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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 b5i/LMP7 and b2i/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, E1B192-200, required immunoproteasome-mediated antigen processing. CD8+ T cell responses to a second epitope derived from this antigen, E1A234-243, 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-dipheria toxin receptor knock in (Kt) – 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 E1A234-243 (SGPSNTPPEI) and E1B192-200 (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 (Innogenet).Recipient mice were irradiated with 9 Gy as a single dose from an X-ray irradiator and reconstituted with 10^5 BM cells. They were allowed to reconstitute for 6 weeks. C57BL/6 J mice were purchased from Charles River, B6.129S2-Cd207tm3(DTR/GFP)B6.129S2-CD207tm3(DTR/GFP)Mal/J from Jackson and B6.SJL mice and 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).

2.3. LC Depletion

Depletion of LC in bone marrow chimeric mice in which B6.129S2-Cd207tm3(DTR/GFP)B6.129S2-CD207tm3(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 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 E1B192-200 VNIRNCYI or E1A234-243 SGPSNTPPEI for 6 h at 37 °C in RPMI 1640 medium supplemented with 10% FCS-HI (Lonza), 2 mM L-glutamine, 30 μM 2-mercaptopoethanol (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; Biologend), CD45.2-FITC (clone 104; Biologend) 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). MxB was 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 Student’s 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 pDNA 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 \[^{b2i/MECL1^\(-/-)}^{b5i/}\] 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\(_{192-200}\)-specific CD8\(^+\) T cells were quantified in the spleen (Fig. 1A) using IFN\(_\gamma\) 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

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**Fig. 1.** E1 cDNA tattoo immunization only elicits E1B-specific CD8\(^+\) T cells in immunoproteasome competent mice. (A) C57BL/6 (WT) and \[^{b2i/MECL1^\(-/-)}^{b5i/}\] 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 IFN\(_\gamma\) CD8\(^+\) T cells was measured. (C) Percentages of E1A or E1B-specific IFN\(_\gamma\) CD8\(^+\) T cells in the spleen of immunoproteasome competent C57BL/6 (WT, filled circles) or immunoproteasome deficient \[^{b2i/MECL1^\(-/-)}^{b5i/}\] LMP7\(^{-/-}\) (KO, filled squares) mice, immunized using E1 cDNA tattoo immunization, were measured by re-stimulation *ex vivo* with peptides and detected by IFN\(_\gamma\) ICS and flow cytometry. Every dot represents an individual mouse, corrected for IFN\(_\gamma\) 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).
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-dependent E1A234-243 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 E1B192-200 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 E1B192-200.
sugesting 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 IFN-γ 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
immunoproteasome-dependent E1B_{52-200} 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 LIN+ 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.

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