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Dissecting antigen processing and presentation routes in dermal vaccination strategies

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ABSTRACT

The skin is an attractive site for vaccination due to its accessibility and presence of immune cells surveilling this barrier. However, knowledge of antigen processing and presentation upon dermal vaccination is sparse. In this study we determined antigen processing routes that lead to CD8$^+$ T cell activation following dermal DNA tattoo immunization, exploiting a model antigen that contains an immunoproteasome-dependent epitope. In agreement with earlier reports, we found that DNA tattoo immunization of wild type (WT) mice triggered vigorous responses to the immunoproteasome-dependent model epitope, whereas gene-deficient mice lacking the immunoproteasome subunits β5i/LMP7 and β2i/MECL1 failed to respond. Unexpectedly, dermal immunization both of irradiated bone marrow (BM) reconstituted mice in which the BM transplant was of WT origin, and of WT mice transplanted with immunoproteasome subunit-deficient BM induced a CD8$^+$ T cell response to the immunoproteasome-dependent epitope, implying that both BM and host-derived cells contributed to processing of delivered model antigen. Depletion of radiation-resistant Langerhans cells (LC) from chimeric mice did not diminish tattoo-immunization induced CD8$^+$ T cell responses in most mice, illustrating that LC were not responsible for antigen processing and CD8$^+$ T cell priming in tattoo-immunized hosts. We conclude that both BM and non-BM-derived cells contribute to processing and cross-presentation of antigens delivered by dermal DNA tattoo immunization.

1. Introduction

The earliest successful vaccination against smallpox was accomplished by cutaneous vaccination. Nowadays most vaccines are administered intramuscularly, but the skin remains a very attractive target for vaccination, because of its accessibility and possibilities for lower antigen doses. Currently, a number of cutaneous delivery methods are being tested, including different types of microneedles and tattoo immunization. While these methods have been demonstrated to induce both humoral and cellular responses, the underlying mechanisms contributing to cellular immune activation have only partially been explored. Vaccination-induced priming of CD8$^+$ T cell responses requires the cross-presentation of intradermally delivered antigens by professional antigen presenting cells (pAPC), to CD8$^+$ T cells in the draining lymph nodes. Different studies have defined a variety of pAPC subsets as responsible for the interaction with vaccine antigen-specific CD8$^+$ T cells, including dendritic cells (DC) residing in the lymph nodes, langerin$^+$ dermal DC, and Langerhans cells (LC), although LC may either have a stimulatory or inhibitory role [1–4]. Moreover, while induced CD8$^+$ T cell responses are primed by either of these DC subsets, it remains unclear whether these DC process the epitopes they present, or acquire them from other, non-dendritic, cells.

The epitopes, presented on (p)APC to CD8$^+$ T cells, are processed mainly by proteasomes, which are multi-catalytic enzyme complexes present in the cellular cytosol and nucleus. Proteasome' catalytic activity is displayed by three subunits, β1, β2 and β5, present in the inner two β rings of the 20S proteasome catalytic core particle. Exposure of cells to inflammatory cytokines induces the

Abbreviations: DTR, diphtheria toxin receptor (DTR); KO, knock out; LC, Langerhans cell; KI, knock in; WT, wild type.

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expression of three facultative catalytic sites, β1i/LMP2, β2i/MECL1 and β5i/LMP7, which replace their constitutively expressed homologues in newly assembled proteasomes, leading to the formation of intermediate-type proteasomes and immunoproteasomes [5]. Depending on the presence of either the inducible subunits or their constitutive homologues, proteasomes display different catalytic pocket conformations and peptide transport dynamics [6], which quantitatively alters the pool of peptides produced by proteasomes [7–9].

In contrast to most cell types, pAPCs express the proteasome immunosubunits continuously and contain relatively high quantities of immunoproteasomes. In previous studies using β2i/MECL1 and β5i/LMP7 double gene-deficient (β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$) KO mice [10], we showed that priming of CD8$^+$ T cell responses specific for an adenovirus model antigen-derived epitope, E1B$_{192-200}$, required immunoproteasome-mediated antigen processing. CD8$^+$ T cell responses to a second epitope derived from this antigen, E1A$_{234-243}$, were unaffected by the absence of immunosubunit expression in these mice. We decided to use this model system to determine antigen processing and presentation routes that lead to the priming of the CD8$^+$ T cell response after dermal DNA tattoo immunization [11]. Using BM chimeric mice, composed of WT -, CD207-diptheria toxin receptor knock in (KI) – and β2i/MECL1$^{-/-}$-β5i/LMP7$^{-/-}$ (KO) recipients, reconstituted with WT – or KO BM, we show that both BM- and non-BM-derived cells contribute to the processing of pAPC-presented, dermally delivered vaccine antigen, and that radiation-resistant LC are not responsible for the CD8$^+$ T cell activation.

2. Material and methods

2.1. DNA vaccine

To generate the E1 DNA vaccine, the sequences coding for the Adenovirus early-1-region (E1) derived epitopes E1A$_{234-243}$ (SGPSNTPPEI) and E1B$_{192-200}$ (VNIRNCCYI), each flanked by their natural flanking sequences (encoding 15 amino acids, both N- and C-terminally) [10], were inserted into the pVAX1 vector (Invitrogen), 3′ of and in frame with a tetanus toxin fragment C domain 1 (TTFC)-encoding region [12,13].

2.2. Mice and dermal DNA tattoo immunization

For construction of chimeric mice, bone marrow was flushed from the femurs of donor mice, depleted of mature B and T cells by incubation with a mixture of 10 μg/mL anti-mouse CD4 (clone GK1.5; made in house), CD8 (clone YTS-169; made in house), CD3 (12A2 clone; made in house) and CD19 (clone ID3; made in house), and subsequent incubation with guinea pig complement 4.5 μg/mL for 30 min (Invitrogen). Recipient mice were irradiated with 9 Gy as a single dose from an X-ray irradiator and reconstituted with 10^7 BM cells. They were allowed to reconstitute for 6 weeks. C57BL/6 J mice were purchased from Charles River, B6.129S2-CD207$^{tm3(DTR/GFP)Mal}$/J from Jackson and B6.SJL mice and β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ mice were bred in the animal facility of Utrecht University. The efficacy of reconstitution in mixed bone marrow chimeric mice was evaluated by staining splenocytes with anti-mouse CD11c-APC (clone N418; Biolegend), MHC-II-PE (clone M5/114.15.2; Biolegend), CD45.1-PerCPcy5.5 (clone A20; Biolegend) and CD45.2-FITC (clone 104; Biolegend) and percentages of host-derived DC was measured by FACS (Supplementary Fig. 1).

All mice were immunized at day 0, 3 and 6 with 15 μl cDNA (2 μg/μl) in TE buffer with a 9-needle bar mounted on a tattoo rotary device (Cheyenne) on 100 Hz, at 1 mm depth for 1 min [11]. All animal experiments were approved by the Animal Ethics Committee from Utrecht University (DEC 2013.II.07.084).

2.3. LC Depletion

Depletion of LC in bone marrow chimeric mice in which B6.129S2-Cd207$^{tm3(DTR/GFP)Mal}$/J mice had been reconstituted with B6.SJL bone marrow or β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ BM, was performed by *i.p.* injection of 7.5 ng/gr body weight diphtheria toxin (Sigma) in PBS at day −2, 0 and 6. Efficiency of depletion was measured by FACS analysis at day 0 (Supplementary Fig. 3).

2.4. rLM-E1 Infection

Recombinant *L. monocytogenes* rLM-E1 was grown in brain-heart infusion medium (BD Biosciences) supplemented with 250 μg/ml spectinomycin and harvested while in log phase. Mice were inoculated *i.v.* in the tail vein with a sub-lethal dose of 5000 CFU in 100 μl PBS.

2.5. Analysis of specific CD8$^+$ T cell responses

2.5.1. Intracellular cytokine staining (ICS)

Donor derived CD8$^+$ T cell responses were quantified as reported [8]. Briefly, 2.5×10^6 erythrocyte depleted splenocytes were incubated with or without 1 μg/ml synthetic E1B$_{192-200}$ VNIRNCCYI or E1A$_{234-243}$ SGPSNTPPEI for 6 h at 37 °C in RPMI 1640 medium supplemented with 10% FCS-HI (Lonza), 2 mM L-glutamine, 30 μM 2-mercaptoethanol (Gibco), 10 μM monensin (eBioscience) and penicillin/streptomycin. In case of splenocytes from mice infected with rLM-E1, 50 μg/mL gentamycin (Gibco) was added to the medium as well. Cells were stained with anti-mouse CD45.1-PerCPcy5.5 (clone A20; Biolegend), CD45.2-FITC (clone 104; Biolegend) and CD8-APC (clone 53–6.7; eBioscience) in the presence of anti-mouse CD16/CD32 (clone 2.4G2; made in house), fixed and stained with IFNγ-PE (clone XMG1.2; eBioscience) and analyzed on a FACS Canto II (BD Biosciences) using FlowJo software (Tree Star).

2.5.2. IFNγ ELISPOT

MAIP ELISPOT plates (Millipore) were coated with 2 μg/ml anti-mouse IFNγ (clone AN18; made in house) in PBS overnight at 4 °C. Wells were washed and blocked with RPMI 1640 medium (Life Technologies) containing FCS HI (Lonza). 5×10^5 or 2.5×10^5 erythrocyte depleted splenocytes were plated with or without 1 μg/ml synthetic peptide for 6 h in 1 ml FCS-HI and 2-mercaptoethanol (Gibco) supplemented RPMI at 37 °C. Plates were washed with PBS plus 0.01% tween 20 (PBST), and IFNγ was detected with biotinylated anti-mouse IFNγ (clone XMG1.2; BD), followed by alkaline phosphatase-conjugated streptavidin (Jackson Immuno Research Laboratories), in PBST supplemented with 2% BSA. The assay was developed with the Vector blue substrate kit (Vector Laboratories) and analyzed using an ELISPOT plate reader and scanner (AELVIS).

2.5.3. Statistical analysis

To compare donor-derived responses to individual epitopes between the different groups of mice, epitope specific responses of every mouse were corrected for background IFNγ level as measured in samples incubated without peptide, in both IFNγ- ELISPOT and IFNγ ICS. Differences in CD8$^+$ T cell responses detected by ICS or ELISPOT in C57BL/6 (WT) or β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ mice (KO) mice that were tattooed or infected, were tested for significance using Students T test. The variance homogeneity was tested using Levene's test. A Two-Way ANOVA, corrected for multiple comparisons using Tukey's correction was used to test for differ-

ences in responses of the different chimeric mice. P values <.05 were considered significant.

3. Results

3.1. Both infection with rLM-E1 and dermal E1 cDNA tattoo immunization elicit CD8⁺ T cell responses towards E1B$_{192-200}$ in immunoproteasome competent mice only

To determine which cells process the antigens that prime CD8⁺ T cell responses following dermal DNA tattoo immunization, a p/DNA vaccine was constructed encoding the adenovirus-derived E1B$_{192-200}$ and E1A$_{234-243}$ epitopes [14] in context of their natural flanking sequences, and preceded by TTFC, to enhance the immunogenicity of this construct [15]. Earlier studies using the same E1 sequences expressed by recombinant *Listeria monocytogenes* (rLM-E1) [10] showed that E1B$_{192-200}$ elicits a vigorous CD8⁺ T cell response in infected immunoproteasome competent wild type (WT) mice but, due to inefficient proteasome-mediated processing, fails to prime E1B$_{192-200}$-specific CD8⁺ T cells in β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) mice. E1A$_{234-243}$ is less immunogenic but triggers comparable responses in both mouse strains [10].

To test whether tattoo immunization with constructed DNA vaccine primes E1-specific CD8⁺ T cells, WT and KO mice were immunized three times, within six consecutive days (Fig. 1A). Seven days after the last immunization, E1B$_{192-200}$ and E1A$_{234-243}$-specific CD8⁺ T cells were quantified in the spleen (Fig. 1A) using IFNγ ICS (Fig. 1B). Consistent with our previous studies [10], we found that all WT mice mounted vigorous CD8⁺ T cell responses to E1B$_{192-200}$, while KO mice failed to respond to this epitope (Fig. 1C). Responses to the control epitope E1A$_{234-243}$ were similar in the two strains (Fig. 1C). Thus, similar to infection with rLM-E1 [10], priming of E1B$_{192-200}$ – specific CD8⁺ T cell responses by dermal DNA tattoo immunization requires immunoproteasome-mediated E1 antigen processing, while both immuno - and constitutive proteasomes produce the E1A$_{234-243}$ epitope with sufficient efficiency to prime a CD8⁺ T cell response.

3.2. In infection with rLM-E1 the presence of proteasome immunosubunits in BM derived cells, and not the periphery, is essential for the processing of E1B$_{192-200}$

To determine whether the pathogen-derived CD8⁺ T cell epitopes, presented by pAPC in rLM-E1 infection, are generated solely by BM-derived cells or whether also non-BM-derived cells contribute to epitope generation, bone marrow (BM) chimeric mice were created. To this end, WT and KO recipient mice were lethally irradiated and then reconstituted with either WT or KO BM. Six weeks later, mice were infected *i.v.* with a sub-lethal dose of rLM-E1 (Fig. 2A). Quantification of E1-specific CD8⁺ T cells in the spleen at day 8 post infection showed that all mice reconstituted with WT BM responded to the E1B$_{192-200}$ epitope, although responses detected in KO recipients were significantly lower than these in WT recipient mice (Fig. 2B). In contrast, we did not detect any response to E1B$_{192-200}$ in either WT or KO recipient mice, reconstituted with KO BM (Fig. 2B). As expected, CD8⁺ T cell responses to the immunoproteasome-independent E1A$_{234-243}$ epitope were detected in all mouse groups, including chimeric mice reconstituted with KO BM. Since rLM-E1-infected chimeric mice that expressed the proteasome immunosubunits in all cells except BM-derived cells failed to respond to the immunoproteasome-dependent E1B$_{192-200}$ epitope while mice reconstituted with WT

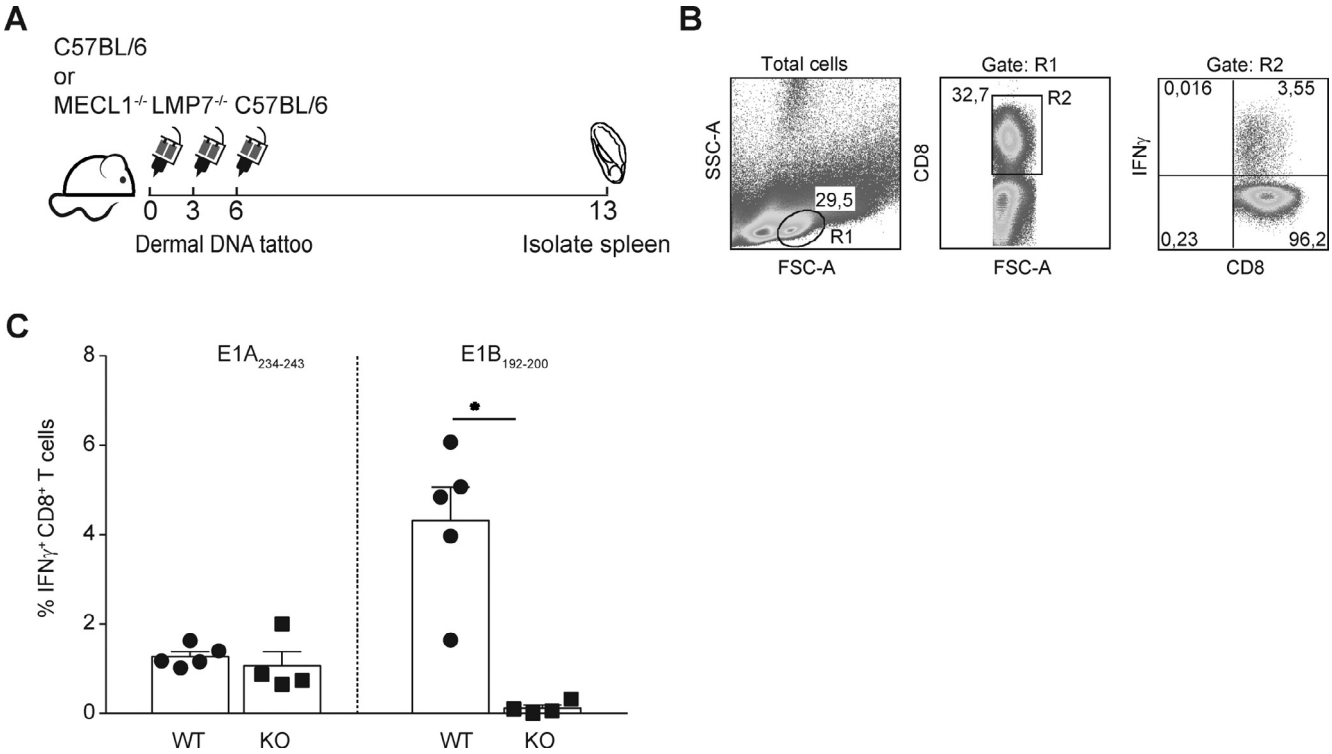

Fig. 1. E1 cDNA tattoo immunization only elicits E1B-specific CD8⁺ T cells in immunoproteasome competent mice. (A) C57BL/6 (WT) and β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) mice were immunized using E1 cDNA tattoo immunization at day 0, 3 and 6 and at day 13 the splenocytes were harvested. (B) Gating strategy; in the total cell population, lymphocytes were gated (R1). R1 was gated on CD8⁺ T cells (R2). In R2 the percentage of IFNγ⁺CD8⁺ T cells was measured. (C) Percentages of E1A or E1B-specific IFNγ⁺CD8⁺ T cells in the spleen of immunoproteasome competent C57BL/6 (WT, filled circles) or immunoproteasome deficient β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO, filled squares) mice, immunized using E1 cDNA tattoo immunization, were measured by re-stimulation *ex vivo* with peptides and detected by IFNγ ICS and flow cytometry. Every dot represents an individual mouse, corrected for IFNγ background level as measured in samples incubated with medium, and means (bars) ± SEM per peptide are indicated. Data are representative of two independent experiments (n ≥ 5 animals per group), analyzed using a students' T test (* P < .05).

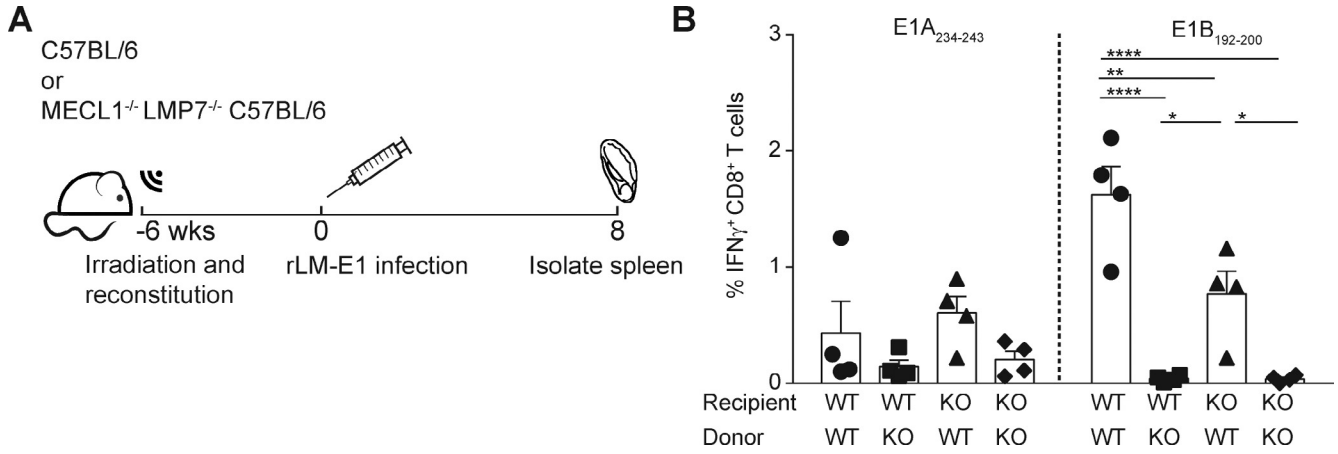

Fig. 2. Infection with rLM-E1 is dependent on immunoproteasome competent BM-derived cells. (A) C57BL/6 (WT) and β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) bone marrow chimeric mice were infected with rLM-E1 at day 0 and at day 8 the splenocytes were harvested. (B) Percentages of E1A or E1B-specific CD8$^+$IFNγ$^+$ T cells in the spleen of mixed immunoproteasome competent C57BL/6 (WT) or immunoproteasome deficient β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) individual BM chimeric mice, infected with rLM-E1, were measured by re-stimulation *ex vivo* with peptides and detection by IFNγ ICS and flow cytometry. Four different chimeric mice were used; B6.SJL (WT) → C57BL/6 (WT) chimera's (filled circles), β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) → B6.SJL (WT) chimera's (filled squares), B6.SJL (WT) → β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) chimera's (filled triangles) and β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) → β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) chimera's (filled diamonds). Every dot represents an individual mouse, corrected for IFNγ background level as measured in samples incubated with medium, and means (bars) ± SEM per peptide are indicated. Data are representative of one independent experiment (n = 4 animals per group), analyzed using a two-way ANOVA with Tukey's corrections (* P < .05, ** P < .01, *** P < .001, **** P < .0001).

BM responded, these data lead us to infer that BM-derived cells play an essential role in processing of pAPC-presented epitopes that prime the rLM-E1-specific CD8$^+$ T cell response following rLM-E1 infection.

3.3. In E1 cDNA tattoo immunization, the presence of proteasome immunosubunits in both BM derived cells as well as the periphery is essential for the processing of E1B$_{192-200}$

To determine processing routes of antigens delivered by dermal DNA tattoo immunization, WT and KO recipients reconstituted with WT or KO BM were tattoo-immunized with E1-DNA. Six days after the last immunization, E1-specific CD8$^+$ T cell responses were quantified in the spleen using IFNγ ICS (Fig. 3A) and IFNγ ELISPOT (Fig. 3B, Fig. S2). Confirming results from the infection studies shown in Fig. 2B, all chimeric mice responded to the immunoproteasome-independent E1A$_{234-243}$ control epitope (Fig. 3A), albeit some variation in responses ($p < .05$) was observed using ELISPOT (Fig. 3B) but not using IFNγ ICS (Fig. 3A) as read out. As expected, the immunoproteasome-dependent E1B$_{192-200}$ epitope was recognized by spleen-derived CD8$^+$ T cells of WT BM chimeric WT (control) recipients, while background responses measured for KO mice reconstituted with KO BM were barely detectable (Fig. 3A and B). In KO mice reconstituted with WT BM, compared to WT recipients, approximately five (IFNγ ICS) to seven-fold (ELISPOT) less splenic CD8$^+$ T cells responded to E1B$_{192-200}$,

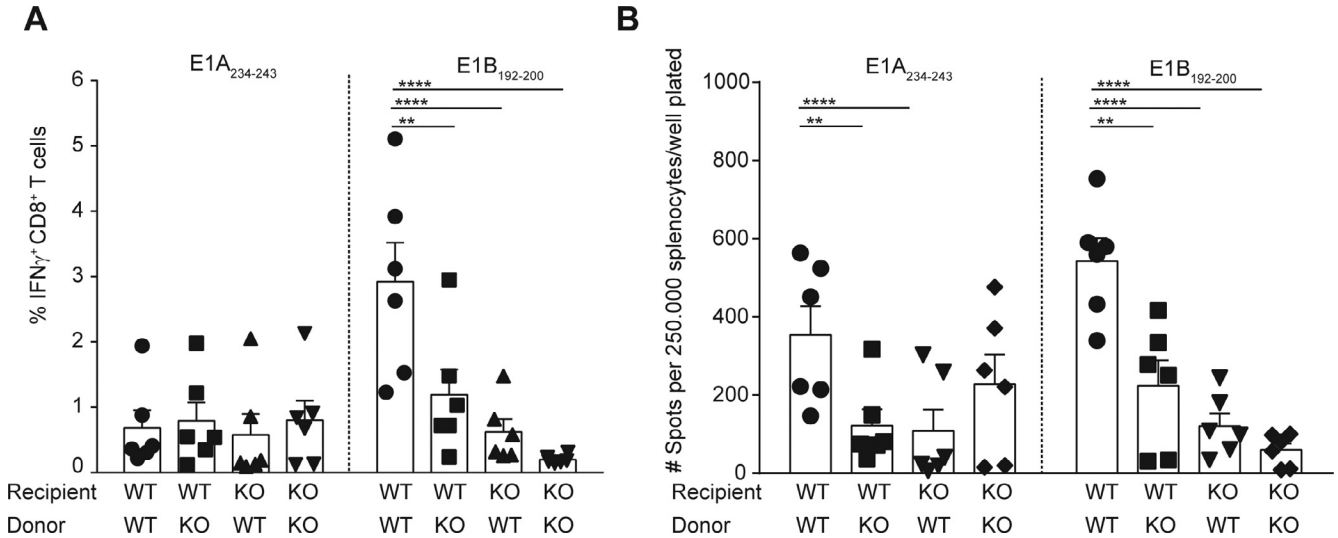

Fig. 3. Dermal DNA immunization is dependent on immunoproteasomes in both BM- and non-BM-derived cells. (A and B) Percentages of E1A or E1B-specific CD8$^+$IFNγ$^+$ T cells or numbers of IFNγ$^+$ cells in the spleen of immunoproteasome competent C57BL/6 (WT) or immunoproteasome deficient β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) bone marrow chimeric mice, immunized using E1 cDNA tattoo immunization, were measured by re-stimulation *ex vivo* with peptides and detection by (A) IFNγ ICS using flow cytometry (B) IFNγ ELISPOT. (A and B) Four different chimeric mice were used; B6.SJL (WT) → C57BL/6 (WT) chimera's (filled circles), β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) → B6.SJL (WT) chimera's (filled squares), B6.SJL (WT) → β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) chimera's (filled triangles) and β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) → β2i/MECL1$^{-/-}$β5i/LMP7$^{-/-}$ (KO) chimera's (filled diamonds). Every dot represents an individual mouse, corrected for IFNγ background level as measured in samples incubated with medium, and means (bars) ± SEM per peptide are indicated. Percentages of responding cells detected in ELISPOT are shown in Supplementary Fig. 2. Data are representative of three independent experiments (n > 4 animals per group), analyzed using a two-way ANOVA with Tukey's corrections (* P < .05, ** P < .01, *** P < .001, **** P < .0001).

suggesting that BM pAPC contributed but were not sufficient to induce a robust $E1B_{192-200}$-specific $CD8^+$ T cell response. Unexpectedly, $E1B_{192-200}$-specific $CD8^+$ T cells were detected also in WT mice reconstituted with KO BM, with percentages of responding $CD8^+$ T cells amounting to approximately 1%, in both assays (Fig. 3A, B). Since the sum of both KO into WT and WT into KO approximates the percentage of response observed in WT into WT animals, we conclude that in DNA tattoo immunized mice, most likely, both BM and non-BM-derived cells contribute to the processing of pAPC-presented antigens.

3.4. Langerhans cells are not responsible for dermal DNA tattoo immunization-induced $CD8^+$ T cell responses

LC are a radiation-resistant DC population [16] that has been reported to contribute to antigen processing and $CD8^+$ T cell priming [17,18]. Thus, the observed $E1B_{192-200}$-specific $CD8^+$ T cell responses in tattoo-immunized WT recipients, reconstituted with KO BM, may be explained by E1 antigen processing by the remaining WT LC population. In order to examine the contribution of LC in our model, WT and knock in (KI) mice expressing the diphtheria toxin receptor (DTR) from the $CD207^+$ promoter were reconstituted with either WT or KO BM. Prior to and following tattoo immunization, $CD207^+$ DTR^+ LC were ablated by *i.p.* injection of diphtheria toxin (for efficiency of LC ablation, see Fig. 4A and Supplementary Fig. 3, showing ablation in *non-chimeric* CD207/DTR KI mice [1]. Of note, in the chimeric mice all $CD207^+$ DC subsets, except for LC, are irradiation sensitive and have been replaced by $CD207^+$ DTR^- cells of the WT or KO BM donor at the start of the experiment). A comparison of $E1B_{192-200}$-specific $CD8^+$ T cell frequencies between WT BM reconstituted CD207/DTR KI chimeras that were treated with DT and WT BM reconstituted WT control mice showed that LC ablation enhanced rather than decreased $E1B_{192-200}$-specific responses in most mice, as measured in both IFNγ ICS and ELISPOT analysis (Fig. 4B, C, Supplementary Fig. 4). A similar pattern was observed for $E1A_{234-243}$ - specific responses in LC-ablated chimeric mice, compared to non-ablated control groups (Fig. 4B, C). Overall, the magnitudes of E1-specific $CD8^+$ T cell responses detected in this experiment were lower than in Fig. 3. Taken together, we conclude that in this experimental setup (Fig. 4), LC are not responsible for the processing and priming of $CD8^+$ T cells specific for antigens delivered by dermal DNA tattoo immunization.

4. Discussion

While accessibility and the demonstrated efficacy of cutaneous vaccination turn the skin into an attractive barrier for vaccine delivery, the antigen processing pathways underlying T cell priming by skin-delivered vaccines remain poorly characterized. Here we show that following local skin immunization, both BM- and non BM-derived cells are involved in antigen cross presentation and priming of vaccine antigen-specific $CD8^+$ T cells, in contrast to systemic immunization/infection where mainly BM-derived cells play a role. Langerhans cells were shown not to be responsible for priming of $CD8^+$ T cell responses upon local skin immunization.

In our study we were able to dissect different antigen processing and presentation routes by exploiting an immunoproteasome-dependent antigen in combination with BM transplanted mice, in which either the BM donor or the recipient lacked immunoproteasomes, and measuring induced antigen-specific $CD8^+$ T cell responses. Our experiments showed that in case of systemic rLM-E1 infection, $CD8^+$ T cell responses to the

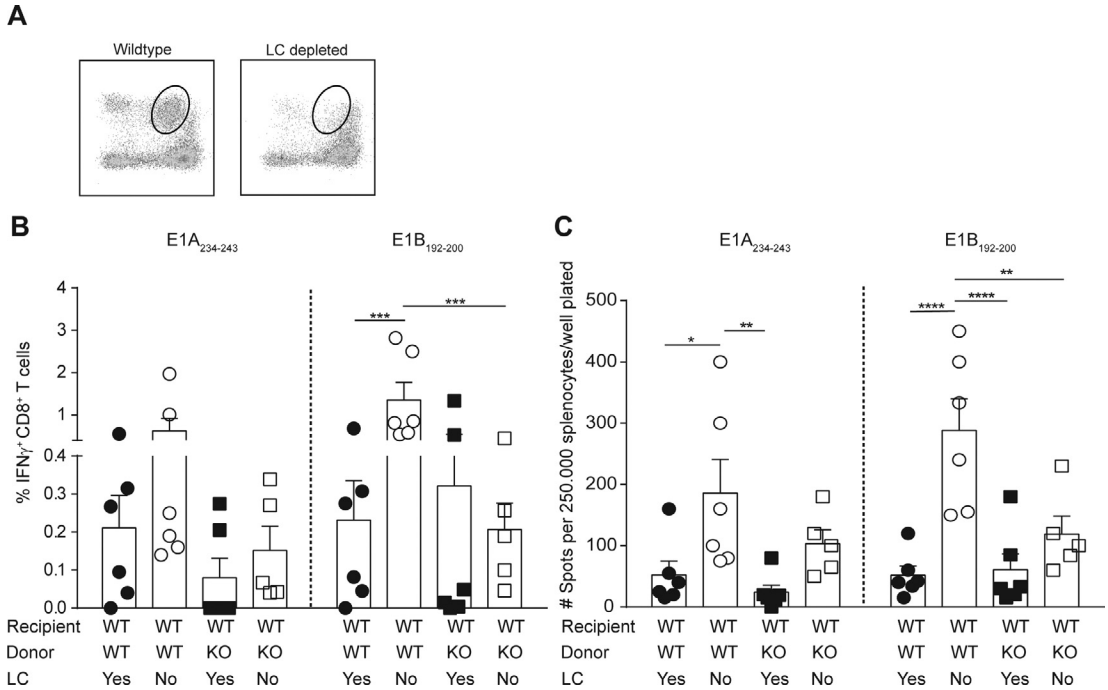

Fig. 4. LC are not responsible for the priming of $CD8^+$ T cell response upon dermal DNA tattoo immunization. (A) LC were ablated from B6.129S2-$CD207^{tm3(DTR/GFP)Mal}$/J mice by a single DT injection. Depletion efficiency was measured two days later. (B and C) Four chimeric mouse groups were made: B6.SJL (WT) → C57BL/6 (WT) chimera's (filled circles), B6.SJL (WT) → LC depleted B6.129S2-$CD207^{tm3(DTR/GFP)Mal}$/J (WT) chimera's (open circles) squares), β2i/$MECL1^{-/-}$β5i/$LMP7^{-/-}$ (KO) → B6.SJL (WT) chimera's (filled squares) and β2i/$MECL1^{-/-}$β5i/$LMP7^{-/-}$ (KO) → LC depleted B6.129S2-$CD207^{tm3(DTR/GFP)Mal}$/J (WT) chimera's (open squares). Percentages of E1A or E1B-specific $CD8^+IFN\gamma^+$ T cells or numbers of $IFN\gamma^+$ cells in the spleens of mixed BM chimeric mice, immunized using E1 cDNA tattoo immunization, were measured by re-stimulation *ex vivo* with peptides and detection by (B) IFNγ ICS using flow cytometry and (C) IFNγ ELISPOT. (B and C) Every dot represents an individual mouse, corrected for IFNγ background level as measured in samples incubated with medium, and means (bars) ± SEM per peptide are indicated. Percentages of responding cells in ELISPOT are shown in Supplementary Fig. 4. Data are representative of one experiment (n = 6 animals per group), analyzed using a two-way ANOVA with Tukey's corrections (* P < .05, ** P < .01, *** P < .001).

immunoproteasome-dependent $E1B_{192-200}$ epitope were induced only in mice in which BM−derived cells contained immunoproteasomes (Fig. 2B), confirming earlier studies [14,19,20]. Thus, in Listeria infection, the non-lymphoid tissues, *i.e.* the liver, despite being a significant harbor of pathogen, do not serve as antigen donor for BM-derived pAPC that prime antigen-specific $CD8^+$ T cells. This is in contrast to mice immunized by dermal DNA tattoo immunization with a TTFC-E1-encoding vector, in which we measured a ~50% reduction in E1B-specific responses if either donor or recipient were deficient in immunoproteasomes (Fig. 3A, B). $CD8^+$ T cell activation in mice transplanted with immunoproteasome-deficient BM could not be explained by the presence of radiation-resistant WT LC (Fig. 4B, C), on the contrary, absence of LC seemed to enhance the responses. These data point at a role for both BM and non BM-derived cells in processing of DNA tattoo-delivered vaccine antigens.

The notion that non-BM-derived cells contribute to the processing of pAPC-presented antigens implies a transport of processed peptides from these cells to DC prior to presentation. One option for such peptide transport would be by cross-dressing, which is a way of cross presentation [21] in which intact p-MHC-I from the surface of a donor cell are transferred to that of an APC [22,23]. Cross dressing can take place via different ways [24], *e.g.* trogocytosis [25], exosomes [26] or tunneling nanotubes [27]. In virus infection, cross-dressed DC were reported to have a crucial role in activating memory, but not naïve T cells [23]. On the contrary, following dermal gene-gun vaccination, cross-dressed DC which presented keratinocyte derived MHC class I – peptide complexes, were shown to activate both naïve and memory $CD8^+$ T cells [28]. Since the threshold for peptide amount required for activation is higher for naïve- than memory $CD8^+$ T cells, the observed discrepancy between the two studies might be related to antigen levels. These levels might be higher in local (gene gun) immunization, resulting in effective contribution of cross dressing to the activation of naïve $CD8^+$ T cells. Such differences in local antigen load might also explain our observation that, in systemic infection, BM-derived cells perform the antigen processing steps required for $CD8^+$ T cell priming, while the absence of immunoproteasomes in the peripheral tissues marginally influences this process, in contrast to cutaneous DNA tattoo immunization where non BM-derived cells contribute significantly.

Next to how vaccine antigens are processed and presented, the presence of cells at the site of immunization that may either support or inhibit immune activation should be considered in vaccine design. In our study, LC appeared to interfere with T cell activation, in agreement to data obtained in a study by Flacher and colleagues [29], as well as in contact hypersensitivity models [4]. Nevertheless, in the same models, LC also have been shown to support $CD8^+$ T cell priming [2,3]. The discrepancy in this seemingly conflicting data potentially lies in another subset of langerin-positive DC located in the dermis, the $CD11b^-CD24^+$ dermal cDC, also called cDC1, $XCR1^+$ DC or Ln^+ dDCs [30–32]. They are capable of presenting keratinocyte-dependent antigens leading to $CD8^+$ T cell activation [30] and due to their CD207 expression, they resemble LC very closely which might have influenced the outcome of these studies.

Thus by studying antigen processing and presentation in mice that lack the processing machinery in specific cell subsets or miss LC, we have provided evidence for multiple ways of cross presentation upon dermal DNA tattoo immunization, with not only a role for DCs but also for cells from the periphery. This knowledge may be exploited to optimize vaccines that are administered in the skin.

Competing financial interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.10.044.

References

[1] Kissenpfennig A, Henri S, Dubois B, Laplace-Builhé C, Perrin P, Romani N, et al. Dynamics and function of langerhans cells in vivo: Dermal dendritic cells colonize lymph node areasdistinct from slower migrating langerhans cells. Immunity 2005;22(5):643–54.
[2] Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity 2005;23(6):611–20.
[3] Bennett CL, Van Rijn E, Jung S, Inaba K, Steinman RM, Kapsenberg ML, et al. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. J Cell Biol 2005;169(4):569–76.
[4] De Agüero MG, Vocanson M, Hacini-Rachinel F, Taillardet M, Sparwasser T, Kissenpfennig A, et al. Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8 + T cells and activating Foxp3 + regulatory T cells. J Clin Invest 2012;122(5):1700–11.
[5] Aki M, Shimbara N, Takashina M, Akiyama K, Kagawa S, Tamura T, et al. Interferon-γ induces different subunit organizations and functional diversity of proteasomes. J Biochem 1994;115(2):257–69.
[6] Liepe J, Holzhütter HG, Bellavista E, Kloetzel PM, Stumpf MPH, Mishto M. Quantitative time-resolved analysis reveals intricate, differential regulation of standard- and immuno-proteasomes. eLife 2015;4:e07545 2015.
[7] Mishto M, Liepe J, Textoris-Taube K, Keller C, Henklein P, Weberruß M, et al. Proteasome isoforms exhibit only quantitative differences in cleavage and epitope generation. Eur J Immunol 2014;44(12):3508–21.
[8] Platteel ACM, Mishto M, Textoris-Taube K, Keller C, Liepe J, Busch DH, et al. CD8+ T cells of Listeria monocytogenes-infected mice recognize both linear and spliced proteasome products. Eur J Immunol 2016;46(5):1109–18.
[9] Textoris-Taube K, Keller C, Liepe J, Henklein P, Sidney J, Sette A, et al. The T210M substitution in the HLA-A*02:01 gp100 epitope strongly affects overall proteasomal cleavage site usage and antigen processing. J Biol Chem 2015;290(51):30417–28.
[10] Deol P, Zaiss DMW, Monaco JJ, Sijts AJAM. Rates of processing determine the immunogenicity of immunoproteasome- generated epitopes. Journal of Immunology 2007;178(12):7557–62.
[11] Bins AD, Jorritsma A, Wolkers MC, Hung C-, Wu T-, Schumacher TNM, et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. Nat Med 2005;11(8):899–904.
[12] Oosterhuis K, Hlschläger P, Van Den Berg JH, Toebes M, Gomez R, Schumacher TN, et al. Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. Int J Cancer 2011;129(2):397–406.
[13] Oosterhuis K, Van Den Berg JH, Schumacher TN, Haanen JBAG. DNA vaccines and intradermal vaccination by DNA tattooing. Curr Top Microbiol Immunol 2012;351(1):221–50.
[14] Sijts AJAM, Standera S, Toes REM, Ruppert T, Beekman NJCM, Van Veelen PA, et al. MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. Journal of Immunology 2000;164(9):4500–6.
[15] Platteel ACM, De Groot AM, Keller C, Andersen P, Ovaa H, Kloetzel PM, et al. Strategies to enhance immunogenicity of cDNA vaccine encoded antigens by modulation of antigen processing. Vaccine 2016;34(42):5132–40.
[16] Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol 2002;3(12):1135–41.
[17] Stoitzner P, Tripp CH, Eberhart A, Price KM, Jung JY, Bursch L, et al. Langerhans cells cross-present antigen derived from skin. Proc Natl Acad Sci U S A 2006;103(20):7783–8.
[18] Mi Q-, Xu Y-, Qi R-, Shi Y-, Zhou L. Lack of microRNA miR-150 reduces the capacity of epidermal langerhans cell cross-presentation. Exp Dermatol 2012;21(11):876-7.
[19] Zaiss DMW, Standera S. Kloetzel P-, Sijts AJAM. PI31 is a modulator of proteasome formation and antigen processing. Proc Natl Acad Sci U S A 2002;99(22):14344–9.
[20] Toes REM, Offringa R, Blom RJJ, Brandt RMP, Van der Eb AJ, Melief CJM, et al. An adenovirus type 5 early region 1B-encoded CTL epitope-mediating tumor eradication by CTL clones is down-modulated by an activated ras oncogene. J Immunol 1995;154(7):3396–405.
[21] Norbury CC. Defining cross presentation for a wider audience. Curr Opin Immunol 2016;40:110–6.

[22] Dolan BP, Gibbs Jr KD, Ostrand-Rosenberg S. Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells. J Immunol 2006;177(9):6018–24.
[23] Wakim LM, Bevan MJ. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. Nature 2011;471(7340):629–31.
[24] Campana S, De Pasquale C, Carrega P, Ferlazzo G, Bonaccorsi I. Cross-dressing: an alternative mechanism for antigen presentation. Immunol Lett 2015;168(2):349–54.
[25] Hudrisier D, Riond J, Mazarguil H, Gairin JE, Joly E. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. J Immunol 2001;166(6):3645–9.
[26] Andre F, Schartz NEC, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. Lancet 2002;360(9329):295–305.
[27] Watkins SC, Salter RD. Functional connectivity between immune cells mediated by tunneling nanotubules. Immunity 2005;23(3):309–18.
[28] Li J, Kim S, Herndon JM, Goedegebuure P, Belt BA, Satpathy AT, et al. Cross-dressed CD8a+/CD103+ dendritic cells prime CD8+ T cells following vaccination. Proc Natl Acad Sci U S A 2012;109(31):12716–21.
[29] Flacher V, Tripp CH, Mairhofer DG, Steinman RM, Stoitzner P, Idoyaga J, et al. Murine Langerin+ dermal dendritic cells prime CD8+ T cells while Langerhans cells induce cross-tolerance. EMBO Mol Med 2014;6(9):1191–204.
[30] Henri S, Poulin LF, Tamoutounour S, Ardouin L, Guilliams M, De Bovis B, et al. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. J Exp Med 2010;207(1):189–206.
[31] Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol 2009;10(5):488–95.
[32] Henri S, Guilliams M, Poulin LF, Tamoutounour S, Ardouin L, Dalod M, et al. Disentangling the complexity of the skin dendritic cell network. Immunol Cell Biol 2010;88(4):366–75.