Laser-Assisted Intradermal Delivery of Adjuvant-Free Vaccines Targeting XCR1 + Dendritic Cells Induces Potent Antitumoral Responses

Dorothea Terhorst, Even Fossum, Anna Baranska, Samira Tamoutounour, Camille Malosse, Mattia Garbani, Reinhard Braun, Elmira Lechat, Reto Crameri, Bjarne Bogen, Sandrine Henri and Bernard Malissen

*J Immunol* 2015; 194:5895-5902; Prepublished online 4 May 2015;
doi: 10.4049/jimmunol.1500564
http://www.jimmunol.org/content/194/12/5895

Supplementary Material  http://www.jimmunol.org/content/suppl/2015/05/01/jimmunol.1500564.DCSupplemental

References  This article cites 47 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/194/12/5895.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

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Laser-Assisted Intradermal Delivery of Adjuvant-Free Vaccines Targeting XCR1⁺ Dendritic Cells Induces Potent Antitumoral Responses

Dorothea Terhorst,*,†,‡,§,¶ Even Fossum,*,¶ Anna Baranska,*,†,‡ Samira Tamoutounour,*,†,‡ Camille Malosse,*,†,‡ Mattia Garbani,‖ Reinhard Braun,# Elmira Lechat,# Reto Crameri,‖ Bjarne Bogen,*,‡,‡,*** Sandrine Henri,*,†,‡,2 and Bernard Malissen*,†,‡,2

The Journal of Immunology, 2015, 194: 5895–5902.

Dendritic cells (DCs) capture Ags in body barriers and migrate to lymph nodes (LNs), where they trigger the differentiation of Ag-specific, naive T cells into effector T cells. Recent studies, based on ontogeny and global gene-expression profiles, showed that a few DC subsets exist in the mouse. Functional specialization probably accounts for their existence, and they can be aligned with functionally equivalent human subsets (1–4). The XC-chemokine receptor 1 (XCR1) binds to a single ligand known as XCL1 and is expressed by a DC subset that was characterized previously by the expression of CD8α, CD207, or CD103 (5–7). Mouse XCR1⁺ DCs excel in cross-presentation of self-Ags (8, 9) and dead tumor cell–associated Ags, a feature that is probably owing to their expression of the C-type lectin CLEC9A (also known as DNGr1), a receptor for damaged and apoptotic cell materials (10, 11). Targeting Ags to DCs has been used successfully to generate strong immune responses and recently entered clinical trials (12). For instance, when delivered i.v. in the presence of adjuvant, Ags chemically or genetically conjugated to CLEC9A Ab or to XCL1 elicited potent cytotoxic CD8⁺ T cell responses capable of destroying tumors (13–17). Owing to its high numbers of DCs, the skin is a particularly attractive site for vaccine administration. However, for ease of application, most of the skin vaccines that are used in mass-vaccination programs are not delivered into the epidermis or the dermis but into the hypodermis, which is a layer of fat and connective tissue just below the dermis. Several delivery systems were developed recently to exploit the potential of dermal DCs. For example, vaccination with microneedles, made from a biocompatible polymer, has been used to introduce influenza virus vaccine (18) or live recombinant human adenovirus type 5 (19) into the dermis. These approaches generated robust humoral and cellular immune responses. A portable laser, the Precise Laser Epidermal System (P.L.E.A.S.E.), was also used to create micropores in the stratum

*Centre d’Immunologie de Marseille-Luminy, UM2 Aix-Marseille Université, 13288 Marseille Cedex 9, France; †INSERM U1104, 13288 Marseille Cedex 9, France; ‡Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7280, 13288 Marseille Cedex 9, France; †Department of Dermatology, Charité University Medicine, 10117 Berlin, Germany; ‡K.G. Jøbsen Centre for Influenza Vaccine Research, University of Oslo, Oslo 0424, Norway; §Department of Molecular Allergology, Swiss Institute of Allergy and Asthma Research, University of Zürich, Davos 7270, Switzerland; ¶Pancee Biosolutions, 9491 Ruggell, Liechtenstein; and ‡Center for Immune Regulation, Institute of Immunology, University of Oslo and Oslo University Hospital Rikshospitalet, Oslo 0424 Norway

D.T. and E.F. contributed equally to this work.

S.H. and B.M. contributed equally to this work.

Received for publication March 10, 2015. Accepted for publication April 15, 2015. This work was supported by institutional funding from Centre National de la Recherche Scientifique and INSERM, grants from European Communities Framework Program 7 (NANOSIF Euronanom Project and EE-ASI European Collaborative Research Project to B.M.), European Research Council (Grant 2012-AdG no. 322465 to B.M.), Agence Nationale de la Recherche (Skin-Dendritic Cells to S.H. and Anti-Bacterial Immune Regulation and DCBiol Labex to B.M.), and the Swiss National Science Foundation (to B.M). The Innate Immunocytes in Health and Diseases Centre d’Immuno-immunomed de Marseille-Luminy–SANOFI collaborative project (to D.T.), MASTERSWITCH (to S.H.), and Anti-Bacterial Immune Regulation (to A.B.), the Norwegian Research Council (GLOBVAC, to E.F.), and the K.G. Jøbsen Centre for Influenza Vaccine Research (to B.B.). Address correspondence and reprint requests to Dr. Bernard Malissen and Dr. Sandrine Henri, Centre d’Immuno-immunomed de Marseille-Luminy, Parc Scientifique de Luminy, 13288 Marseille Cedex 9, France. E-mail addresses: bernardm@cinmi.univ-mrs.fr (B.M.) and henri@cinmi.univ-mrs.fr (S.H.)

The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/J6; CTV, CellTrace Violet; DC, dendritic cell; LC, Langerhans cell; LN, lymph node; mDC, monocyte-derived DC; P.L.E.A.S.E., Precise Laser Epidermal System; STING, stimulator of IFN genes; XCR1, XC-chemokine receptor 1.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/525/00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500564
corneum, the superficial impermeable layer of the skin, and the epidermis, allowing topicaly applied Ags to diffuse into the dermis and to induce potent immune responses (20).

Vaccibodies are homodimeric chimeric proteins consisting of a targeting unit, a hinge domain, and an Ag moiety (21). XCL1-based vaccibodies were developed recently to target Ags to XCR1+ DCs (22). When used in a DNA format, flu hemagglutinin–containing vaccibodies improved CD8+ T cell responses and protected mice against a lethal challenge with influenza A virus (22). In the current study, we used the P.L.E.A.S.E. portable laser to deliver XCL1-based vaccibodies, in a protein format, within the dermis and demonstrated that they permit the harnessing of unique T cell immunostimulatory properties of XCR1+ dural DCs. Using B16F10 (B16), a highly aggressive metastatic and poorly immunogenic melanoma and its OVA-expressing variant (B16-OVA), we demonstrated the in vivo antitumor efficacy of a single round of laser-assisted, dermal delivery of OVA-containing XCL1-based vaccibodies in both prophylactic and therapeutic settings and in the absence of intentionally added adjuvants.

Materials and Methods

Mice

Mice were used at 8–12 wk of age, housed under specific pathogen–free conditions, and handled in accordance with French and European directives. All mice were maintained on a B6 background. OT-I mice (23), OT-II mice (24), Ccr7−/− mice (25), Myd88−/−/Trif−/− mice deficient in both MyD88 and Trif (Ticam1) (called Myd88−/− Trif−/− mice hereafter) (26), and Xcr1−/− mice (Xcr1−/− mice hereafter) (6) were described previously. C57BL/6J (B6) mice were purchased from Janvier (Le Genest-Saint-Ise, France).

Isolation of skin DCs, monocytes, monocyte-derived DCs, and macrophages

To extract skin myeloid cells, ears were split into dorsal and ventral parts and incubated with a solution of PBS containing 1 mg/ml Dispase (Roche) for 2 h at 37˚C or overnight at 4˚C. The dorsal and ventral parts were then cut into small pieces and incubated for 90 min at 37˚C with RPMI 1640 culture medium containing 1 mg/ml DNase and 1 mg/ml Collagenase IV. To extract skin myeloid cells, ears were split into dorsal and ventral parts and incubated with a solution of PBS containing 1 mg/ml Dispase (Roche) for 2 h at 37˚C or overnight at 4˚C. The dorsal and ventral parts were then cut into small pieces and incubated for 90 min at 37˚C with RPMI 1640 culture medium containing 1 mg/ml DNase and 1 mg/ml Collagenase IV.

Flow cytometry

Cells were stained and analyzed using a FACS LSR II system with DIVA software (BD Biosciences). Cell viability was evaluated using SYTOX (Invitrogen), according to the manufacturer’s protocol. Anti–NK1.1 (PK136), anti–CD3 (17A2), anti–Ly-6G (1A8), anti–CD19 (6D5), and anti–CD64 (54-5/7.1) were from BioLegend. Anti–CD11c (N418), anti–MHC class II (I-A/I-E) (M5/114.15.2), anti–CD45.2 (A20), anti–CD24 (M1/69), and anti–CD5 (5-7.3) were from eBioscience. Anti–Ly-6C (AL21), anti–CD4 (RM4-3), and anti–CD8a (53-6.7) were from BD Pharmingen. Prior to the analysis of monocytes, macrophages, DCs, B cells, T cells, NK cells, and neutrophils were systematically gated out using a ‘dump-channel’ corresponding to cells positive for B220, CD3, NK1.1, or Ly-6G. Analysis was performed using FlowJo software (TreeStar).

Generation of vaccibodies

XCL1-OVA vaccibodies contain the XCL1 chemokine and the OVA Ag linked through a dimerization domain consisting of the hinge and CH3 domain of human IgG3. XCL1-OVA vaccibodies were produced in HEK293E cells and purified using an affinity column containing an Ab directed against the vaccibody dimerization domain (22). After elution, vaccibodies were dialyzed against PBS.

FIGURE 1. XCL1-mCherry vaccibodies specifically target XCR1+ dermal DCs in vitro. (A) XCL1-based vaccibodies are homodimeric chimeric proteins consisting of the XCL1 chemokine, a dimerization unit made of the hinge and CH3 domain of human IgG3, and an Ag moiety, such as OVA. To determine whether XCL1-based vaccibodies specifically bind to XCR1+ dermal DCs, the antisense moiety was replaced by mCherry, a red monomeric fluorescent protein. (B) Flow cytometry analysis of CD45+ cells from the ear skin of mice after gating out granulocytes, T cells, B cells, and NK cells. Analysis of CD45+ MHCII+CD11c+ cells (left panel) for the expression of CD24 and CD11b required to identify XCR1+ DCs (CD11b+CD24+) and LCs (CD11b+CD24–). Analysis of CD45+ CD11b+CD24+ monocytes (right panel) for the expression of Ly-6C and CD64 required to identify CD11b+ DCs (Ly-6C–CD64+) and monocytes, moDCs, and macrophages (Ly-6C– to low CD64+ to high).

(C) Single-cell suspensions prepared from the ear skin of B6 WT and Xcr1−/− mice were incubated with XCL1-mCherry vaccibodies and analyzed by flow cytometry, as described in (B). Shaded line graphs represent control, XCL1-mCherry–unstained cell samples. Numbers represent the percentage of XCL1-mCherry+ cells (%).
8%. P.L.E.A.S.E.-assisted skin microporation was performed on the ear of anesthetized mice, and 20 μl sterile PBS, sterile PBS containing 1.5 μg (low) or 3.1 μg (high) OVA, or sterile PBS containing 2.5 μg (low) or 5 μg (high) XCL1-OVA was applied evenly on the microporated ear surface. The PBS solution percolated inside the micropores in <10 min.

**Histology**

For histological analysis, ears were harvested after laser poration and embedded in paraffin. Sections (5 μm) were stained with H&E for microscopic examination.

**Preparation of CellTrace Violet–labeled T cells**

OT-I and OT-II T cells were isolated from pooled LNs and spleen of OT-I or OT-II mice kept on a Rag-2⁻/²-B6 (CD45.1) background using Dynal CD8⁺ and CD4⁺ T Cell Negative Isolation Kits (Invitrogen), respectively. Purity was determined by staining with CD4, CD8, CD5, and TCR Vα2.

For labeling with CellTrace Violet (CTV; Molecular Probes), purified OT-I and OT-II T cells were resuspended in PBS containing 2.5 mM CTV for 20 min at 37°C. A total of 10⁶ CTV-labeled OT-I and OT-II T cells was adoptively transferred into the specified mice. At the indicated times, single-cell suspensions were prepared from the auricular LNs draining the immunized ears, and OT-I and OT-II T cells were analyzed by FACS.

**In vivo cytotoxicity assay**

Splenocytes from B6 mice were pulsed with the SIINFEKL peptide or left untreated and labeled with a low (0.25 mM) or high (2.5 mM) dose of CTV. A total of 10⁷ splenocytes of each preparation was adoptively transferred into mice that had been immunized for 6 d. Thirty-six hours later, a single-cell suspension was prepared from spleen, and the ratio of CTV⁺/CTV⁻ cells was determined by FACS.

**Tumor model**

Mice were injected s.c. into the flank with 10⁵ B16-OVA or B16 melanoma cells (29). Tumor size was assessed 14–16 d later using a caliper. Determining tumor volume after sacrificing the mice and careful dissection of the tumor mass constituted a more accurate and reproducible measure of tumor size compared with the measurement of s.c. tumor volume on live mice with a caliper. To adhere to institutional ethical guidelines, mice inoculated with B16 melanoma cells were sacrificed 14 d (therapeutic protocol) or 16 d (prophylactic protocol) after tumor inoculation. The presence of T cells infiltrating the tumor was assessed after enzymatic treatment of tumor mass with collagenase 2 (Worthington) and Percoll gradient (Amersham-Pharmacia).

**Intracellular staining**

T cells harvested from the tumor mass were incubated for 6 h at 37°C in the presence of PMA (5 ng/ml) and ionomycin (250 ng/ml). Monensin (GolgiStop; BD Pharmingen) was added to the suspension for the last 5 h. Cells were stained with anti-CD5, anti-CD4, anti-CD8, and H-2 Kb tetrarmers loaded with the SIINFEKL peptide (iTag MHC tetramers; Beckman Coulter) and then permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Intracellular cytokines were detected by staining with anti–IFN-γ (XMG1.2; BD Pharmingen).

**Statistical analysis**

The magnitude of the experimental effect observed in preliminary experiments led to the estimation of the proper sample size in each experiment. Because some of the experimental groups did not pass the D’Agostino and Pearson omnibus normality test, a Mann–Whitney U test was used to assess the statistical significance within the different immunization settings. Exact probability values are shown in the figures. A p value > 0.05 was considered nonsignificant.

**Results**

Laser-assisted, intradermal delivery of XCL1-based vaccibodies permits the specific targeting of XCR1⁺ dermal DCs

Prior to targeting XCR1⁺ dermal DCs in situ, we characterized the specificity of the XCL1-based vaccibodies on single-cell suspensions of the ear skin, and the binding of XCL1-mCherry to XCR1⁺ DCs, LCs, CD11b⁺ DCs, monocytes, moDCs, and macrophages was analyzed by flow cytometry, as in Fig. 1B. Shaded line graphs represent control, XCL1-mCherry–unstained cell samples. Numbers represent the percentage of XCL1-mCherry⁺ cells (n = 3).
The antigenic moiety of XCL1-based vaccibodies was replaced by mCherry, a red monomeric fluorescent protein (Fig. 1A) that allows binding specificity to be assessed by flow cytometry. By combining CD24 and CD11b expression, XCR1+ dermal DCs (also known as CD103+ or CD8α+ DCs) and LCs can be readily identified among the CD45+MHCIICD11c+ cells found in the skin of the ear (Fig. 1B) (6, 7, 27). Analysis of CD45+CD11b+CD24low dermal cells for the expression of Ly-6C and CD64 further identified CD11b+ DCs on the basis of their Ly-6C+CD64+ phenotype (27). The remaining CD45+CD11b+CD24low non-DCs included monocytes, moDCs, and macrophages (Fig. 1B). XCL1-mCherry vaccibodies specifically stained XCR1+ DCs, whereas no staining was observed on cells isolated from the ear of mice deficient in XCR1 (Fig. 1C).

To target XCL1-based vaccibodies to XCR1+ dermal DCs in a needle-free manner, we used a P.L.E.A.S.E. portable laser. Application on the mouse ear skin using 75 μs pulse duration with two pulses/pore and an energy of 11.9 J/cm2 resulted in the formation of an array of micropores via laser ablation (Fig. 2A). Histological analysis of ear sections indicated that the stratum corneum and the epidermis were removed, whereas the integrity of the dermis was preserved (Fig. 2B). Therefore, the regimen chosen created pores whose depth was compatible with topically applied XCL1-based vaccibodies reaching the dermis. Analysis of single-cell suspension prepared from ear skin 24 h after laser-assisted delivery of XCL1-mCherry vaccibodies showed that ~15% of XCR1+ dermal DCs were stained; using Xcr1+/− mice, this staining was shown to depend on XCR1 expression (Fig. 2C). Therefore, the combination of laser microporation and XCL1-based vaccibodies permits targeting of XCR1+ dermal DCs in a specific and needle-free manner.

Twenty-four hours after laser microporation, analysis of the ear skin showed a mild inflammation that occurred irrespective of the application of XCL1-OVA vaccibodies and involved infiltrates made of neutrophils and CD45+CD11b+CD24low cells (Supplemental Fig. 1A, 1B). Resolution of the different subsets present among the infiltrating CD45+CD11b+CD24low cells (6, 7, 27) showed that they were dominated by Ly-6Cm-ch high monocytes and moDCs. In contrast, 24 h after laser microporation the numbers of LCs, XCR1+ and CD11b+ dermal DCs, and macrophages remained unchanged (Supplemental Fig. 1C). Therefore, despite the rapid infiltration of laser microporated skin by neutrophils, monocytes, and moDCs, the topically applied XCL1-based vaccibodies still succeeded at targeting XCR1+ dermal DCs.

XCL1-OVA vaccibodies are more effective at eliciting T cell responses on a per-molecule basis than free OVA

To test whether targeting XCR1+ dermal DCs with Ag-loaded XCL1-based vaccibodies induced the activation of Ag-specific T cells in vivo, the model Ag OVA was inserted into XCL1-based vaccibodies. Mice were adoptively transferred with CTV-labeled OT-I CD8+ T cells and OT-II CD4+ T cells; they express a TCR specific for SIINFEKL, an OVA-derived peptide presented by H-2Kb, and for an OVA-derived peptide presented by H2-Ab, respectively. One day later, the ears of the mice were subjected to laser-assisted microporation and topical application of XCL1-OVA vaccibodies. To compare the potency of the XCL1-OVA formulation with that of free OVA and, thus, determine the gain resulting from targeting OVA to XCR1+ DCs through XCL1-OVA vaccibodies, ears also were subjected to laser-assisted microporation and topical application of OVA in amounts equimolar to that present in XCL1-OVA vaccibodies. Three days after immunization, single-cell suspensions were prepared from ear-draining auricular LNs, and the extent of OT-I and OT-II cell proliferation was determined by analysis of OVA-specific CD4+ and CD8+ T cell proliferative responses. (A) Timeline of adoptive transfer of CTV-labeled OT-I and OT-II T cells, laser-assisted dermal Ag delivery, and analysis of OT-I and OT-II T cell proliferation within auricular, ear-draining LNs. (B) Single-cell suspensions were prepared from ear-draining Ag delivery, analysis of OT-I and OT-II T cell proliferation within auricular ear-draining LNs from B6 (WT) and Xcr1−/− mice that received OT-I and OT-II T cells and were immunized by applying 20 μl of PBS containing OVA (3.1 μg), XCL1-OVA vaccibodies (5 μg), XCL1 (1.8 μg) plus OVA (3.1 μg), or PBS alone as a control to laser-microporated ears. Seventy-two hours after Ag delivery, the extent of proliferation was determined by CTV dilution. Numbers represent the percentage of divided T cells (n = 3). (C) Quantification of data shown in (B). Absolute numbers of proliferating OT-I and OT-II T cells recovered per auricular LN from B6 (WT) and Xcr1−/− mice; each symbol represents an individual mouse. The mean (horizontal line) is indicated for each condition.
was determined by CTV dilution (Fig. 3A). XCL1-OVA vaccibodies triggered a higher proliferation of OT-I and OT-II cells than did free OVA (Fig. 3B). Quantification of the data confirmed that XCL1-OVA vaccibodies were, on a per-molecule basis, 15 times more effective at eliciting CD8+ T cell proliferation and 3 times more effective at eliciting CD4+ T cell proliferation than was free OVA (Fig. 3C). When the same experiments were repeated with Xcr1−/− mice, the beneficial effect observed following XCL1-OVA treatment was lost: the magnitude of proliferation induced with XCL1-OVA was comparable to that of OVA (Fig. 3C).

The XCL1 chemokine present in XCL1-OVA vaccibodies was shown to retain its chemotactic function (22). Therefore, the higher potency noted for XCL1-OVA Abs compared with OVA may not result from the targeting of OVA to XCR1+ DCs but from the ability of XCL1 to promote encounters between XCR1+ DCs and T cells (5, 30). However, coadministration of OVA and XCL1 in free forms and in amounts similar to those used in XCL1-OVA treatment resulted in levels of T cell proliferation similar to those elicited by OVA alone (Fig. 3C). Therefore, the physical linkage between OVA and XCL1 provided by the vaccibody format was essential to maximize the immunogenic potential of XCR1+ dermal DCs, and it is likely that XCR1 merely functioned as a target for delivery of Ag.

**T cell responses elicited by laser-assisted intradermal delivery of XCL1-OVA vaccibodies require migratory XCR1+ DCs and occur in a MyD88-Trif–independent manner**

Skin DCs capture incoming Ags and after 2–5 d, depending on the DC subset, migrate to skin-draining LNs to elicit T cell responses (31–33). However, Ags that enter the dermis and are small enough to penetrate the lymphatic vessels can reach the LN subcapsular sinus in a free form (34). Such lymph-borne Ags can be captured by DCs that line the subcapsular sinus (35). This led to a more rapid generation of effector T cell responses, independent of skin-derived migratory DCs. DC-free drainage of skin-delivered Ags is amplified during needle-based, intradermal, and s.c. immunization as a result of the excessive interstitial hydrostatic pressure created by fluid injections (34). To evaluate whether T cell responses elicited by laser-assisted, intradermal delivery of XCL1-OVA vaccibodies required the migration of skin-derived XCR1+ DCs, rather than the capture of free XCL1-OVA vaccibodies by the XCR1+ DCs that permanently reside in skin-draining LNs (36), we used Ccr7−/− mice, in which the CCR7-dependent migration of DCs from the skin to the draining LNs is impaired (25). Analysis of T cell responses of wild-type and Ccr7−/− mice 3 d after immunization showed that OT-I and OT-II responses were reduced 10–20-fold in the absence of CCR7 (Supplemental Fig. 2A, 2B). Therefore, CD4+ and CD8+ T cell activation induced by laser-assisted intradermal delivery of XCL1-OVA vaccibodies is primarily due to migration of OVA-presenting XCR1+ dermal DCs to draining LNs. Consistent with the view that following laser-assisted intradermal delivery free XCL1-OVA vaccibodies do not diffuse away from the ear immunization site via the blood, adoptively transferred OT-I and OT-II T cells present in the spleen and in LNs that drain territories distinct from the ear showed no sign of proliferation (Supplemental Fig. 2C, 2D).

Although XCL1-OVA vaccibodies were affinity purified and reconstituted in endotoxin-free PBS prior to delivery, we determined whether the extensive proliferation of Ag-specific T cells observed after targeting XCR1+ dermal DCs with XCL1-OVA vaccibodies persisted in Myd88−/−/Trif−/− double-deficient mice that are deprived of two adaptors used in all of the TLR signal-transduction networks. Laser-assisted, intradermal delivery of XCL1-OVA vaccibodies in Myd88−/−/Trif−/− double-deficient mice resulted in levels of OT-I and OT-II T cell proliferation similar to those elicited in wild-type mice (Supplemental Fig. 3). Therefore, T cell responses triggered by laser-assisted intradermal delivery of XCL1-OVA vaccibodies require the migration of dermal XCR1+ DCs and occur irrespective of TLR signals.

**FIGURE 4.** Laser-assisted, dermal delivery of XCL1-OVA vaccibodies protects mice against melanoma tumor growth in therapeutic and prophylactic settings. (A) Timeline of the therapeutic setting involving s.c. inoculation of the B16-OVA melanoma, laser-assisted dermal delivery of PBS, OVA, or XCL1-OVA vaccibodies, and tumor volume measurement. (B) A cohort of mice, treated as in (A) with 20 μl of PBS containing OVA (low dose: 1.5 μg; high dose: 3.1 μg) or XCL1-OVA vaccibodies (low dose: 2.5 μg or high dose: 5.0 μg), was analyzed for tumor volume. Control mice were treated with 20 μl of PBS. Tumor volume is shown for each individual mouse, and the mean (horizontal line) is indicated for each condition; one representative experiment of three is shown. (C) Timeline of the prophylactic setting involving laser-assisted, dermal delivery of 20 μl of PBS or PBS containing OVA (1.5 μg) or XCL1-OVA vaccibodies (2.5 μg); s.c. inoculation of the B16-OVA melanoma; and tumor volume measurement. (D) A cohort of mice, treated as specified in (C) with OVA or XCL1-OVA, was analyzed for tumor volume. Control mice were treated with 20 μl of PBS. Tumor volume is shown for each individual mouse, and the mean (horizontal line) is indicated for each condition (n = 3). n.s., nonsignificant.
Intradermal delivery of XCL1-OVA vaccibodies protects mice against melanoma tumor growth in prophylactic and therapeutic settings

The B16-OVA melanoma is not rejected by immune-competent syngeneic B6 mice unless they have been subjected to prophylactic or therapeutic immunization. To evaluate the capacity of laser-assisted, intradermal delivery of XCL1-OVA vaccibodies to inhibit the growth of B16-OVA tumors, B6 mice were inoculated s.c. in the flank with B16-OVA cells (Fig. 4A). Three days later, the ears of the mice were subjected to laser-assisted, dermal delivery of XCL1-OVA vaccibodies, and tumor growth was monitored 14 d after tumor inoculation. For comparison, control mice received PBS or equimolar amounts of free OVA. A single intradermal immunization with a low or a high dose of XCL1-OVA vaccibodies significantly suppressed tumor growth compared with the OVA and PBS groups, and tumor growth was further reduced with the high dose of XCL1-OVA vaccibodies (Fig. 4B). To investigate the prophylactic effects on tumor growth induced by laser-assisted intradermal XCL1-OVA immunization, B6 mice were immunized with XCL1-OVA vaccibodies and inoculated s.c. with B16-OVA tumor cells 30 d later (Fig. 4C). Monitoring tumor growth 16 d after tumor inoculation showed that XCL1-OVA vaccibodies significantly slowed tumor growth compared with OVA and PBS (Fig. 4D). As expected, XCL1-OVA vaccibodies induced an OVA-specific immunity, and mice immunized with XCL1-OVA vaccibodies were not able to control the growth of B16 melanoma that did not express OVA (Fig. 5A). Moreover, the capacity of XCL1-OVA vaccibodies to inhibit B16-OVA tumor growth was dependent on the expression of XCR1 (Fig. 5B).

To assess whether the reduction in melanoma tumor growth observed upon treatment with XCL1-OVA vaccibodies was associated with the induction of endogenous, Ag-specific CD8+ T cells, wild-type and Xcr1−/− mice were immunized via laser-assisted intradermal delivery of XCL1-OVA vaccibodies. Six days after immunization, the magnitude of OVA-specific T cell cytotoxicity was assessed using an in vivo cytotoxic assay (Fig. 5C). XCL1-OVA vaccibodies induced a significant T cell cytotoxicity compared with PBS or OVA treatment. The absence of detectable lysis in Xcr1−/− mice indicates that such T cell cytotoxicity was dependent on the expression of XCR1. Moreover, analysis of the CD8+ and CD4+ T cells that infiltrated the B16-OVA vaccibodies showed that they were capable of producing IFN-γ (Fig. 5D). In contrast, OVA treatment did not induce IFN-γ+ T cells over PBS control, a finding consistent with the observation that OVA treatment was unable to slow tumor growth (Fig. 4). In addition, staining of CD8+ T cells that infiltrated the regressing B16-OVA tumor mass found in XCL1-OVA–treated mice with H-2 Kb tetramers loaded with the OVA-derived, SIINFEKL peptide showed an ∼100-fold increase in the numbers of OVA-specific, IFN-γ–producing CD8+ T cells compared with OVA-treated mice (Supplemental Fig. 4). Therefore, laser-assisted, intradermal delivery of XCL1-OVA vaccibodies induced antitumoral responses that can be correlated with the presence of OVA-specific IFNγ+ CD8+ T cells in the regressing tumor mass.

Discussion

In the current study, we show that, by creating laser-generated micropores in the stratum corneum and epidermis of the skin, it is possible to target XCL1-OVA vaccibodies to XCR1+ dermal DCs and to induce Ag-specific CD8+ and CD4+ effector T cells. This process required migration of XCR1+ dermal DCs to draining LNs.
and occurred irrespective of TLR signals. Moreover, a single intradermal immunization with XCL1-OVA vaccibodies protected mice against melanoma tumor growth in both prophylactic and therapeutic settings and in the absence of intentionally added adjuvant. Whether Ag targeting to DC results in tolerance or immunity depends on parameters such as the immunogenicity of the targeting Ab (16) and the coadministration of adjuvants (37, 38). Adjuvants are intended to trigger the pattern-recognition receptors that are expressed by the targeted DCs and that are normally used to detect invading microorganisms or endogenous “danger” signals. In contrast to other studies that targeted XCR1+ DCs via needle-based, i.v., or cutaneous injection (14, 15, 39), we achieved Ag-specific protection against the B16-OVA melanoma in the absence of intentionally added adjuvant. Moreover, TLR signals were dispensable for the Ag-specific T cell responses resulting from laser-assisted intradermal delivery of XCL1-OVA vaccibodies. The mode of Ag delivery itself by skin laser microporation most likely explains such a marked difference in adjuvant requirement. The fractional Er:YAG laser operating in the P.L.E.A.S.E. portable laser creates microcogulated areas in the skin that include dying cells (40). STING (stimulator of IFN genes) is a protein that resides in the endoplasmic reticulum of many cells, including DCs. It cooperates with the nucleotide-transferase eGAS to trigger the production of type I IFNs in response to the presence of pathogen- or self-derived DNA in the cytosol. It was shown recently that XCR1+ DCs contribute to trigger T cell responses against tumors in a STING-dependent manner (41–43). XCR1+ DCs use STING to sense the self-DNA released by dying tumor cells (44) and, as a result, produce type I IFN that contributes to boost their Ag-presenting function and T cell costimulatory properties. Therefore, it is likely that, in our model, the death of keratinocytes induced by laser microporation acts as a STING-dependent adjuvant. We also noted that skin microporation triggers a rapid infiltration of the treated skin area with granulocytes, monocytes, and moDCs. Therefore, altogether, the adventitious phenomena resulting from skin laser microporation itself create an inflammatory milieu that most likely favors the development of potent T cell immune responses in the absence of exogenous adjuvants. In a mouse model, a rat anti-CLEC9A Ab used to deliver OVA to XCR1+ DCs induced CD4+ T cell and humoral responses against OVA in the absence of adjuvant (16), a property resulting from the presence of helper epitopes on the rat Ab that were recognized as foreign by the mouse immune system. Along the same line, the dimerization unit derived from human IgG3 domains and present in vaccibodies likely contributes to enhance mouse immune responses against the antigenic cargo (21). Finally, considering that the use of adjuvants in vaccines is often associated with safety issues, the ability to induce protective responses against melanoma tumor growth independently of the administration of exogenous adjuvants should facilitate the development of safer vaccines. Migratory DCs originating from tissues such as the skin and intestine are thought to “instruct” Ag-specific naïve T cells in a way that confers them with a propensity to home to the tissue from which the migratory DCs originated (45). Such tropism allows primed T cells to exert their effector functions in the tissue subjected to the Ag challenge. When injected i.v., Ag-conjugated anti-CLEC9A Abs target the XCR1+ DCs that permanently reside in the spleen and, thereby, initiate T cell responses in this organ (14). In contrast, following laser-assisted, intradermal delivery of XCL1-OVA vaccibodies, the onset of T cell responses depended on the migration of XCR1+ dermal DCs and remained limited to the skin-draining LNs. Whether skin-derived XCR1+ DCs confer to Ag-specific T cells a skin tropism superior to that elicited by spleen-resident XCR1+ DCs constitutes an important issue when treating conditions such as cutaneous melanoma. Although the T cell priming resulting from laser-assisted, intradermal delivery of XCL1-OVA vaccibodies is limited to the LN draining the treated skin area, a systemic T cell response ensued that was capable of protecting against cutaneous melanoma developing at a site distant from the one used for immunization. When translated to humans, this approach should limit the systemic side effects resulting from the administration of i.v. vaccines while achieving systemic protective immunity. In conclusion, using laser-assisted intradermal delivery and a model Ag fused to the XCL1 chemokine, we showed that it is possible to target dermal XCR1+ DCs and harness their cross-presentation capacity. Although the efficiency of many vaccines relies on multiple rounds of administration in the presence of adjuvants, we showed that a single intradermal immunization with XCL1-based vaccibodies is sufficient to protect mice against melanoma tumor growth in the absence of exogenous adjuvants. Therefore, topic, laser-assisted intradermal delivery of Ags targeting XCR1+ DCs constitutes a promising route for the delivery of vaccines. In humans, XCR1 expression defines a DC subset that shows a similar anatomical distribution to mouse XCR1+ DCs and is endowed with cross-presentation capacity (30, 46–48), a feature that should facilitate the translation of the present mouse model to human settings.

Acknowledgments
We thank M. Dalod, A.-M. Schmitt-Verhulst, and C. Coscia for discussions, R.J. Driscoll for the Xcr1pinDgeo mice, L. Alexeopoulo for the Myd88−/−Trif−/−Ddeo2 mice, M. Dalod for importing the Xcr1pinDgeo mice, and L. Chasson for assistance with histology.

Disclosures
R.B. holds shares in Pantec Biosolutions. B.B. is the inventor on United States patent 8,932,603 B2 on vaccibody filed by Oslo University Hospital, according to institutional rules. B.B. and E.F. are inventors on a patent application on XCL1 vaccibodies. B.B. is head of the scientific advisory board and hold shares in Vaccibody. The other authors have no financial conflicts of interest.

References


