h following skin treatments. Based on previous publications, the following markers were used to identify LCs from DDCs and other DC populations homing to sDLNs (32, 36): 1) surface CD11c (a pan DC marker in mice); 2) surface CD11b (expressed by myeloid DCs and not by CD8α+ DCs); 3) surface MHC-IIhigh (DC activation marker highly expressed by skin derived DCs); and 4) intracellular langerin (CD207 (expressed by LCs and not by DDCs)). Skin migratory LCs, expressing CD11c, CD11b, MHC-IIhigh, and langerin were the main skin DC subset that increased in the sDLNs after GG immunization in the presence of the NK1R agonist (Fig. 5I). Langerin-negative skin migratory DDCs expressing CD11c, CD11b, and MHC-IIhigh were increased following GG treatments alone but reduced following GG plus NK1R agonist treatments. The high increase of LCs homing to sDLNs was observed 24 h after skin treatment and decreased gradually thereafter (48- and 96-h follow-up; data not shown). Additionally, a population of langerin+CD11b+ DCs were detected in the sDLNs (Fig. 5I), which was not affected by any treatment group. These cells likely represent the population of CD8α+ langerin+ LN resident DCs previously described as blood-derived langerin+ DCs (36). Taken together, these results indicate that the GG in combination with i.d. injections of the NK1R agonist preferentially favors the LN homing of activated
LCs, which are the only skin DC-subset expressing tg Ag under our experimental conditions.

To further confirm that the enhanced expression of transgenes observed was dependent on the activation state of LN-homing LCs, which have translocated NF-κB into their nuclei, we GG transfected the skin (abdomen) of mice with the plasmid pNFnB-Luc encoding Luc under control of a promoter containing upstream four tandem copies of the NF-κB consensus sequence. Mice were sacrificed 24 h later, and Luc assays were performed on sDLN cells. GG transfections of mouse skin i.d. injected with the NK1R agonist resulted in a 9-fold increase in NF-κB promoter activity in sDLNs, compared with Luc expression in sDLNs of control mice transfected with GG alone (Fig. 5J). This latter result further confirms that the addition of NK1R agonist to GG immunizations enhances transgene expression in sDLNs by favoring the arrival of LCs from skin transfected sites. These LCs were highly activated and consequently have translocated efficiently NF-κB into their cell nuclei.

**Signaling via NK1R enhances OVA-specific IgG production and secretion of IFN-γ by T cells stimulated by cutaneous GG immunizations**

Our results indicate that genetic immunizations of the skin with the GG in combination with the NK1R agonist 1) targets epidermal LCs, 2) increases the homing of epidermal LCs to the sDLNs, and 3) enhances the capability of migrating LCs to express tg proteins driven by promoters with NF-κB-responsive elements (i.e., CMVp). An important function of an adaptive immune response is the production of Ag-specific Igs, particularly relevant for the purpose of antiviral vaccines. Thus, we analyzed whether the NK1R agonist favors high levels of Ag-specific IgG. For this purpose, mice were immunized with the GG on the abdominal skin (one priming plus two boosting doses, 7 days apart) with pCMV-OVA-TR in the presence (or not; control) of NK1R agonist. Results are expressed as the means of spot-forming cells ± 1 SD of triplicates. Data are representative of three experiments. Insets are representative images of the IFN-γ ELISPOT wells. E. Ag-specific cytotoxic in vivo killing assays performed in B6 mice preimmunized with GG on the abdominal skin with pCMV-OVA-TR and one boosting performed in B6 mice preimmunized with GG on the abdominal skin with pCMV-OVA-TR and one boosting (Fig. 5J). This latter result further confirms that the enhanced expression of transgenes to promote the development of CD8 T cells secreting IFN-γ or IL-5 in response to restimulation with syngeneic BMDCs pulsed with either IAg2,323–339 or H-2Kb, OVA257–254-restricted peptides was measured by ELISPOT assays. The NK1R agonist significantly increased the number of IFN-γ-secreting CD4+ and CD8+ T cells when compared with T cells from mice immunized with GG alone (Fig. 6, C and D). Secretion of IL-5 remained at background levels in all groups (Fig. 6, C and D). As controls, splenic CD4+ and CD8+ T cells from untreated mice or mice immunized with the irrelevant pCMV-Luc did not secrete detectable levels of IFN-γ or IL-5 in response to restimulation (data not shown). Additionally, we tested whether the NK1R agonist augmented the capability of skin GG immunizations to promote the development of CD8+ effector T cells with...
cytotoxic function by in vivo killing assays. B6 mice were GG immunized on the abdominal skin with pCMV-OVA-TR as aforementioned in the presence (or not; control) of the NK1R agonist. Five days following the last immunization, target cells (CFSEhigh SIINFEKL-pulsed B6 splenocytes) and control cells (CFSElow nonpulsed B6 splenocytes) were adoptively transferred (i.v.) into immunized mice. Four hours later, the relative percentage of splenic CFSEhigh and CFSElow cells was assessed by flow cytometry. %SCL was then calculated by using the formula: %SCL = 100 × (1 − (CFSElow:CFSEhigh of splenocytes from untreated mice)/(CFSElow:CFSEhigh of splenocytes from immunized mice)).

Induction of robust CTL responses by genetic immunizations delivered with the GG requires an aggressive immunization scheme consisting of one priming dose, followed by two or more boosting doses (27). Using such an immunization protocol, under our experimental conditions, the GG induced a robust CTL function in vivo (as high as 79% of Ag-specific cell killing), which was augmented by combining GG with the NK1R agonist (as high as 92% of specific cell killing) (data not shown). To further determine whether the NK1R agonist adjuvant activity favors robust CTL responses during a suboptimal GG immunization protocol, we analyzed the Ag-specific CTL function induced in B6 mice immunized by one priming and one boosting dose in the presence or absence of NK1R agonist. Under these suboptimal immunization conditions, the NK1R agonist induced a significantly higher CTL response compared with GG immunizations alone (Fig. 6E), demonstrating that the NK1R agonist promotes both Th1 and CTL-Tc1 immune responses. Signaling via NK1R favors skin effector cellular immune responses

The potential of the NK1R agonist to promote differentiation of effector T cells under our experimental conditions was investigated by DTH assays. Mice were immunized on the abdomen with pCMV-OVA-TR delivered by GG immunizations in the presence (or not; control) of the NK1R agonist. Three days after the last immunization, DTH responses were elicited by GG delivery of pCMV-OVA-TR to the ear pinna. The effector cellular immune response was assessed by measuring the increase of ear thickness 24, 48, and 72 h following elicitation. Negative controls included
mice immunized with pCMV-Luc (irrelevant gene) and animals not sensitized but elicited with pCMV-OVA-TR 24 h before measuring of ear thickness. Immunization with the GG in the presence of the NK1R agonist increased significantly the intensity of the DTH reaction compared with GG immunizations alone (Fig. 7A). Interestingly, the DTH response elicited by the GG was almost completely abrogated by the NK1R antagonist L733060 administered i.d., indicating that, in the absence of exogenous [Sar9Met(O2)11]-SP, immunization of the skin with the GG stimulates the release of low levels of endogenous NK1R agonists (likely SP) (Fig. 7A). Likewise, blockade of CD40-CD40L interaction by the neutralizing Ab CD154 (MR1), injected i.p. before immunizations, abrogated completely the induction of DTH responses (data not shown). This result indicates the relevance of CD4+ Th cells for the effector cellular immunity induced by our immunization approach.

The characterization and severity of the cellular immune infiltrate of DTH responses were assessed in ear skin sections obtained 72 h after elicitation. Histological analysis demonstrated a dramatic increase in the thickness of the epidermis and dermis in those animals immunized with GG in the presence of NK1R agonist compared with GG alone or control groups (Fig. 7, B–E). Increase of skin thickness correlated with the presence of severe cellular infiltrates composed of mononuclear cells localized mainly in the epidermis where the tg Ag was expressed (Fig. 7E). Compared with control mice, GG alone induced significant mononuclear inflammatory infiltrate, which localized mainly in the dermis (Fig. 7, B–D). Phenotypic analysis by immunofluorescent microscopy demonstrated that the inflammatory infiltrate observed with GG in the presence or absence of the NK1R agonist was a Th1-mediated DTH response composed mainly of CD4+ and CD8+ T cells and macrophages (Fig. 7, F–K). Importantly, quantification of the inflammatory infiltrate demonstrated a significantly higher number of CD8+ T cells and macrophages in the skin of mice treated with GG and NK1R agonist compared with GG alone (Fig. 7L).

Discussion

Rational vaccine designs must induce long-lasting Th1- and Tc1-biased immunity, both critical for eradication of intracellular pathogens and tumor cells. Skin genetic immunizations have been proven to elicit potent T cell immune responses characterized by high levels of transgene expression combined with rapid activation and mobilization of cutaneous DCs. For genetic immunizations in vivo, delivery of pDNA is preferred over recombinant vectors, which provide the immune system with additional immune-dominant epitopes (39, 46, 48, 49). Using the GG as a method to deliver pDNA to the skin results in high, efficient transfection of epidermal cells. Importantly, our laboratory and others (24, 26, 27, 50) have demonstrated that cutaneous immunizations with the GG are followed by CTL responses that depend on the activation and migration of directly transfected DCs, as well as of DCs that have acquired tg proteins from neighboring cells. Nevertheless, regardless of the ability of the GG to promote CTL differentiation, the critical development of a Th1-biased immune response necessary to support cellular immunity and CTL memory after GG immunization is, to some extent, lacking (28–31).

In this study, we have investigated two of the main reasons by which cutaneous immunizations with the GG fail to induce efficient cellular immunity: 1) the possibility that the GG transfects mostly epidermal LCs that, unlike the DDCs, have been described recently as APCs with regulatory function (32, 36–38); and 2) the lack of or insufficient Th1-biases adjuvant effect.

Currently, the individual contributions of epidermal LCs and DDCs to the outcome of skin-related immune responses are controversial. Although, several studies have described LCs as potent APCs (51–56), recent observations propose a rather tolerogenic role for LCs. Using pathogenic infections or LC-depleted tg mice, recent studies implicate LCs as having an immune suppressive role, and they point toward DDCs and CD8α+ blood-derived DCs as the main inducers of efficient antiviral, allogeneic, and hypersensitivity immune responses (32, 36, 37, 41–43).

Different from these observations, our results demonstrate that GG immunization delivers pDNA to the epidermis and support the idea that epidermal LCs, instead of DDCs, are the main skin DCs transfected by the GG. Epidermal LCs transfected with tg Ag migrated rapidly to the sDLNs, as shown by the presence of DCs containing gold particles and by the expression of the intact tg proteins in the inguinal LNs of mice GG-immunized on the abdomen 24 h earlier. The possibility that the tg protein expression observed in sDLNs may have been caused by free naked DNA or secreted tg protein mobilizing from the skin via lymph is very unlikely. The GG delivers pDNA either into the cytoplasm/nucleus of cells or in the extracellular compartment. pDNA delivered to the extracellular compartment is highly unstable and becomes degraded by tissue DNases soon after being released (57, 58). Under our experimental conditions, the possibility that tg protein secreted by transfected keratinocytes could have reached the sDLNs has been excluded because tg Luc remains intracellular and cervical sDLNs distant from the skin transfected area did not express tg proteins.

Our results demonstrated that, following a rather aggressive protocol of GG immunizations, targeting exclusively the epidermis induced a robust CTL response accompanied by a weak Th1 biasing of CD4+ T cells, an indication that under our experimental conditions LCs are immunostimulatory rather than tolerogenic APCs. Thus, the second question addressed was whether a potent proinflammatory Th1-biasing mediator administered during GG immunizations enhances the ability of LCs to generate/amplify Ag-specific CD4+ Th1 responder cells and the CTL function of CD8+ Tc1 cells. We chose the proinflammatory neuropeptide NK1R agonist [Sar9Met(O2)11]-SP as a Th1-Tc1-biasing adjuvant based on the fact that signaling T cells via the NK1R favors Th1-biased responses and local injection of NK1R agonist prevents the tolerogenic effects exerted by irradiation of the skin with UV-B light (23). In addition, the NK1R agonist mimics the endogenous secretion of SP during the initial stages of acute inflammation of the skin (59, 60). Likewise, our results demonstrated that the NK1R agonist increased the ability of the GG to trigger acute inflammation of the skin at the site of immunization and favored a more effective homing of activated epidermal LCs to sDLNs.

Although the proinflammatory effects that follow cutaneous administration of the NK1R agonist can be attributed to the secretion of mediators by mast cells and keratinocytes as previously described (61, 62), other reports (63) have shown that murine DCs in secondary lymphoid organs and commercially available human DCs express NK1R. Importantly, our data demonstrate for the first time constitutive expression of the NK1R by murine LCs, indicating that signaling LCs directly via the NK1R may affect their APC function and ability to express transgenes. Accordingly, neuropeptides such as SP and calcitonin gene-related peptide, secreted by cutaneous δ fibers, exert important immune-modulation of LCs (13) and previous reports (23, 64) have shown that the NK1R agonist down-regulates the migratory function of LCs. However, our results clearly indicate that the NK1R agonist triggers a massive and rapid LC mobilization of the epidermis. These discrepancies can be ascribed to the use of different experimental models and time points analyzed. Nevertheless, in our studies, we should not overlook that besides epidermal LCs, keratinocytes and skin-resident leucocytes (i.e., mast cells, DDCs) might also be direct
targets of the NKIR agonist. In this scenario, the beneficial effects of the NKIR agonist on GG immunizations might be the result of the release of inflammatory mediators by bystander cells combined to the direct signaling of LCs during the initiation of the acute skin inflammatory response.

The observation that local administration of the NKIR antagonist L733060 decreased the ability of the GG immunization to induce the expression of transgenes in skin and sDLNs and to promote a potent DTH response against the tg Ags indicates that the GG treatment by itself releases endogenous NKIR agonists, likely SP. However, the levels of endogenous NKIR agonist released by GG immunizations alone were not sufficient to trigger the inflammatory changes observed in the skin and sDLNs after administration of the NKIR agonist alone or in combination with the GG. Accordingly, administration of exogenous NKIR agonist induced the highest expression of transgenes driven by transpro- 
motors containing NF-kB responsive elements (i.e., CMV promoter) by migrating LCs (39, 45). This phenomenon was due to a combination of the following factors: 1) a high level of activation of LCs, which have translocated NF-kB into their nuclei; and 2) an efficient homing of LCs to sDLNs. The rapid homing of LCs to the sDLNs observed here is in contrast to previous reports, describing a slow migration of LCs even 96 h after treatment of the skin with an irritant and the irritant tetramethylrhodamine-5-(and 6)-isothiocyanate (36). In this regard, we have previously shown that the treatment of the skin with the GG induces a rapid and massive mobilization of human epidermal LCs that begins as soon as 3 h following GG treatment (24). Accordingly, Porgador et al. (26) have demonstrated homing of directly transfected DCs expressing tg reporter proteins in sDLNs 24 h following GG immunization of mouse skin. Moreover, this study shows that the proinflammatory effects of the NKIR agonist combined with the GG induced immuno- logically relevant changes in sDLNs, as demonstrated by significant expansion of lymphatic vessels expressing CCL21/SLC and by extensive sinus hyperplasia. Thus, we can conclude that the rapid mobilization of LCs from the epidermis to sDLNs observed in our study was caused by a combination of factors, including the damage triggered by the GG in the skin and the proinflammatory effects exerted by the NKIR agonist in the skin and sDLNs. In agreement with our data, a recent report (65) has demonstrated a higher DC mobilization from the periphery into the sDLNs follow- ing inflammatory expansion of lymphatic vessels after skin treatments with keyhole limpet hemocyanin in the presence of CFA.

The NKIR agonist neuropeptide [Sar^9Met (O^2)_{3}]-SP seems to represent a safe adjuvant to promote Th1-Tc1-mediated cell immunity. Our results strongly support the concept that, under the appropriate proinflammatory environment, epidermal LCs are capable of eliciting potent T cell immune responses, which can be exploited for vaccine development.

Disclosures

The authors have no financial conflict of interest.

References