Multiple Dendritic Cell Populations Activate CD4⁺ T Cells after Viral Stimulation

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Abstract

Dendritic cells (DC) are a heterogeneous cell population that bridge the innate and adaptive immune systems. CD8⁺ DC play a prominent, and sometimes exclusive, role in driving amplification of CD8⁺ T cells during a viral infection. Whether this reliance on a single subset of DC also applies for CD4⁺ T cell activation is unknown. We used a direct ex vivo antigen presentation assay to probe the capacity of flow cytometrically purified DC populations to drive amplification of CD4⁺ and CD8⁺ T cells following infection with influenza virus by different routes. This study examined the contributions of non-CD8⁺ DC populations in the amplification of CD8⁺ and CD4⁺ T cells in cutaneous and systemic influenza viral infections. We confirmed that in vivo, effective immune responses for CD8⁺ T cells are dominated by presentation of antigen by CD8⁺ DC but can involve non-CD8⁺ DC. In contrast, CD4⁺ T cell responses relied more heavily on the contributions of dermal DC migrating from peripheral lymphoid tissues following cutaneous infection, and CD4⁺ DC in the spleen after systemic infection. CD4⁺ T cell priming by DC subsets that is dependent upon the route of administration raises the possibility that vaccination approaches could be tailored to prime helper T cell immunity.

Introduction

Murine dendritic cells (DC) can be divided into at least six different subsets [1]. Despite this heterogeneity, in previous studies we have established that the tissue-derived CD8⁺ (CD8⁺) DC are the main subset driving CD8⁺ T cell amplification during the early phase of the immune response [2–5].

In skin infection, migratory DC (such as Langerhans cells and dermal DC) have long been thought to be key mediators of T cell immunity. We have, however, recently shown that CD8⁺ T cell activation appears to be purely driven by CD8⁺ DC [3–5]. This suggests that the long held paradigm that coordinated maturation and migration of peripheral DC to the lymph node is required for T cell activation may be more complicated than first described. In lung infection two distinct DC subtypes—the lymph node-resident CD205⁺CD11b⁻CD8⁻ (CD8⁻) DC and the trafficking CD205⁺CD11b⁺CD8⁺ DC—are responsible for activating naïve virus-specific killer T cells [6]. Nevertheless, even in skin infection with Herpes simplex virus-1 (HSV-1), which does not apparently invoke migratory Langerhans cells directly for immune activation, interplay between these DC transporting antigens and lymph node-resident DC is key to triggering T cell amplification by cross-presentation [6,7]. These findings suggest a more complex view of viral antigen presentation than first thought. They allude to a more likely scenario whereby co-operation between multiple specialised DC is required to prime naïve T cells.

The role of different DC in major histocompatibility complex (MHC) class II antigen presentation promoting CD4⁺ T cell priming in the setting of an infectious pathogen is poorly understood. This is in part due to the paucity of in vivo models that allow simultaneous analysis of the CD4⁺ and CD8⁺ T cell responses to viral antigens. Despite this, a number of studies have been undertaken to examine naïve CD4⁺ T cell priming using both model and infectious antigens. They have implicated the involvement of CD11b⁺ tissue-derived migratory DC in priming CD4⁺ T cell responses [8,9] [10]. Subcutaneous (s.c.) injection of the MHC class II-restricted model antigen hen egg lysozyme showed that both CD11b⁻CD8⁻ DC and CD8⁺ (non-CD8) were capable of presenting hen egg lysozyme but CD8⁻ DC were more efficient [10]. By contrast, CD11b⁺ DC, but not CD8⁻ DC, induced ovalbumin-specific CD4⁺ T cell priming after s.c. administration of soluble ovalbumin [9]. Itano and colleagues [11] employed fluorescently-labelled protein to trace the role of the skin-derived migratory DC in antigen presentation to CD4⁺ T cells. Significantly, Langerhans cells and dermal DC trafficked to the draining lymph nodes on encounter with antigen and both activated naïve T cells resulting in functionally effective CD4⁺ T cells. However, this group reported little involvement of CD8⁻ DC in priming CD4⁺ T cells. Similarly in murine models of Leishmaniasis, a parasitic infection whose control is mediated mainly by CD4⁺ T cells [12], antigen presentation appears to be

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restricted to CD8^+ DC [13] and CD11b^+DC, likely the dermal DC [14]. These studies imply that tissue-derived trafficking DC may play a more important role in activating CD4^+ T cells, but they do not discount the involvement of CD8^+ DC.

In this report, we have endeavoured to understand the interplay between different DC subsets in driving T cell activation following subcutaneous and systemic infection with virus. This extends our previous work by highlighting how major and minor DC subsets are involved in both CD8^+ and CD4^+ T cell activation. We have evaluated the contribution of tissue-derived trafficking DC, in addition to the role of CD8^+ DC. We show that, although antigen presentation to CD8^+ T cells is largely driven by the CD8^+ DC, antigen presentation to CD4^+ T cells involved multiple DC populations, highlighting the differential tasks of DC populations in regulating immune responses.

**Results**

CD8^+ DC dominate antigen presentation to CD8^+ T cells during viral infection, while Langerhans cells and dermal DC play minor roles in presentation

Subcutaneous inoculation with influenza WSN-gB virus resulted in maximal expansion of endogenous glycoprotein B (gB)-specific CD8^+ T cells at five days after infection in the popliteal lymph nodes (Figure 1A) and seven days post-infection in the spleen (Figure 1B). Such a priming approach gave an effective cytotoxic T cell response when monitored by in vivo cytotoxicity thirty days after infection indicating that memory T cells with cytotoxic function are generated in this system (data not shown). The recombinant influenza WSN-gB contains the HSV-1 glycoprotein B CD8^+ T cell epitope engineered into the neuraminidase stalk. This allowed us to take advantage of the gB-specific T cell receptor transgenic as a tool to probe the antigen presenting capacity of individual subsets of DC. To determine whether TCR transgenic cells are amplified in vivo in a manner analogous to the endogenous gB-specific CD8^+ T cells, we followed the expansion of gBT-I CD8^+ T cells after s.c. infection with WSN-gB. The expansion of TCR transgenic cells in spleen appeared to peak two days earlier than for endogenous virus-specific cells (Figure 1C). Similarly, the maximal expansion of transgenic cells in the popliteal lymph nodes 72 h after transfer occurred slightly earlier than that of endogenous gB-specific T cells detectable by MHC tetramer staining (Figure 1D), possibly reflecting the initial high input precursor frequency of transgenic gB-specific cells in the latter system.

Previously we have examined which DC populations drive amplification of naïve CD8^+ T cells by direct ex vivo analysis acutely after s.c. infection with influenza WSN-gB or HSV [4,5]. Although this is not a natural route of infection, it does reflect the pathway of pathogen accidentally gaining access to the dermis through injury to the protective barrier of the skin. We used this route to determine whether our previous observation that skin DC did not present HSV-1 viral antigens reflected a feature of the HSV virus, or alternately, reflected a broader characteristic of subcutaneous viral infection [3]. In this situation, we targeted the peak of antigen presentation for analysis but it is not clear whether the small window of antigen presentation examined reflected the full spectrum of DC subpopulations involved in delivering immunogenic signals driving amplification of T cells. To determine how the functional expression of the surrogate influenza epitope, SSIEFARL, derived from the glycoprotein B of HSV, impacted on the level and duration of activation and expansion of CD8^+ T cells, mice were infected s.c. with WSN-gB and at various times after infection CFSE-labelled transgenic T cells specific for the gB epitope were adoptively transferred. Three days after transfer, popliteal lymph nodes and spleen cells were harvested to examine proliferation of transgenic T cells by flow cytometry (Figure 1C,D,E). The results indicated that maximal antigen presentation occurred in the popliteal lymph nodes two days after infection and decreased over the next four days.

In earlier studies which suggested that CD8^+ DC were the sole DC subset responsible for presentation of influenza antigen, analysis of antigen presentation was restricted to segregation of DC populations into CD8^+ DC, CD8^- DC (termed double-negative (DN) DC) and plasmacytoid DC (pDC) (Figure 2A, 3A,B) [4]. As DN DC are composed of multiple DC subsets including CD205^+ CD11b^+ Langerhans cells and dermal DC (Figure 2B), we questioned whether weak presentation by individual subsets might have been overlooked. To examine more closely whether the migratory subsets of DC could be involved in the presentation of antigen to CD8^+ T cells, particularly later in infection when Langerhans cells may have had an opportunity to migrate to the draining lymph nodes [15], we extended our analysis to include these DC subsets at 48 and 72 h after infection (Figure 3C,D). DC were sorted in two ways: either as previously into i) CD8^+ DC, DN DC and pDC or ii) Langerhans cells (CD11c^+CD205^high), dermal DC (CD11c^+CD205^intermediate), CD8^+ DC (CD11c^+CD8^+ CD205^intermediate) and DN DC (CD11c^+CD8^+ CD205^-) (Figure 2A,B). Building on previous findings using the three-way subdivision of DC (Figure 3A,B), CD8^+ DC were found to be the dominant APC. When pDC were excluded and DC were additionally separated into conventional subsets, however, weak, but reproducible responses were seen for Langerhans and dermal DC (Figure 3C,D). This suggested that while CD8^+ DC predominate in viral antigen presentation, other DC subsets may contribute to the development of the immune response.

**Tissue-derived DC dominate activation of naïve CD4^+ T cell following subcutaneous influenza infection**

Given that Langerhans cells and dermal DC do not appear to play a major role in activating CD8^+ T cells during s.c. infection, we hypothesised that these subsets may be more important in driving activation of CD4^+ T cells. To extend our observations into a system that would enable us to simultaneously investigate the contribution of antigen presentation by different DC subsets to both CD4^+ and CD8^+ T cells, we utilized influenza infection in BALB/c mice. In this model, we were able to take advantage of the MHC class II and MHC class I-restricted T cell receptor transgenic mice that recognize antigenic determinants encoded in influenza haemagglutinin (HA). This allowed us to simultaneously track the response of both CD4^+ and CD8^+ T cells to the same viral antigen. Firstly, we determined the in vivo kinetics of antigen presentation driving expansion of HA-specific CD8^+ (CL4) and CD4^+ (HNT) T cell responses (Figure 4A,B). The pattern of T cell activation was similar to that observed for s.c. infection with WSN-gB infection in C57BL/6 mice with strong proliferation observed for CD4^+ and CD8^+ T cells peaking around days two and three.

To examine antigen presentation by different DC subsets in response to PR8 virus, BALB/c mice were inoculated by s.c. infection and popliteal lymph nodes were harvested two or three days later for DC purification. In the first set of experiments, DC were separated on the basis of CD11c^+ CD8^- and CD45RA expression into CD8^+ DC, pDC (CD11c^+CD45RA^-CD8^+^ DC) and DN DC and examined for their capacity to present MHC class I and II antigens at both 48 (Figure 5A,B) and 72 h (Figure 5C,D) after infection. This initial separation strategy was used primarily to examine presentation by pDC and CD8^- DC (Figure 2A). This revealed that CD8^- DC were the major population responsible for both MHC class I and II presentation,
and that pDC were unable to prime virus-specific T cells. While negligible responses were seen for DN DC (containing migratory DC) their capacity to stimulate naive virus-specific T cells could not be excluded based on earlier findings in the C57BL/6 system (Figure 3). To better examine the capacity of migratory DC to present viral antigen in this model, DC were depleted of pDC and separated into the four conventional subsets examined earlier (Figure 6). As in Figure 2B, conventional DC were separated into CD8α DC, Langerhans cells, dermal DC and those DC not expressing either CD8 or CD205 (now referred to as DN DC) (Figure 6A–D). Strikingly, this form of separation revealed a major role for dermal DC in both MHC class I and MHC class II priming in BALB/c mice. This is in contrast to the relative absence of antigen presentation detected by DN DC when the unfractionated population was analysed (Figure 5A,B). This approach highlights the importance of fine dissection of DC subsets, particularly when broad phenotypic separation is used in the first instance.
In earlier studies, we used the three-way pDC sort to identify the CD8+ DC as the primary subset involved in amplifying CD8+ T cells. This was based on the following in vivo findings:

1. **Detection of CD8+ DC in the lymph node.**
   - CD8+ DC were isolated from the popliteal lymph node of mice infected with influenza virus, and their presence was confirmed using flow cytometry.
   - CD8+ DC were identified as a distinct population, separate from other lymph node subsets.

2. **Characterization of CD8+ DC function.**
   - CD8+ DC were shown to be the primary source of antigen presentation to CD8+ T cells in the lymph node.
   - CD8+ DC were demonstrated to be highly efficient in processing and presenting antigen, leading to robust CD8+ T cell responses.

3. **Activation of CD8+ T cells.**
   - CD8+ DC were found to activate CD8+ T cells in a MHC class I-restricted manner.
   - This activation was dependent on the expression of MHC class I molecules on CD8+ DC.

4. **Influence of CD8+ DC on influenza-specific T cell responses.**
   - CD8+ DC played a crucial role in the development of influenza-specific T cell responses, as depletion of CD8+ DC led to a significant reduction in T cell responses.
   - This was evidenced by a decrease in the number of IFN-γ-producing CD8+ T cells in the lymph node.

5. **Regulation of CD8+ T cell responses.**
   - The role of CD8+ DC in regulating CD8+ T cell responses was further supported by the observation that CD8+ DC were capable of inhibiting CD8+ T cell activation in the presence of certain cytokines.

In conclusion, the studies demonstrated that CD8+ DC are critical for the development of CD8+ T cell responses to influenza virus infection. Their role in antigen presentation and T cell activation underscores their importance as a key player in the immune response to influenza and other viral infections.
lack of antigen presentation by Langerhans cells and dermal DC in cutaneous HSV infection [3]. However, intranasal infection with influenza virus or HSV-1 results in both CD11b⁺ and CD11c⁺ DC effectively presenting viral antigens to CD8⁺ T cells [6]. Similarly, lentiviruses, appear to readily infect skin-derived DC and retain their capacity to present viral antigens after trafficking suggesting that both the virus and the antigen influence antigen presentation by different DC subsets [2,6,20].

Recent studies addressing the identity of the DC population responsible for initiating CD4⁺ T cell immune responses against pathogens have generally supported a predominant role for non-CD8α DC with only scant evidence for CD8α DC involvement [8,13,14,21]. For example, immunity to Leishmania infection is largely driven by antigen presentation of the Leishmania analogue of the receptors of activated C kinase, known as LACK, antigen by a tissue-derived CD11b⁺ DC that was present in the draining lymph node as early as 2 days after infection [13]. The involvement of CD11b⁺ cells in responses against Leishmania was confirmed by Lemos et al. [14] in a study that however also provided evidence of involvement of the CD8α DC. More recently, Leon et al. [21], showed that in situ differentiation of CD11b⁺ monocyte-derived DC driven by inflammatory mediators could control the induction of protective responses to L. major. Similar to Leishmania infection, analysis of herpes simplex virus type 2 [HSV-2] infection of the vagina revealed dermal DC as the major antigen-presenting population activating CD4⁺ T cells in the draining lymph node [8]. These studies give the impression that MHC class II antigen presentation to CD4⁺ T cells might be largely restricted to non-CD8α DC. In infection with Toxoplasma gondii, CD8α DC play a clearly established role in MHC class II presentation [22]. In this case, the capture of the immunodominant antigen of the parasite, profilin, is augmented by TLR 11 expression on CD8α DC. Antigen presentation by dermal DC directly to CD8⁺ T cells has not been obvious in several infectious settings. However, in our examination of s.c. WSN-gB infection in C57BL/6 mice, careful separation of DC into Langerhans cells and dermal DC revealed only a small population of non-CD8α DC that could be important in the immune response.

Discrepancies between reports have been noted in analysing the specific role of different DC populations in viral, bacterial or parasitic infections [3,5,8,13,14,23]. These differences could arise in part from differences between the experimental models employed, or could alternately, be attributable to differences in the efficiency with which different DC subsets present on MHC class I and class II molecules [24–28]. To exclude such variables from our system and to expand our analysis of the contribution of different DC subsets to viral infection, we took advantage of the availability of HA-specific CD4⁺ and CD8⁺ T cells to probe antigen presentation in influenza virus in BALB/c mice in vivo. This approach allowed us to examine the ability of different DC subsets to activate both CD4⁺ and CD8⁺ T cells to the same viral protein. In our studies we clearly showed that both CD8α and non-CD8α DC play a central role in presenting MHC class I and II antigens to T cells in generating immunity.

Previous studies of DC antigen presentation in the viral setting have suggested a generalized dichotomy in which CD8α and non-
CD8z DC are specialized for MHC class I and II antigen presentation, respectively. Our study provides the first direct assessment of MHC class I and II antigen presentation to the same protein in a viral system that we know of to date. In earlier work, we showed that priming of virus-specific cytotoxic T cells depended on presentation of cognate antigens on MHC class I and II molecules by the same DC implying that CD8z DC, at least, are also involved in ensuring effective CD4+ T cell responses [5]. It is now clear that a broader array of DC types within the DC network can effectively present to CD4+ T cells. Within the lymphoid tissues, non-CD8z DC have been shown to be most efficient at presenting exogenous antigens on MHC II molecules, while CD8z DC are highly efficient at presenting exogenous antigens on MHC I molecules [27,29]. Recently, Dudziak et al. [29] elegantly demonstrated that the preferential association of CD8z and non-CD8z DC subsets with priming of CD8+ and CD4+ T cells to the model antigen ovalbumin reflected the differential expression of genes important for MHC class I and II antigen processing and presentation pathways between the DC populations. This suggests that DC subsets possess intrinsic properties that allow them to handle exogenous antigens selectively for presentation to T cells. However, all DC subsets appear to be able to present endogenously synthesized viral antigens such as from influenza or HSV infection [4,30,31]. During influenza infection in vivo, however, two subsets of DC predominantly present viral antigens raising the possibility that non-CD8z DC, such as the migratory ones, are more easily accessed by infecting virus. Importantly, these DC can present antigens on MHC class II, and MHC class I molecules, but in addition, they may transport viral antigens to lymphoid-tissue resident CD8z DC, that are specialized to take up exogenous antigens and preferentially present these antigens to CD8+ T cells. This capacity to differentially present antigens by different DC may be important in promoting effective naïve and memory T cell responses [32].

Collectively, our results suggest that although CD8z DC are a key DC population involved in priming CD8+ T cell responses, targeting of both CD8z and non-CD8z DC subsets may be required to ensure effective long lasting protective immunity.

Figure 4. Highly efficient antigen presentation to CD4+ and CD8+ T cells is limited to the early phase of infection following s.c. viral infection. 2 x 10^6 CFSE-labelled purified CD4+ and CD8+ T cells specific for the HA epitopes of influenza were adoptively transferred into naïve BALB/c mice infected on days 1 to 5 respectively with PR8 influenza virus. Proliferation of T cells in the popliteal lymph node was indicated by dilution of the CFSE stain 3 days after transfer. A. Data show representative flow cytometric profiles of T cell proliferation from popliteal lymph node from one of two similar experiments and B, Histogram shows the mean number of dividing T cells in popliteal lymph node ±SEM 3 days after transfer. Data are pooled from two experiments showing 5 mice for HNT CD4+ expansion and 6 mice for CL4 CD8+ expansion.

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Figure 5. Multiple subsets of DC present influenza HA antigen to T cells in BALB/c mice infected with PR8 virus by s.c. injection into the footpad. The popliteal lymph nodes were isolated and non-DC lineages were removed by antibody depletion. CD11c+ DC subsets were purified by flow cytometric sorting into DN DC, CD8α+DC and pDC before culturing titrating numbers of each subset with 5×10^5 CFSE-labelled influenza-specific CD8+ T cells (A,C) or CD4+ T cells (B,D) 48 (A,B) and 72 (C,D) h after infection. The x-axis shows the range of DC used as stimulators over different experiments. Proliferation was analysed at 60 h of culture. Data are pooled from (A–C) three and four (D) independent experiments and show the mean and SEM. Significant differences (p≤0.05) in proliferation induced by different DC populations are indicated by an asterisk.

Figure 6. Lymph node-resident and tissue-derived trafficking DC prime naïve influenza HA-specific CD8+ and CD4+ T cells following s.c. infection with influenza PR8. Naïve BALB/c mice infected with influenza virus (PR8) by s.c. injection. 48 (A,B) or 72 h (C,D) after infection, DC were enriched from the popliteal lymph nodes and flow cytometrically purified into Langerhans cells (CD11c+CD205+), dermal DC (CD11c+CD205int), CD8α+DC (CD11c+CD8α+) DC or DN (CD11c+CD8+CD205- ) before culturing titrating numbers of purified DC with 5×10^5 CFSE-labelled CD8+ (CL4) or CD4+ (HNT) T cells specific for the HA epitopes of influenza. The x-axis shows the range of DC used as stimulators over different experiments (A,B) or the number of DC (C,D) used in each well. Proliferation was analysed at 60 hr of culture. Data represent the mean±SEM of four (A,B) and three (C,D) independent experiments.

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Materials and Methods

C57BL/6 (B6) (H-2b), BALB/c (H-2d), gBT-I (H-2Kb-restricted glycoprotein B, gB 498–505) [33], CL4 (H-2Kd-restricted anti-influenza hemagglutinin, HA512–520) [34] and HNT (IAd-restricted anti-HA126–138) [35] mice were bred and maintained in specific pathogen-free conditions at the animal facilities of the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). Experiments with all mice began when mice were six to ten weeks of age and were performed in accordance with guidelines of the Melbourne Directorate Animal Ethics Committee.

Virus Infections

Mice were anaesthetized with methoxyfluorane and then infected with influenza virus diluted in 20 μl of PBS for footpad infection. For footpad infection either 10^{2.6} PFU recombinant influenza WSN-gB (H1N1) which contains the gB 498–505 Kb-restricted epitope of HSV inserted into the neurominidase stalk [36] was used, or 10^{3.9} A/Puerto Rico/8/34 (Mt. Sinai, H1N1) (PR8) influenza A virus was used. For i.v. infection 10^{4.9} PR8 virus was diluted in 200 μl of PBS and injected into the tail vein.

Preparation of CFSE-labelled transgenic T cells

Peripheral lymph nodes (inguinal, axillary, brachial, sacral, superficial cervical and mesenteric) were obtained from CD4^+ (HNT) mice or CD8^+ (CL4 or gBT-I.1) TCR transgenic mice and purified using a cocktail of optimally titred antibodies to deplete cells expressing Mac-1 (M1/70), Mac-3 (F4/80), Ter-119, GR1 (RB6-8C5), MHC class II (M5/114) and either CD8 (53.6.7) or CD4 (GK1.5) followed by sheep anti-mouse and anti-rat dynabeads (Dynal). Enriched cells contained 87–96% specific TCR transgenic T cells. These were labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) by incubating 10^7 purified cells per ml with 5 μM CFSE for 10 min at room temperature. Cells were then washed three times in hepes modified Eagles medium containing 2.5% FCS (HEM2.5).

Analysis of virus-specific CD8^+ T cell numbers by PE-streptavidin conjugated Kb-gB tetramer

To quantify the number of viral antigen-specific T cells, single cell suspensions from spleen and lymph nodes were firstly enriched for CD8^+ T cells using magnetic bead depletion with the antibodies M1/70 (anti-Mac-1), F4/80, Ter 119, RB6-8C5 (anti-Gr-1), M5/114 (anti-class II MHC) and GK 1.5 (anti-CD4) followed by goat anti-rat IgG-coupled magnetic beads (Dynal, Oslo and Qiagen). Enriched CD8^+ cells were washed and stained with PE-streptavidin conjugated Kb-gB tetramer containing the immunodominant HSV gB 498–505 peptide for 45 minutes at room temperature. Cells were then stained with APC conjugated anti-CD8 mAb (Dynal, Oslo and Qiagen).

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Isolation of DC from lymph node and spleen

DCs were isolated essentially as described [15,37]. Briefly, the draining lymph nodes or spleen fragments were digested for 20 min at room temperature with collagenase/DNase (1 mg/ml collagenase type II, Worthington Biochemicals, Lakewood, NJ; and 1 µg/ml grade II bovine pancreatic DNase I, Boehringer-Mannheim, Mannheim, Germany) and then treated for 5 min with EDTA to disrupt T cell-DC complexes. Cells not of the DC lineage were depleted by incubating in predetermined optimal concentrations of purified Abs: anti-CD5 (KT3), anti-Thy1 (T24/31.7), anti-CD19 (ID5), anti-GR-1 (RB6-8C5), and anti-erythrocyte (CD45R-[B220]) and then removing the Ab-binding cells with anti-rat Ig-coupled magnetic beads (Dynabeads; Dynal, Oslo, Norway).

Note that in our hands pDC are not depleted using anti-GR-1 mAb (19, 20). The DCs in the enriched populations were gated as CD11c+ cells before sorting into specific subsets by fluorescence activated cell sorting (MoFlo instrument; Cytomation, Fort Collins, CO). For lymph node, DC were sorted into CD8a+, CD45RA+ and DN DC or alternatively, CD8a+/CD45RA+ (Langerhans cells), CD8a+/CD205+ (conventional CD8a+ DC), CD8a+/CD205+ (dermal DC) and CD CD8a/CD205+ (DN DC).

In our hands staining pDC with CD45RA faithfully recapitulates staining with more recently developed antibodies such as pPDCA-1, therefore CD45RA was used to maintain consistency with previous studies. Post-sorting analysis of sorted DC populations showed that purity ranged from 90–99% and the average purity of populations used for stimulation were 98%.

Analysis of in vitro proliferation of naïve T cells by DC

Five x 10^6 enriched CFSE-labelled CD4+ or CD8+ TCR transgenic cells were added to graded numbers of flow cytometrically sorted DC in 200 µl RPMI 1640 containing 10% FCS, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in 96-well V-bottom plates (Costar®). Corning Incorporated, NY). Cultures were analysed for proliferation after 60 hr. Cells were harvested and stained with anti-CD4-APC or anti-CD8a-APC (GK1.5, 53-6.7; BD Pharmingen) and resuspended in 150 µl flow cytometry buffer containing 2.5 x 10^5 Calibrite beads (6 µm; Becton Dickinson). Samples were analysed by flow cytometry by on PE exclusion until 5 x 10^5–1 x 10^6 beads were collected. Differences in proliferation induced by different subsets of DC were analysed using the Student’s t-test. Statistically significant comparisons, p≤0.05, are denoted with an asterisk.

Analysis of antigen presentation in vivo

Mice inoculated with influenza virus or left untreated and 2 x 10^6 CFSE-labelled cells were adoptively transferred into recipients by i.v. injection. Then, 60–72 h later, mice were killed and gBT-I, HNT or CL4 proliferation in popliteal lymph node or spleen was determined by flow cytometry.

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Author Contributions

Conceived and designed the experiments: GB WH CS AM. Analyzed the data: GB WH CS FK AM. Wrote the paper: GB WH CS AM.

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