Batf3 selectively determines acquisition of CD8+ dendritic cell phenotype and function

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Batf3 is a transcription factor that impacts the development of CD103+ tissue-resident dendritic cells (DCs). However, whether Batf3 is absolutely required for the development of CD8+ DCs remains controversial. Id2 is required for CD8+ DC development. Here we show that bone marrow chimeric mice with a deletion of Id2 in the CD11c compartment lose the ability to reject a skin graft expressing a non-self protein antigen or mount a delayed hypersensitivity response. In contrast, Batf3−/− mice remained competent for skin graft rejection and delayed hypersensitivity, and retained a CD8+ DC population with markers characteristic of the CD11b+ DC lineage, including CD11b, CD4 and CD172α, as well as the key regulator transcription factor IRF4, but lacked IRF8 expression. CD8+ DCs in Batf3−/− mice took up and cleaved protein antigen and larger particles but were unable to phagocyte dying cells, a characteristic feature to the CD8+ DC lineage. These data clarify a requirement for CD8+ lineage DCs to induce effectors of neo-antigen-driven skin graft rejection, and improve our understanding of DC subtype commitment by demonstrating that in the absence of Batf3 CD8+ DCs can change their fate and become CD11b+ DCs.

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INTRODUCTION

Professional antigen-presenting cells (APCs), including dendritic cells (DCs) in the skin and draining lymph nodes, play a critical role in maintaining tolerance of the adaptive immune system to beneficial skin microbiota, and conversely in priming T cells specific to proteins from viruses infecting the skin, including herpes-simplex and human papillomavirus.1-4 According to our current understanding, conventional DCs (cDCs) can be categorized into two main lineages, namely CD8+ DCs and CD11b+ DCs,5 with the CD8+ DC lineage being critical in promoting immunity to viral skin infection.1 Both CD11b+ and CD8+ lineages develop from common DC precursors in the bone marrow, but their functional maturation depends on different transcription factors. Development of CD8+ DCs requires Irf8, Id2, Nfil3 and Batf3, whereas development of CD11b+ DCs depends on RelB, Notch2 and Irf4.6 The CD8+ DC lineage is of particular interest because of their ability to take up cellular material, cross-present antigen and activate CD8+ cytotoxic T cells.1,7-11 This lineage includes tissue-migratory CD103+ DCs as well as the lymphoid tissue-resident CD8+ DCs.

Mice lacking the CD8+ DC lineage, including IRF8−/−, Batf3−/− and Id2flx/flox-CD11cCre+ mice, have demonstrated the importance of these DCs in a variety of disease models.12-20 However, recent reports suggest that while Batf3 may be crucial in the development of CD103+ DCs, its requirement for the development of CD8+ lymphoid tissue-resident DCs can be bypassed by other factors. For example, Flt3L-driven bone marrow cultures from Batf3−/− mice give rise to CD8+ but not CD103+ DCs.21 Furthermore, infection with Mycobacterium tuberculosis was shown to restore both CD8+ and lung-resident CD103+ DCs in Batf3−/− mice via induction of IL-12 and enabled control of the intracellular bacterium comparable to wild-type mice.22 Recently, development of CD8+ DCs in Batf3−/− mice was shown to differ between different animal housing facilities.23 Petersen et al. showed that a CX3CR1-Langerin-CD8+ DC subset could be found in Batf3−/− mice, which they proposed to be a non-functional precursor of mature Langerin+ CD8+ DCs.24 In a recent study, bone marrow-derived pre-CD8 DCs treated with GM-CSF did not complete their development into functional CD8+ DCs in the absence of Batf3, but instead diverted to the CD11b+ lineage through the expression of CD172α,25 an observation that allows speculation about plasticity within different pre-committed DC precursors.

In the present report, we used two mouse models to determine the role of CD8+ lineage DCs in neo-antigen-driven skin graft rejection and delayed type hypersensitivity (DTH) to broaden our understanding on immune responses to skin tropic viral diseases or skin-targeted autoimmunity.26,27 Here, we used Id2flx/flox-CD11cCre+ chimeric mice, which lack CD8+ lineage DCs, to show that these are required for neo-antigen-driven skin graft rejection and DTH. We show further that a residual CD8+ DC population exists in Batf3−/− mice that are competent for skin graft rejection and DTH. We characterized the difference in functional capacity of the different APCs in Batf3−/− animals, and provide evidence to show that in the

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The fate of CD8+ DCs in the absence of Batf3

J Chandra et al

RESULTS AND DISCUSSION

Induction of DTH and rejection of neo-antigen-expressing skin grafts are driven by CD8+ lineage DCs, but are Batf3 independent

To investigate the importance of CD8+ lineage DCs, including CD103+ migratory and CD8+ lymphoid tissue-resident DCs, in the process of neo-antigen-driven skin graft rejection, we employed two transgenic mouse models with deficits in APC function. We grafted OVA-expressing skin from K5mOVA transgenic mice onto mice chimeric for an Id2-deleted CD11c+ lineage (Id2fox/fox-CD11cCre+ chimeras), or onto mice deleted of the Batf3 transcription factor (Batf3−/− mice). Id2fox/fox-CD11cCre+ chimeras were unable to reject K5mOVA skin grafts, whereas there was no difference in skin graft rejection between Batf3−/− and control mice (Figure 1a). To assess the OVA-specific CD8+ T-cell response generated in response to K5mOVA skin grafting, we restimulated lymph node cells of K5mOVA skin-grafted mice with the MHC class I-restricted OVA peptide SIINFEKL, and determined the number of primed OVA-specific CD8+ T cells by IFNγ ELISPOT. Lymph node cells from K5mOVA skin-grafted Id2fox/fox-CD11cCre+ chimeras showed a significantly reduced number of OVA-specific CD8+ IFNγ-secreting T cells when compared with grafted control mice, in keeping with their failure to

Figure 1 Batf3-independent but not Id2-independent CD8+ DCs contribute to neo-antigen-driven skin graft rejection and DTH. (a) Ear skin of C57BL/6 (left) and K5mOVA (right) mice was grafted onto Id2fox/fox-CD11cCre+ (fox/fox) and control chimeras or Batf3−/− and control mice. Grafts were assessed and measured weekly for 7–8 weeks. Shown are representative photographs of one mouse per group. The size of grafts was depicted using imaging software. Each point represents one animal, with indication of mean ± s.d. of the whole group (n=5-6). Graft survival curves depict loss of grafts defined as necrosis and ulceration. (b) At termination of the grafting assessment, lymph node cells were restimulated with SIINFEKL and IFNγ-producing T cells were counted by ELISPOT. Each point represents one animal with indication of mean ± s.d. of whole group (n=5,6). (c, d) Id2fox/fox-CD11cCre+ (fox/fox) and control chimeras (c) or Batf3−/− and control mice (d) were immunized intradermally with OVA and QuilA. Seven days later, mice were challenged with OVA delivered to ear skin. Ear thickness was measured before and up to 72 h post OVA injection. Each point represents mean ± s.e.m. of the whole group (n=10 of two independent pooled experiments for c, n=5 of one independent experiment for d) at 24, 48 and 72 h post OVA challenge. (e) Id2fox/fox-CD11cCre+ (fox/fox) and control mice were immunized intradermally with OVA and QuilA. Seven days later, mice received a 1:40 ratio of CFSE labelled naked reference and SIINFEKL-pulsed target cells delivered intravenously. Spleens were analysed for killing of target cells 18 h after transfer by flow cytometry. The graph indicates the number of target cells per 500 reference cells. CU: Control Unimmunized. Each point represents one animal with indication of mean ± s.d. of the whole group (n=5). Shown is one of two independent experiments. For statistics, transgenic mice were compared with controls. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (unpaired two-tailed t-test). A full colour version of this figure is available at the Immunology and Cell Biology journal online.
reject K5mOVA grafts (Figure 1b). Batf3−/− mice also had reduced numbers of OVA-specific CD8+ IFNγ-secreting T cells in lymph nodes after receipt of a K5mOVA skin graft, although graft rejection was comparable to control mice (Figure 1b).

In a DTH model we tested the immune response to antigens delivered intradermally further. In line with the previous data, we found that Id2fl/fl-CD11cCre+/− chimeras were significantly restricted in their ability to mount a DTH response (Figure 1c), whereas Batf3−/− mice responded in comparison to the control mice (Figure 1d). To investigate if cytotoxicity was affected in response to the intradermally delivered antigens in the absence of CD8+ lineage DCs, we assessed the killing of SIINFEKL antigen-pulsed target cells in OVA-immunized mice. We found that, similar to the limited IFNγ response, both Id2fl/fl-CD11cCre+/− chimeras and Batf3−/− mice were significantly restricted in their ability to kill target cells (Figure 1e).

Our data suggest that expression of the Id2 transcription factor in CD8+ lineage DCs is critical for CD8+ T-cell priming, DTH and antigen-driven graft rejection, and that also the Batf3 transcription factor is required for APCs to induce a maximal cytotoxic CD8+ T-cell response. However, in the absence of Batf3, both DTH and neo-antigen-driven skin graft rejection can be induced. In a previous study, minor antigen-driven (H-Y) skin graft rejection was dependent on Batf3-dependent APCs. Differences in either the type of antigen, soluble or membrane located, or the distribution of antigen expression, on keratinocytes or on all somatic cells, might explain these differences. However, previous findings showing that a contact hypersensitivity response is Batf3-independent is consistent with the DTH we describe here.12

**Batf3−/− mice contain a residual population of CD8+ DCs**

A possible lack of CD8+ DCs in Batf3−/− mice has been proposed, with unclear experimental findings. We therefore analysed the peripheral DC compartment in Batf3−/− mice and Id2fl/fl-CD11cCre+/− mice and observed a significant reduction, but not a complete absence, of CD8+ DCs in the spleen of both these mice, when compared to control animals (Figure 2a). However, the reduction of CD8+ DCs in Id2fl/fl-CD11cCre+/− chimeras was more pronounced than in Batf3−/− mice. Additionally, we observed a significant increase of CD11b+ DCs in the spleen of Batf3−/− mice, and a lesser but significant reduction in Id2fl/fl-CD11cCre+/− chimeras. CD103+ DCs in skin were absent in both the mouse models (Figure 2b). In lymph nodes of Batf3−/− mice, we found a significant reduction in CD8+ DCs and CD103+ DCs, and a significant increase in CD11b+ DCs (Supplementary Figure 2).

**In the absence of Batf3, CD8+ DCs acquire a phenotype characteristic to the CD11b+ DC lineage**

Diversification of bone marrow-derived CD8+ pre-DCs to the CD11b+ DC lineage, mediated by GM-CSF and in the absence of Batf3, has recently been described.25 We therefore examined DCs for a range of characteristic DC membrane proteins, and observed that the residual CD8+ DCs in Batf3−/− spleens expressed the surface markers that characterize the CD11b+ DC lineage. In detail, CD8+ DCs in Batf3−/− spleens expressed various and significantly higher levels of CD11b and CD172α compared to CD8+ DCs in control mice, and were partly CD4+ (Figure 3a–c). This could be clearly observed by visual inspection of flow cytometry data where we plotted CD8 against CD11b, CD4 and CD172α on DCs (Figure 3a), and by comparing mean fluorescent intensities (MFI) of these markers expressed on CD8+ DCs (Figure 3b and c). In contrast, no up-regulation of CD11b was observed in the small population of CD8+ DCs in Id2fl/fl-CD11cCre+/− chimeras. These data suggest that, in the absence of Batf3, peripheral CD8+ DCs in secondary lymphoid tissues divert to the CD11b+ DC lineage, and no such phenomenon can be observed in DCs lacking Id2.

**Batf3−/− CD8+ DCs process antigen and take up particles but lose the capacity to phagocytose dying cells**

Next, we investigated whether the residual CD8+ DC population in Batf3−/− mice could process exogenous antigen effectively. We incubated splenocytes from Batf3−/− and control mice with a self-quenching dye coupled to OVA (DQ-OVA), which fluoresces on tryptic cleavage, and analysed cleavage of the DQ-OVA in CD8+ and CD11b+ DCs (Figure 4a). We observed that both CD8+ and CD11b+ DCs cleaved DQ-OVA, and that CD8+ DCs showed greater MFI than CD11b+ DCs (Figure 4a). DQ-OVA processing in CD11b+ DCs was comparable in Batf3−/− and control splenocytes. Surprisingly, the residual population of CD8+ DCs in Batf3−/− mice processed more DQ-OVA than control CD8+ DCs, shown both by an increased percentage and by MFI, suggesting that these residual Batf3−/− CD8+ DCs can process antigen efficiently. We further assessed phagocytic function by incubating splenocytes with dye-labelled liposomes. We found that both CD8+ DCs and CD11b+ DCs of control and Batf3−/− mice took up liposomes in comparable amounts, whereas no uptake was observed when samples were incubated on ice (Figure 4b).

A characteristic feature of CD8+ DCs is their superior capacity to endocytose dying cells. We therefore determined whether the residual CD8+ DC population in Batf3−/− mice was able to take up dead cells. We injected labelled dying cells into Batf3−/− and control mice and found that endocytosis of dying cells was restricted to CD8+ DCs in control mice, while CD11b+ DCs showed no uptake of dying cells (Figure 4c). Interestingly, the residual population of CD8+ DCs in Batf3−/− mice were largely unable to take up dying cells (Figure 4c), demonstrating a loss of function and suggesting that these DCs do not functionally resemble the classical CD8+ DCs found in immunocompetent control mice. In support of this hypothesis, we recognized that a small fraction of the Batf3−/− CD8+ DCs that had endocytosed dying cells were CD11b negative, and no Batf3−/− CD8+ DCs had endocytosed cells (Figure 4d). These data further suggest a process of DC divergence from the CD8+ to the CD11b+ DC lineage in the absence of Batf3, with loss of specific function of dead cell phagocytosis but not of antigen uptake and processing or uptake of particles in general.

In conclusion, functional commitment of APC precursors to either the conventional CD8+ lineage or the CD11b+ lineage DCs had originally been held to be irreversible, with development of CD8+ lineage DCs dependent on transcription factors IRF8 and Id2, whereas...
Figure 2 Batf3−/− mice contain a residual population of CD8+ DCs and increased numbers of CD11b+ DCs. (a) Splenocytes of Batf3−/−, Id2flx/flx-CD11cCre+ chimeras and control mice were analysed by flow cytometry. CD11c+ MHCII+ DCs (pre-gated on live singlets, CD19neg, TCRβneg) were assessed for expression of CD8 and CD11b. Each row of flow cytometry plots stems from one representative mouse per group. Each point in the graph represents one animal with indication of mean ± s.d. of the whole group (n=5–10, pooled from up to three independent experiments). (b) Ear skin of Batf3−/−, Id2flx/flx-CD11cCre+ chimeras and control mice was separated into dermis and epidermis. Live singlets, CD45+ cells were assessed for a population of MHCII+ CD11c+ DCs that was further gated onto CD103+ DCs by flow cytometry. In the graph, each point represents one animal with indication of mean ± s.d. of the whole group. For Batf3−/− mice shown are data pooled from two independent experiments (n=7). For Id2flx/flx-CD11cCre+ chimeras shown is one representative from two independent experiments, n=3. For statistics, transgenic mice were compared with controls. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (unpaired two-tailed t-test).
Figure 3 Batf3-independent but not Id2-independent CD8⁺ DCs express CD11b, CD4 and CD172α. (a) DCs in spleens of Id2floxflox-CD11cCre⁺ chimeras (floxflox) and controls, or Batf3⁻⁻ and controls were gated as live singlets, CD19neg, TCRβneg, MHCII⁺ and CD11c⁺ as depicted in Figure 2. From these cells, expression of CD8 was plotted against CD11b, CD4 and CD172α. Shown are flow cytometry plots for one representative mouse per group. (b) DCs gated as depicted in (c) were further gated onto CD8⁺ DCs and CD11b⁺ DCs according to the gating strategy used in Supplementary Figure 1. Overlay histograms represent expression of CD11b, CD4 and CD172α of CD8⁺ DCs (black line) and CD11b⁺ DCs (grey line). (c) The median fluorescent intensity of CD11b, CD4 and CD172α of CD8⁺ DCs in Id2floxflox-CD11cCre⁺ (floxflox), Batf3⁻⁻ and control mice from the data shown in C and D was compared. Each point represents one animal with indication of mean ± s.d. of the whole group (n=4–5, pooled from 2 of 4 independent experiments). For statistics, transgenic mice were compared with controls. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (unpaired two-tailed t-test).
The fate of CD8⁺ DCs in the absence of Batf3

J Chandra et al

Immunology and Cell Biology

the Batf3 transcription factor enabled DC survival. A recent study showed that development in the bone marrow of CD8⁺ lineage DCs requires auto-activation of IRF8, and that Batf3 was required to maintain IRF8 activation and that, in the absence of Batf3, pre-CD8⁺ DCs lost IRF8 expression and diverted to the CD11b⁺ DC lineage. We show here that this process applies not only to pre-DCs in the bone marrow, but also to mature CD8⁺ DCs in secondary lymphoid tissues. We show further that phenotypically mature CD8⁺ lineage DCs

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The fate of CD8⁺ DCs in the absence of Batf3

J Chandra et al

Immunology and Cell Biology

develop and survive in the absence of Batf3, and that these DCs can contribute to neo-antigen-driven skin graft rejection and to development of delayed type hypersensitivity. As both antigen-specific IFNγ release and CD8+ T cell cytotoxic functions are significantly reduced in the absence of Batf3, the mechanisms by which DCs lacking Batf3 induce these reactions is unclear. Batf3−/− CD8+ lineage DCs express surface markers CD11b, CD172α and CD4, and key regulator transcription factor IRF4, all characteristic of CD11b+ lineage DCs, but lack expression of IRF8, characteristic of the CD8+ lineage, and fail to endocytose dying cells. Further studies are required to investigate the stability of these characteristics, and whether they can be further altered in the periphery in response to inflammation, which would have implications for our understanding of the function of particular DC subsets in health and disease.

**METHODS**

**Mice and chimeras**

C57BL/6 and C57BL/6-SJL were obtained from the Animal Resources Centre (Perth, Western Australia, Australia). K5mOVA mice were obtained from H Azukizawa (Osaka, Japan)27 and bred in house. OT-I mice were obtained from F Cabone (Melbourne, Victoria, Australia)19 and bred in house with CD11cCre+ mice to obtain Id2flox/flox mice, but lack expression of IRF8, characteristic of the CD8+ lineage, and fail to induce these reactions is unclear. Batf3−/− mice were used as Batf3+/- or C57BL/6-SJL mice (Supplementary Figure 1). All mice were kept under specific pathogen-free conditions at the Biological Research Facilities of Translational Research Institute and Otto Hirschfield Facility of the University of Queensland. All animal procedures and experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia, with approval from the IMVS Animal Ethics Committee and the University of Queensland Animal Ethics Committee (#367-13).

**Skin grafting**

Ear skin was transplanted onto recipient flanks as described previously.30 Briefly, donor ear skin was split into dorsal and ventral surfaces (−1 cm²) and placed onto the thoracic flank region of anaesthetized recipients. Grafts were covered with antibiotic-impregnated gauze (Bactigras, Smith and Nephew, London, UK) and bandaged with a micropore tape and Flex-wrap (Lyppard, Queensland, Australia). Bandages were removed 7 days later and grafts were monitored for 7–8 weeks. Photographs including a ruler were taken weekly and grafts were analysed by measurement using Fiji Imaging software. Graft rejection was defined as loss of distinct border, signs of ulceration and necrosis.

**DTH**

Mice were immunized intradermally to the flank with 100 µg OVA (Sigma) and 20 µg QuilA in a volume of 200 µl. Seven days later, mice were challenged with 10 µg OVA delivered intradermally to the right ear in a volume of 20 µl. Ear thickness was determined by caliper measurement before OVA challenge and 24, 48 and 72 h after OVA challenge. Phosphate-buffered saline (PBS) injection to the left ear was followed as negative control.

**In vivo cytotoxicity assay**

Mice were immunized intradermally to the flank with 100 µg OVA (Sigma) and 20 µg QuilA in a volume of 200 µl. Seven days later, mice received a 1:40 ratio of CFSE labelled CD45.1+ reference splenocytes and CD45.2+ SIINFEKL-pulsed target splenocytes (total number 10 x 10⁶) by intravenous injection. Target cells were pulsed with 500 ng ml⁻¹ SIINFEKL for 1 h at 37 °C in medium containing 10% FCS. Spleens were harvested 18 h after target cell transfer, prepared as single cell suspension and assessed by flow cytometry for the presence of CFSE+ CD45.1+ and CD45.2+ cells.

**Figure 4** IRF4-expressing CD8+ DCs in Batf3−/− mice are able to process antigen but lose capacity to take up dying cells. (a) Splenocytes of Batf3−/− and control mice were incubated with DQ-OVA for 3 h and subsequently analysed by flow cytometry for DQ-OVA processing. CD8+ and CD11b+ dendritic cell subsets were gated as shown in Figure 2. Shown are representative histograms of green fluorescence (DQ-OVA) of treated versus untreated CD8+ and CD11b+ DCs in Batf3−/− and control splenocytes. The percentage and MFI of DQ-OVA+ CD8+ or CD11b+ DCs as well as of untreated samples (−) was compared. Each point represents a sample of one animal with indication of mean ± s.d. of the whole group (n=4). Shown is one of two independent experiments. (b) Splenocytes of Batf3−/− and control mice were incubated with Dil-labelled liposomes for 90 min and subsequently analysed by flow cytometry for liposome uptake. Shown are representative histograms of red fluorescence (Dil+) of CD8+ and CD11b+ DCs in Batf3−/− and control splenocytes incubated at 37 °C or on ice. The percentage of Dil+ cells of CD8+ or CD11b+ DCs was compared. Each point represents a sample of one animal with indication of mean ± s.d. of the whole group (n=5). (c, d) CellTrace Violet-labelled, UV-irradiated dying cells were injected into Batf3−/− and control mice. Three hours later, splenic CD11b+ and CD8+ DCs were analysed for endocytosis of dying cells. (c) Shown are flow cytometry plots that were pre-gated on DCs as shown in Supplementary Figure 1, then CellTrace Violet was plotted against CD8+ and CD11b+. CellTrace Violet+ cells depict DCs that endocytosed dying cells. In the graph, shown are percentages of CellTrace Violet+ cells of CD8+ and CD11b+ DCs. Each point represents one animal with indication of mean ± s.d. of the whole group (representative of three independent experiments, n=4). (d) Whole DCs (grey) and CellTrace Violet+ CD8+ DCs (black) of one representative Batf3−/− mouse from (c) was plotted against CD11b to indicate that CellTrace Violet+ CD8+ DCs are CD11b+. (e, f) Splenocytes of Batf3−/− and control mice were surface stained for DC markers and intracellular stained for IRF4 and IRF8. Splenocytes were pre-gated on live singlets, CD19neg, TCRneg, MHCIId+ and CD11c+, CD8+ or CD11b+ (as depicted in Figure 2). (e) Shown are representative histograms of IRF4 and IRF8 expression of CD8+ (black line) and CD11b+ DCs (grey line) of one mouse per group. (f) Mean fluorescent intensities of IRF4 and IRF8 in CD8+ and CD11b+ DCs are depicted. Each point represents one animal with indication of mean ± s.d. of the whole group. Shown are the data of two pooled independent experiments (n=7). For statistics, transgenic mice were compared with controls. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (unpaired two-tailed t-test).
Cell isolations
Mice were euthanized in CO₂ and organs were removed. For DC experiments, spleens were injected with or lymph nodes (axial, inguinal) were cut into small pieces and digested with 0.5 mg ml⁻¹ collagenase D (Roche, Berlin, Germany) diluted in PBS 10% FCS for 30 min at 37 °C. Spleens and lymph nodes were disintegrated by straining through a 70 μm cell strainer (BD Pharmingen, North Ryde, NSW, Australia). Splenocytes were red blood cell lysed using ACK buffer. For adoptive transfer of OT-I- KbNzegFP CD8⁺ T cells and ELISPOT assays single cell suspensions of lymph nodes (axial, inguinal and ear-draining) and spleens were prepared by mechanically disrupting the tissue and straining through a 70 μm cell strainer. CD8⁺ T cells were isolated using EasySep Mouse CD8⁺ T Cell Isolation Kit (StemCell Technologies) according to the manufacturer protocol. Cell counts were performed using Countess Automated Cell Counter (Invitrogen). For cell isolations from skin, ear skin was split with forceps into dorsal and ventral sides and floated epidermis facing down in 1.2 mg ml⁻¹ dispase diluted in PBS (Roche) for 60 min at 37 °C. The thin epidermal layer was stripped from dermis using forceps. Dermis and epidermis were further processed separately and cut into small pieces followed by digestion with 0.5 mg ml⁻¹ of collagenase D (Roche) diluted in PBS 10% FCS for 60 min at 37 °C. The remaining tissue was disrupted by straining through a 70 μm cell strainer (BD Pharmingen). Total cell isolations of two ears/one animal was used for flow cytometry analysis.

ELISPOT
OVA-specific CD8⁺ T cell responses were measured using IFNγ ELISPOT. The ELISPOT protocol has been previously described. Briefly, 2 × 10⁵ cells were plated in medium containing 10% FCS in 96 well plates (Millipore) coated with 8 μg capture antibody against IFNγ (AN18, Mabtech AB, Stockholm, Sweden). Cells were restimulated with 10 μg ml⁻¹ OVA peptide SIINFEKL for 24 h. After washing, plates were incubated with a biotinylated antibody against IFNγ (R4-6A2, Mabtech AB, Stockholm, Sweden). For detection, horseradish peroxidase-conjugated strepavidin (Sigma-Aldrich, St Louis, MO, USA) and DAB tablets (Sigma-Aldrich) were used. Spots were counted using an automated ELISPOT reader system ELR02 (Auto-immun Diagnostika GmbH, Strassberg, Germany).

Flow cytometry
Anti-mouse monoclonal antibodies (mAbs) to CD16/32 (clone 2.4G2, no. 553142, BD Pharmingen), CD45.2 (clone 104, no. 103131, Biologend, Australian Biosearch, Wangarra, WA, Australia), CD8 (clone SK1, no. 100734, Biologend), CD11c (clone HL3, no. 55798, BD Pharmingen), CD11b (clone M1/70, no. 101224, Biologend), CD103 (clone M290, no. 121405, Biologend), MHCII (clone M5/114.15.2, no. 107628, Biologend), CD4 (clone GK1.5, no. 553729, BD Pharmingen), CD172a (clone P84, no. 144013, Biologend), CD45.1 (no. 562452, BD Pharmingen), IRF4 (clone 3E4, no. 11-9858, ebioscience, Jomar Life Research, Scoresby, VIC, Australia), IRF8 (clone V3GYWCH, no. 17-9852, ebioscience) and rat IgG1 isotype (clone RTK2071, no. 400407, 400426, 400120, Biologend) were used. For staining of myeloid cells, cells were incubated with mAbs to CD16/32 and live/ dead cell fluorescent reactive dye (Thermo Fisher, Brendale, QLD Australia) for 20 min at 4 °C to block unspecified staining and exclude dead cells. All mAbs were incubated for 30 min at 4 °C at predetermined optimal concentrations. For intracellular staining of IRF4 and IRF8, cells were fixed and permeabilized using the FoxP3 intracellular staining kit according to the manufacturer’s instructions (ebioscience). Cells were acquired on a BD LSR Fortessa X20 flow cytometer and analysed using Kaluza software (Beckman Coulter, Sydney, NSW, Australia).

Antigen processing
Splenic single-cell suspensions were incubated for 3 h at 37 °C with 1 μg ml⁻¹ DQ-OVA (Molecular Probes, Thermo Fisher) in complete media, and subsequently antibody stained for flow cytometry.

Liposome uptake
Liposomes (Control liposomes PBS from Clodronate Liposomes, Netherlands) were labelled with a 1:100 dilution of 25 mg ml⁻¹ Dil dye (Molecular Probes) for 30 min on ice. Spleenic single-cell suspensions were incubated for 90 min at 37 °C or on ice with Dil-labelled liposomes in complete media at a dilution of 1:50, and subsequently stained for flow cytometry.

Endocytosis assay
Single cell suspensions of C57BL/6 spleens and thymi were labelled with 5 μM CellTrace Violet (Life Technologies) for 20 min in PBS at 37 °C. Cells were washed and UV irradiated for 30 min. Death of cells was confirmed using trypan blue. 2.5 × 10⁶ dying cells were injected intravenously into recipients. Three hours later, recipients were euthanized, spleens were taken and analysed by flow cytometry for uptake of dying cells by DC subsets.

Statistics
Statistical analysis were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA). Experimental groups were compared by unpaired two-tailed t-test. Differences are considered significant if P < 0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Author contributions: JC performed experiments, analysed and interpreted the data, performed statistical analysis and drafted the manuscript. PTYK and AMH performed experiments and analysed the data. GTB reviewed the manuscript and interpreted data. IHF supervised the study, interpreted the data, drafted and reviewed the manuscript. GTB and IHF gave final approval of the submitted version.

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)