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

Alicia R. Mathers, Olga A. Tckacheva, Brian M. Janelsins, William J. Shufesky, Adrian E. Morelli and Adriana T. Larregina

J Immunol 2007; 178:7006-7017; doi: 10.4049/jimmunol.178.11.7006
http://www.jimmunol.org/content/178/11/7006

References This article cites 62 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/178/11/7006.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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

Alicia R. Mathers,§† Olga A. Tckacheva,*† Brian M. Janelins,*† William J. Shufesky,‡§ Adrian E. Morelli,§§ and Adriana T. Larregina2*†

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) sensing the presence of proinflammatory mediators in their microenvironment, 2) acquiring/process extracellular Ag, 3) transporting Ag from the skin to the skin-draining lymph nodes (sDLNs), and 4) priming and biasing the differentiation of naive CD4+ and CD8+ T cells into effector cells (8, 10, 11).

In addition to their role during the initiation of acquired immunity, the skin cells and nerve endings release proinflammatory mediators, including cytokines, hormones, and neuropeptides, which influence the immunostimulatory function of resident DCs (10, 12). Proinflammatory neuropeptides from the innate immune system are released immediately following skin injury. Interestingly, epidermal LCs make physical contact with peripheral nerve δ fibers that secrete substance P (SP) and calcitonin gene-related peptide (13, 14).

SP and the more recently described hemokinin-1 belong to the family of proinflammatory neuropeptides known as tachykinins. SP and hemokinin-1 bind with high affinity the neurokinin 1 receptor (NK1R) and with low affinity the NK2R and NK3R (15–18). Binding of the NK1R on naive CD4+ T cells promotes proliferation, Th1 differentiation, and cellular immunity, whereas binding the NK2R inhibits the development of inflammatory responses (15, 19–22). In the skin, signaling through the NK1R with SP or NK1R agonists prevents cutaneous tolerance induced by UV-B light and favors delayed-type hypersensitivity (DTH) reactions through the release of IL-12p70, a potent Th1-biasing cytokine (19, 23). Taken together, these observations highly suggest that signaling via the NK1R during the initiation of skin-mediated immune responses will favor development of Th1 effector immunity.

Due to the accessibility of the skin immune system and the ease of targeting the epidermis, genetic immunizations by the gene gun...
(GG) have been used as an appropriate vaccination method to promote the generation of CTL immunity directed to transgenic (tg) Ag (24–27). However, there is increasing evidence that skin GG immunizations administered without Th1-biasing adjuvants generates Th2 effector cells, which are unable to sustain cellular immunity and CTL memory (6, 28–31). The absence of Th1 responses following skin GG immunizations can be ascribed to either: 1) lacking of a Th1-biasing adjuvant effect; or 2) delivery of tg Ag almost exclusively to epidermal LCs, which have been described recently as tolerogenic/anti-inflammatory skin DCs (32–35). Thus, for the purpose of skin genetic immunizations, it would be relevant to determine whether: 1) administration of proinflammatory Th1-Tc1-biasing adjuvants provides LCs with the appropriate stimulus to favor effector cellular immunity during GG immunizations; or 2) genetic immunizations should target exclusively DCs, described as the most potent skin resident APCs (36–38).

In this study, we tested whether local administration of an NKIR agonist provides a Th1 adjuvant effect to favor the ability of skin DCs to induce Th1-Tc1 immunity during skin GG immunizations. We used the synthetic NKIR agonist [Sar"/Met(O)3]1-SP (NKIR agonist), a highly stable SP derivative that binds exclusively to the NKIR 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 NKIR agonist. Mechanistic studies demonstrated that the NKIR 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 NKIR agonist favored the activation of skin LCs as determined by the high expression of cell surface I-Aβ 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/Tc1-biased, and CTL responses that supported effector cellular immunity as demonstrated in vivo by DTH assays.

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 µ) GG was used to deliver naked plasmid DNA (pDNA) precipitated onto 1-µm gold particles (Bio-Rad) to the skin of B6 mice, as described previously (27). The following plasmids encoding reporter proteins or the model Ag chicken OVA under control of the human IE-CMV promoter used in this study have been previously described (39): 1) pCMV-Luc encoding firefly luciferase (Luc); 2) pCMV-EGFP encoding the enhanced version of the GFP (EGFP); and 3) pCMV-OVA-TR encoding Luc downstream of four tandem copies of the NF-κB consensus promoter, fused to a TATA-like promoter region from the HSV thymidine kinase promoter. After incubation, the epidermis was dissected from the dermis with fine forceps under a microscopic examination. Unlike enzymatic digestions, EDTA treatment of the skin allows a clean separation of the epidermal dermal junction and allows the purification of LCs without contamination with DDCs. To obtain epidermal single-cell suspensions, the epidermis was dissected as described and incubated with 0.25% trypsin/EDTA (Invitrogen Life Technologies) for 15 min at 37°C with continuous shaking. Trypsin activity was neutralized with PBS, and single-cell suspensions were obtained by vigorous pipetting (4°C), followed by filtration through a 70-µm cell strainer (Falcon; BD Diagnostic Systems). Single-cell suspensions from LNs were prepared by teasing apart the tissue with 18-gauge needles, followed by incubation in 1 mg/ml collagenase (Sigma-Aldrich) and 0.02 mg/ml DNase I (Roche) for 30 min at RT. Further cellular LN dissociation was accomplished by adding 1 M EDTA during the last 5 min of incubation, followed by filtration through a 70-µm cell strainer (Falcon; BD Biosciences).

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 tissues, samples of skin and LNs were embedded in Tissue-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 the avidin/biotin blocking kit (Vector Laboratories). For codetection of CD11c and NKIR in skin, cross-sections of mouse ear skin were incubated with biotin anti-CD11c mAb (BD Biosciences Pharmingen) and rabbit anti-mouse NKIR polyclonal Ab (Advance Targeting Systems), followed by Cy2 anti-rabbit IgG and Cy3-streptavidin. For codetection of CD11c and langerin in LNs, sections were incubated with biotin anti-CD11c (BD Pharmingen) and Alexa Fluor 488 anti-langerin 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-CD8α 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 2HCl (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 (BD Pharmingen), 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 NKIR polyclonal Ab, recognizing the N terminus epitope of the human and mouse NKIR (Santa Cruz Biotechnology), 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-IAα, FITC anti-CD11b mAbs, and Alexa Fluor 488 anti-langerin (CD207) polyclonal Ab. All Abs were purchased from BD Biosciences Pharmingen.
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 GG immunized on the abdominal skin with pCMV-OVA-TR (one priming plus two boosting doses, 7 days apart) in the presence (or not) of control (the NK1R 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/PBS. Plates were washed, and 100 μl of an Avidin-HRP (BD Pharmingen) solution diluted in 10% FBS/PBS 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 Emax 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 NK1R 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-2Kb-restricted OVA257–264 peptide (SIINFEKL), 2) 10 μg/ml, the Ia+-restricted OVA123–139 peptide (ISQAVHAAHAEINEAGR), or 3) left untreated (control), BMDCs were then gamma irradiated and cocultured with 2 × 10^5 CD4+ or CD8+ responder T cells (respectively) with or without the NK1R agonist or the nonpeptide NK1R antagonist L733060 (Tocris Bioscience), as well as with the exception of langerin (eBioscience). Appropriate fluorochrome-conjugated species and isotype-matched irrelevant mAbs were used as negative controls.

For ELISPOT, 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 NK1R 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-2Kb-restricted OVA257–264 peptide (SIINFEKL), 2) 10 μg/ml, the Ia+-restricted OVA123–139 peptide (ISQAVHAAHAEINEAGR), or 3) left untreated (control), BMDCs were then gamma irradiated and cocultured with 2 × 10^5 CD4+ or CD8+ responder T cells (respectively) with or without the NK1R agonist or the nonpeptide NK1R antagonist L733060 (Tocris Bioscience), as well as with the exception of langerin (eBioscience). Appropriate fluorochrome-conjugated species and isotype-matched irrelevant mAbs were used as negative controls.

In vivo killing assay

B6 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 (CFSEblue). Control (nonpulsed) B6 splenocytes were labeled with 0.5 μM CFSE (CFSEred). Five days following the last boosting dose, target cells (CFSEblue control cells) were flow-sorted, B6 splenocytes were injected i.v. (2 × 10^7 cells in 300 μl PBS/mouse, 1:1 target:control cell ratio) into immunized and killed (untreated) mice. Four hours later, the relative percentage of splenic CFSEblue and CFSEred cells was assessed by flow cytometry. The percentage of specific cell lysis (%SCL) was then calculated by the formula: %SCL = 100 × (1 − (CFSEred: CFSEblue of splenocytes from untreated mice)/(CFSEblue: CFSEred of splenocytes from immunized mice)).

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) of control (the NK1R agonist or the nonpeptide NK1R antagonist L733060 (Tocris Bioscience), both at 10 mmol/100 μl PBS/dose and administered i.d. locally immediately before sensitization. Three days following the last sensitization, 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 DTH 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 posthoc 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

Immunoassay using the GG restricts expression oftg 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). The 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 (p Backbone). 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 p Backbone (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 p Backbone (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 [Sar9Met(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 sDLN migration.

FIGURE 2. Skin DCs constitutively express the NK1R. A–C and D–F are cross-sections of skin (ear) from two independent experiments showing the expression of the NK1R (green) by keratinocytes, epidermal LCs (arrowheads), and DDCs (arrows) (the latter two identified by their expression of CD11c in red). C and F. The yellow fluorescence is due to the overlap of red (CD11c) and green (NK1R). Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (blue). Immunofluorescence, magnification: ×200. G. The green histogram demonstrates the expression of NK1R by freshly isolated LCs gated on CD11c expression. The gray histogram corresponds to negative control cells. The numbers in the histogram represent the percentage of NK1R-positive LCs and the mean fluorescent intensity (between parentheses). Data are representative of two independent experiments.

FIGURE 3. GG immunizations with the NK1R agonist triggers skin acute inflammation and increases the expression of tg Ag. A–D. Cross-sections of skin (abdomen) obtained from mice untreated (A) and experimental animals 24 h after administration (i.d.) of the NK1R agonist (B), immunization with GG (C), or treatment with GG plus NK1R agonist (D). The arrows indicate the localization of the acute cellular infiltrate composed mainly of PMN cells. The insets detail the characteristics of the acute inflammatory infiltrate. H&E staining: magnification, ×200; insets, ×1000. E and F. Expression of tg Luc in the skin (E) and sDLNs (F) 24 h following GG delivery of pCMV-Luc in the presence (or not; control) of the NK1R agonist or the NK1R antagonist. Data are representative of four independent experiments. E and F. Results are expressed as mean RLU ± 1 SD of the fold increase compared with background levels obtained from nonimmunized control mice. N.D., nondetected.
to the sDLNs. Thus, we analyzed 1) the mobilization of epidermal LCs to sDLNs, and 2) the expression of tGLuc in sDLNs dependent on NF-H9260B 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 neuropeptide 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). These results highly suggest that the combination of GG and the NK1R agonist promotes the 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 4. Effects of skin genetic immunizations with the GG and the NK1R agonist on migration of epidermal LCs. A–D, Distribution of DEC-205⁺ LCs (in red) in epidermal sheets (ear) obtained from nonimmunized 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). The few LCs remaining in the epidermis after treatment became round-shaped (arrows), a feature indicative of LC migration. Immunofluorescence: magnification, ×200. E, Quantification of the density of DEC-205⁺ LCs in epidermal sheets (ear) 24 h posttreatment. Data represent the means ± 1 SD from three independent experiments performed. F, Comparison of the expression of LYVE-1 (red) and the chemokine CCL21/SLC (green) in the sDLNs 24 h following the indicated treatments. Immunofluorescence: magnification, ×100.
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 subcapsular 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 (IAβ)lowlangerin+ epidermal LCs and CD11c+CD11b+ MHC-II (IAβ)highlangerin+ 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 nonimmunized 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 (IAβ)highlangerin+) and DDCs (CD11c+CD11b+ MHC-II (IAβ)highlangerin+) 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-II (IAβ)high (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-II (IAβ)high, 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-II (IAβ)high 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 pNFκB-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 sDLNs 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 the 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 dose performed in B6 mice preimmunized with GG on the peritoneum and one boosting dose 7 days apart. GG immunizations alone increased the production of OVA-specific IgG (detected at a dilution of 1/1,000) (Fig. 6, A and B), whereas the administration of the NK1R agonist during GG immunizations significantly increased the production of OVA-specific IgG (detected at a dilution of 1/16,000) (Fig. 6, A and B).

To further analyze the adjuvant effects of the NK1R agonist, we investigated its ability to promote development of Th1- and Tc1-biased immunity following GG immunizations. Five days after the second boost, the frequency of purified splenic CD4+ and CD8+ T cells secreting IFN-γ or IL-5 in response to restimulation with syngeneic BMDCs pulsed with either IAg1-OVA123–139- or H-2Kb-OVA257–264-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 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 [Sar^9Met^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. 7). 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.

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-biasing 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 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^+ Th responder cells and the CTL function of CD8^+ Tc1 cells. We chose the proinflammatory neuropeptide NK1R agonist [Sar^9Met^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 leukocytes (i.e., mast cells, DDCs) might also be direct...
targets of the NK1R agonist. In this scenario, the beneficial effects of the NK1R 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 NK1R 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 NK1R agonists, likely SP. However, the levels of endogenous NK1R agonist released by GG immunizations alone were not sufficient to trigger the inflammatory changes observed in the skin and sDLNs after administration of the NK1R agonist alone or in combination with the GG. Accordingly, administration of exogenous NK1R agonist induced the highest expression of transgenes driven by transpromoters containing NF-κB 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-κB 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 potent allergens transglutaminase 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 NK1R agonist combined with the GG induced immunologically 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 NK1R 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 following inflammatory expansion of lymphatic vessels after skin treatments with keyhole limpet hemocyanin in the presence of CFA.

The NK1R agonist neuropeptide [Sar³Met(O²)¹]-SP meets the requirements for a Th1-Tc1-biasing adjuvant while also favoring the elicitation of Ab responses. Our data demonstrate that GG immunizations induced the secretion of Ag-specific IgG, as well as cellular immunity mediated by CD8⁺ and CD4⁺ T cells. Importantly, these immunological responses were greatly increased by the presence of NK1R agonist as demonstrated by the significantly higher number of IFN-γ-secreting Ag-specific CD⁴⁺ Th1 and CD⁸⁺ Tc1 cells. Moreover, the NK1R agonist was able to trigger strong CTL function of CD⁸⁺ T cells in vivo following a suboptimal GG immunization protocol. Importantly, the adjuvant effect of the NK1R agonist further tested by DTH assays demonstrated that local administration of the NK1R agonist during sensitization favored the generation of Th1 effector immunity, an effect confirmed by the intense cutaneous infiltrate of CD⁸⁺ T cells and macrophages during the elicitation phase.

To our knowledge, this is the first study that has tested the ability of a NK1R agonist to enhance the generation of Ag-specific humoral and cellular Th1 and Tc1 immune responses elicited by GG immunizations of the skin. The synergistic effect of the NK1R agonist on cutaneous GG immunization was mediated, at least in part, by inducing inflammatory changes in the skin and sDLNs. The use of the synthetic NK1Ra such as [Sar³Met(O²)¹]-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


