Thymic but not splenic CD8\(^+\) DCs can efficiently cross-prime T cells in the absence of licensing factors

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Cross-presentation is an important mechanism to elicit both immune defenses and tolerance. Although only a few DC subsets possess the machinery required for cross-presentation, little is known about differences in cross-presenting capabilities of DCs belonging to the same subpopulation but localized in different lymphoid organs. In this study, we demonstrate that steady-state thymic CD8\(^+\) DCs can efficiently cross-prime naïve CD8\(^+\) T cells in the absence of costimulation. Surprisingly, cross-priming by splenic CD8\(^+\) DCs was dependent on licensing factors such as GM-CSF. In the absence of GM-CSF, antigen–MHC-class-I complexes were detected on thymic but not on splenic CD8\(^+\) DCs, indicating that the cross-presentation capacity of the thymic subpopulation was higher. The observed cross-priming differences between thymic and splenic CD8\(^+\) DCs did not correlate with differential antigen capture or costimulatory molecules found on the surface of DCs. Moreover, we did not detect overall impairment of antigen presentation, as peptide-loaded splenic CD8\(^+\) DCs were able to induce CD8\(^+\) T-cell proliferation. The observation that thymic CD8\(^+\) DCs are more efficient than splenic CD8\(^+\) DCs in T-cell cross-priming in the absence of licensing factors indicates that the requirements for efficient antigen presentation differ between these cells.

Keywords: CD8\(^+\) DC · Cross-priming · Licensing · Spleen · Thymus

Introduction

The priming of CD8\(^+\) cytotoxic T cells requires antigen presentation associated with MHC class I (MHC-I) molecules by antigen-presenting cells, usually dendritic cells (DCs). The endogenous MHC-I pathway is restricted to peptides derived from intracellular antigens. However, when intracellular pathogens do not infect DCs, or when tumors are not DC-derived, CD8\(^+\) T cells can only be primed when DCs process and present extracellular antigen in the context of MHC-I molecules, a process called cross-presentation [1]. Cross-presentation is also required to induce deletion of auto-reactive CD8\(^+\) T cells and, consequently, tolerance to self-antigens [2]. The most relevant cross-presenting DCs in mice are the ones expressing the CD8 and CD103 molecules [3, 4], and DCs that cannot cross-prime efficiently, such as CD4\(^+\) and CD8\(^+\)CD4\(^−\) double negative (DN) DCs, are specialized for presentation in the MHC class II (MHC-II) context, which is important to activate CD4\(^+\) T cells [5]. In addition, CD8\(^+\) DCs present an exceptional capacity to produce bioactive IL-12 when receiving innate and T-cell-derived signals [6] and, inversely under other conditions can induce the conversion of antigen-specific T cells into regulatory T cells [7].

Although functional studies of the different DC subpopulations have taken into consideration the molecular markers cited above, DCs classified as belonging to the same subpopulation may originate from different precursors, depending on the organ where they are localized. Within the thymus, the majority of DCs are CD8\(^+\), and approximately 70% of thymic CD8\(^+\) DCs derive from an intrathyMIC T-cell precursor, characterized by its IgH D-J DNA rearrangements. In the spleen, few if any CD8\(^+\) DCs present such...
rearrangements [8]. Additionally, there is evidence that stromal cells in different microenvironments may influence DC differentiation and function [9, 10]. Moreover, it was recently demonstrated that splenic CD8⁺ DCs are heterogeneous regarding their ability to cross-present antigens in vivo [11], and in a different study, only about 50% of splenic CD8⁺ DCs, those that also expressed CD103, were able to cross-present cell-derived antigens [12]. Interestingly, although tissue-restricted antigens (TRAs) have a rare and limited expression within the thymus, with several individual tissue-restricted antigens being expressed only by a minor (1–3%) subset of medullary epithelial cells (mTECs) [13], central tolerance is nevertheless an efficient process in which a large proportion of the auto-reactive T cells are negatively selected directly by medullary epithelial cells or by antigen acquisition and cross-presentation by DCs. On the other hand, tolerance in the periphery may not be as efficient, as cross-tolerance occurs only if the self-antigen is present at a high concentration [14]. Moreover, it has been argued that auto-reactive lymphocytes are indeed part of the peripheral T-cell repertoire, as best illustrated by the fact that immunization with particular self-antigens can elicit experimental autoimmune diseases. This is especially well documented for auto-antigens of the central nervous system [15]. Such differences in central versus peripheral tolerance raise questions regarding the efficiency of antigen cross-presentation by thymic and splenic CD8⁺ DCs.

While splenic CD8⁺ DCs and their role in cross-presentation are well examined, thymic CD8⁺ DCs have been much less studied. Here, we investigated functional differences between thymic and splenic CD8⁺ DCs isolated from steady-state nontransgenic mice. We show that when isolated and tested in vitro under controlled conditions, cross-presentation of soluble and cell-associated ovalbumin (OVA) by thymic CD8⁺ DCs is independent of licensing factors, such as GM-CSF or CD40 signalization, even with small amounts of antigen or low ratios of DCs/T cells. Surprisingly, splenic CD8⁺ DCs were impaired in antigen presentation in the absence of these factors, even when a large amount of antigen was available or high ratios of DCs/T cells were used. In the absence of GM-CSF, antigen–MHC-I complexes were detected on viable thymic CD8⁺ DCs but not on viable splenic CD8⁺ DCs, indicating that cross-presentation capacity of the thymic subpopulation was higher. These results indicate that the requirements for efficient antigen presentation differ between thymic and splenic CD8⁺ DCs.

Results

Thymic CD8⁺ DCs are more efficient than splenic CD8⁺ DCs in cross-presentation

Murine DCs can be segregated into conventional (cDCs) and plasmacytoid DCs (pDCs). The cDCs can be subdivided into three subsets based on the surface expression of the CD4 and CD8 molecules: (i) CD4⁺ DCs which express the signal regulatory protein alpha (Sirpα, also known as CD172a), (ii) CD8⁺ DCs (Sirpβ⁻), and (iii) CD8⁻CD4⁻ DN DCs (Sirpβ⁺). Thymic cDCs can be segregated into two subpopulations: (i) CD8⁻ DCs (Sirpβ⁻) and (ii) CD8⁻CD4⁻ DN (Sirpβ⁻); CD4⁺ DCs are not found in the thymus [16]. Our gating strategy to sort the different subpopulations of DCs is shown in Fig. 1A. The accuracy of gating was confirmed by the characteristic expression of CD8 and CD11b, with Sirpβ⁻ cells being CD8⁺CD11blow and Sirpβ⁺ cells being CD8⁻CD11bhigh (Fig. 1B). The weak staining of CD8 in thymic Sirpβ⁻ DCs is probably due to CD8 uptake from thymocytes, reinforcing the need of the alternative marker Sirpβ to saturate this population. In addition, viability of both splenic and thymic DCs was similar after sorting, as assessed by Annexin V and propidium iodide (PI, Fig. 1C).

To assess if splenic and thymic CD8⁺ DCs display the same ability in ex vivo antigen cross-priming, we sorted CD8⁺ DCs from spleen and thymus of C57BL/6 mice and assessed their ability to induce proliferation of naïve T cells. After sorting, DCs were loaded with soluble OVA and cocultured with CFSE-labeled CD8⁺ OT-I T cells expressing a TCR specific for OVA in the context of H-2Kb. As a read-out for cross-priming, T-cell proliferation was analyzed by flow cytometry after 3 days. This approach offers the advantage that DCs isolated from the two organs can be incubated with equal amounts of antigen, which would be difficult to achieve in an in vivo scenario. Thymic CD8⁺ DCs displayed high efficiencies in cross-priming T cells in response to soluble antigen, as 65 ± 10% of the OT-I cells in the coculture proliferated. Surprisingly, splenic CD8⁺ DCs did not induce efficient proliferation of OT-I T cells, as only 8 ± 2% of the cells proliferated (Fig. 2A). While thymic DCs were able to cross-prime OT-I T cells even at the lowest concentrations of OVA (Fig. 2B), or at low ratios of DCs/T cells (Fig. 2C), splenic DCs did not induce efficient T-cell proliferation at any concentration of OVA (Fig. 2B) or ratio of DCs/T cells (Fig. 2C). We also compared the ability of thymic and splenic CD8⁺ DCs to cross-prime OT-I cells in response to cell-associated antigen, in particular OVA-coated apoptotic β2m/RAG knockout spleenocytes. These splenocytes are not able to directly present antigen to T cells and did not elicit proliferation of OT-I cells in absence of DCs (data not shown). In this situation, splenic CD8⁺ DCs were able to cross-prime OT-I cells, however, they required much larger amounts of cell-associated antigen than thymic CD8⁺ DCs (Fig. 2D). This indicates that the superior capability of thymic DCs to cross-prime is not limited to soluble antigen. The reason why splenic DCs show improved cross-priming activity with cell-associated OVA compared with the soluble form may be due to the fact that the former was present for the entire coculture period, and that apoptotic cells could possibly activate DCs through receptors such as Clec9A and CD36 [17, 18]. The impaired competence of splenic DCs in T-cell priming was not a result of an overall impairment in antigen presentation, since DCs loaded with low concentrations of OVA peptide were able to efficiently induce T-cell proliferation (Fig. 2E), although also in this scenario the thymic CD8⁺ DCs were more efficient in T-cell priming than the splenic DCs. We also investigated whether the observed functional differences were due to differential capacities...
to internalize antigen. When DCs were incubated with fluorochrome-conjugated OVA, the two cell populations internalized comparable amounts of antigen (Fig. 2F). In summary, these results demonstrate that the thymic CD8\(^+\) DCs are more efficient in cross-priming than the splenic CD8\(^+\) DCs and indicate that this is not a consequence of differences in antigen capture or overall impairment of antigen presentation.

Licensing is dispensable for T-cell priming by thymic but not by splenic CD8\(^+\) DCs

Next, we addressed the effect of costimulation on the cross-priming activity of splenic and thymic CD8\(^+\) DCs. Splenic CD8\(^+\) DCs have been previously described as potent cross-presenting cells both in vivo and ex vivo. However, in our study, splenic CD8\(^+\) DCs were not able to efficiently cross-prime ex vivo under “steady-state” conditions. This discrepancy may be due to the fact that in the previous studies, ex vivo cross-priming assays have been performed in the presence of GM-CSF and/or DCs have been isolated from Flt3 ligand-treated mice. Accordingly, it has been recently demonstrated that, similar to CD40 ligand, GM-CSF is a licensing factor that activates DCs [19]. To explore this possibility, we performed T-cell proliferation assays in the presence of GM-CSF, and found that in this situation splenic CD8\(^+\) DCs were indeed able to efficiently cross-prime and induce proliferation of 80\(\pm\)8% of OT-I cells, which is comparable to the efficiency of the thymic CD8\(^+\) DCs (Fig. 3A). Overall, GM-CSF increased T-cell proliferation by splenic CD8\(^+\) DCs by approximately ten-fold (compare Figs. 2A and 3A), and addition of...
Figure 2. Uptake and in vitro cross-presentation of antigen by thymic and splenic CD8+ DCs. (A) DCs were loaded with soluble OVA and cocultured with CFSE-labeled OT-I cells. The percentage of proliferating T cells was determined as described in Materials and methods section and as shown on the left. Numbers indicate the percentage of proliferating cells; negative, OT-I cells alone. This strategy was used to determine the percentage of proliferating T cells in all experiments. Histograms shown represent results from one out of four independent experiments. The error bars on the right show the range of values obtained in four independent experiments. **p = 0.0013 (Student's two-tailed t-test). (B) Sorted DCs were loaded with the indicated concentrations of soluble OVA and cocultured with CFSE-labeled OT-I cells. The graph shows mean values obtained from two independent experiments. (C) DCs were loaded with soluble OVA, and the indicated numbers of DCs were cocultured with CFSE-labeled OT-I cells. The graph shows mean values obtained from two independent experiments. (D) DCs were loaded with the indicated concentrations of OVA peptide (SIINFEKL) and cocultured with CFSE-labeled OT-I cells. The graph shows mean values obtained from two independent experiments. (E) DCs from thymus (open histograms) and spleen (shaded histograms) were incubated at 37°C with the indicated concentrations of OVA-Alexa Fluor 488. The histograms show endocytosis of OVA-labeled antigen. Control represents the fluorescence obtained by incubation with the highest concentration of OVA-Alexa Fluor 488 on ice. Results shown are representative of one out of two independent experiments.
antibodies that bind and block GM-CSF significantly reduced T-cell proliferation by splenic CD8\(^+\) DCs \((p = 0.0010, \text{Fig. 3B})\), confirming the licensing activity of GM-CSF. Moreover, in the presence of GM-CSF, the efficiency in cross-priming was comparable between thymic and splenic DCs at all ratios of DCs/T cells tested (Fig. 3C). CD40 stimulation also resulted in a much higher cross-priming activity of splenic CD8\(^+\) DCs (Fig. 3D), indicating that these cells can efficiently cross-prime T cells in response to soluble antigen only when a licensing factor is provided. On the other hand, GM-CSF and CD40 stimuli improved cross-priming by thymic CD8\(^+\) DCs only slightly (compare Figs. 2A–D, 3A, C and D), probably because these cells are already efficient in cross-priming T cells in the absence of these factors. Furthermore, in the presence of GM-CSF, splenic DCs induced OT-I cell proliferation as efficiently as thymic DCs at the lowest concentration of cell-associated OVA (Fig. 3E), indicating that licensing is required for efficient cross-priming by splenic CD8\(^+\) DCs independent of the antigen source.

Next, we addressed if the observed differences in cross-priming by thymic and splenic CD8\(^+\) DCs was a consequence of differential antigen cross-presentation and whether GM-CSF can improve the cross-presentation capacity of the splenic subpopulation. For this, DCs were cultured together with soluble OVA or a control Ag (bovine serum albumin, BSA) and stained with the Ab 25-D1.16, which specifically recognizes the OVA peptide SIINFEKL in the context of MHC-I Kb (Kb-OVA257–264). Since we used soluble OVA protein as antigen, the Kb-OVA257–264 complexes represent only a small fraction of all possible OVA peptide–MHC-I complexes.

Figure 3. Licensing factor enables in vitro cross-priming by splenic CD8\(^+\) DCs. (A) This assay was performed as described in Fig. 2A, except that GM-CSF was added to the culture. The percentage of proliferating T cells was determined as shown on the left. Numbers indicate the percentage of proliferating cells; negative, OT-I cells alone. Histograms shown represent results from one out of four independent experiments. The error bars on the right show the range of values obtained in four independent experiments. (B) This assay was performed exactly as described in Fig. 3A, except that neutralizing anti-GM-CSF Ab or isotype control was added to the culture. Results are representative of one out of two independent experiments. (C) This assay was performed as described in Fig. 2C, except that GM-CSF was added to the culture. The graph shows mean values obtained from two independent experiments. (D) This assay was performed as described in Fig. 2A, except that anti-CD40 Ab was added to the culture. Results are representative of one out of two independent experiments. (E) DCs were cultured with 5 \times 10^5 OVA-coated apoptotic \beta2m/RAG knockout splenocytes and CFSE-labeled OT-I cells in the presence of GM-CSF. Results are representative of one out of two independent experiments.
complexes and pronounced differences in staining were therefore not expected. Nevertheless, viable (PI negative) thymic CD8\(^+\) DCs showed clear staining of K\(^b\)-OVA\(_{257-264}\) in the absence of GM-CSF (Fig. 4A). Under the same conditions, no such staining was observed with viable splenic CD8\(^+\) DCs, but the addition of GM-CSF resulted in a marginal increase of staining above background (BSA) in these cells (Fig. 4A). Specificity of the 25-D1.16 Ab was further confirmed using DCs isolated from β2m/RAG knockout mice. As DCs from these mice lack β2m, they are deficient in the expression of MHC class I molecules and, as expected, no staining was observed (Fig. 4A). To appreciate the relevance of the results above, we loaded CD8\(^+\) DCs with increasing concentrations of SIINFEKL peptide or a control peptide (MOG) and determined the sensitivity of the 25-D1.16 Ab for detection of K\(^b\)-OVA\(_{257-264}\) complexes relative to the sensitivity of the cross-priming assay. The lowest concentration of peptide (2 ng/mL) that was sufficient to support OT-I proliferation (Fig. 2E) did not support detection with the 25-D1.16 antibody (Fig. 4A). A tenfold increase of the peptide concentration (20 ng/mL) resulted in only a marginal increase in the intensity of Ab staining above background (MOG peptide; Fig. 4A), indicating that the small difference detected by the addition of GM-CSF reflects increased cross-presentation. A 100-fold increase of the peptide concentration (200 ng/mL) resulted in a more pronounced staining intensity. These results indicate that the observed differences regarding OT-I cell proliferation in the previous experiments reflect, at least in part, differences in OVA cross-presentation between thymic and splenic CD8\(^+\) DCs.

We assessed the viability of thymic and splenic CD8\(^+\) DCs under culture conditions in the presence or absence of GM-CSF. The results revealed that at 20 h after incubation, the thymic cells were more viable than the splenic cells, and addition of GM-CSF decreased cell death of both subpopulations (Fig. 4B). While the reduced viability of the splenic subpopulation may contribute to their inefficiency of cross-priming in the absence of GM-CSF, other factors may play important roles as well because increasing the number of splenic CD8\(^+\) DCs did not increase the efficiency of cross-priming (Fig. 2C), and K\(^b\)-OVA\(_{257-264}\) complexes were detected on viable thymic CD8\(^+\) DCs but not on viable splenic CD8\(^+\) DCs (Fig. 4A). All together, these results indicate that GM-CSF is important for supporting cross-priming of splenic CD8\(^+\) DCs and that this factor is dispensable for thymic DCs.

**Phenotype and activation profile of thymic and splenic CD8\(^+\) DCs**

Given that the ability of DCs to stimulate naive T cells is strongly determined by their maturation state, i.e. by the expression levels of costimulatory molecules, we next investigated whether thymic CD8\(^+\) DCs are constitutively more activated/mature than splenic CD8\(^+\) DCs. Overall, both thymic and splenic CD8\(^+\) DCs expressed high levels of activation markers, including CD80, CD86, CD40, and MHC-II (I-Ab) (Fig. 5A), indicating that the functional differences were not due to activation/maturation in general. We also assessed the expression of specific surface markers found among CD8\(^+\) DCs and related to cross-presenting capabilities. Mannose receptor (MR) (also known as CD206) mediates internalization of antigens into early endosomes and cross-presentation [20]. Although MR is expressed by BM-derived DCs.

![Figure 4](image-url). Cross-presentation and viability of thymic and splenic CD8\(^+\) DCs. (A) DCs isolated from WT (C57BL/6) or β2m/RAG knockout (β2m\(^-\)) mice were incubated with soluble OVA (open histograms) or BSA (shaded histograms, top) in medium alone or in the presence of GM-CSF, or CD8\(^+\) thymic DCs were incubated with OVA peptide (SIINFEKL, open histograms) or MOG control peptide (shaded histograms, bottom) and stained with the 25D1.16 Ab which recognizes SIINFEKL-MHC-I complexes (K\(^b\)-OVA\(_{257-264}\)). Results are representative of one out of two independent experiments. (B) DCs were loaded with soluble OVA and cultured in medium alone (left) or in the presence of GM-CSF (right). The percentage of live cells (PI negative) was determined after 20 h of incubation. The error bars show the range of values obtained in three independent experiments. ***p = 0.0008, *p = 0.0108 (Student’s two-tailed t-test).
(BM-DCs) and macrophages, its expression in splenic DCs is controversial [20, 21], and the MR status of thymic DCs has not previously been examined. We found that, as opposed to BM-DCs, neither thymic nor splenic CD8⁺ DCs expressed MR (Fig. 5B). It has been suggested that collagenase digestion routinely used to isolate DCs from tissues may not be compatible with MR detection [22]; however, in our hands, both DC populations were also MR-negative when prepared without collagenase digestion (data not shown). Antigen endocytosed via CD205 (also known as DEC-205) enters the MHC class I and MHC class II antigen presentation pathways and is subsequently presented to both CD8⁺ and CD4⁺ T cells with high efficiency [23]. Both

**Figure 5.** Maturation and phenotypic characterization of thymic and splenic CD8⁺ DCs. (A and B) Thymic and splenic cells were labeled and gated as shown in Fig. 1A. In addition, the cells were stained for the expression of the indicated molecules (open histograms). The numbers indicate the ratio of the mean fluorescence intensity relative to the (A) unstained or (B) isotype control (shaded histograms). BM cells were cultured in the presence of GM-CSF, and CD11chigh DCs (BM-DCs) were used as a positive control for the MR at day 8 of culture. (C) Thymic and splenic cells labeled and gated as shown in Fig. 1A were stained with a CD103 Ab or isotype control. The numbers indicate the percentage of CD103⁺ cells present in the CD8⁺ DC subpopulations. Results shown are representative of one out of two independent experiments.
Thymic and splenic CD8⁺ DCs showed equal expression of CD205 (Fig. 5B), indicating that this receptor was not responsible for the higher efficiency of antigen presentation by thymic DCs. Furthermore, the monophasic expression of CD205 in both thymic and splenic DCs reinforces the absence of contamination with other cell types in the isolates from both organs.

Recently, it has been shown that CD103⁺ (integrin αE) DCs share some of the properties of the CD8⁺ DCs, including cross-presentation capability and dependence on the Batf3 (Jun dimerization protein p21SNFT) transcription factor for their development [24, 25]. Although it has been described that 50–70% of splenic CD8⁺ DCs coexpress CD103 [12, 26], we and others [27] found much lower proportions of these cells. In both C57BL/6 and Balb/c mice, only 10–20% of splenic CD8⁺ DCs showed weak expression of CD103. Interestingly, around 70% of the thymic CD8⁺ DCs from both mouse strains showed strong CD103 staining (Fig. 5C). This finding indicates that possibly the CD103⁺ status of the subpopulation may correlate with the observed functional differences between thymic and splenic CD8⁺ DCs.

**Thymic CD103⁺ CD8⁺ and CD103⁻ CD8⁺ DCs efficiently cross-prime T cells**

As GM-CSF supported efficient T-cell proliferation by splenic CD8⁺ DCs, we next investigated the effect of GM-CSF on the activation status of DCs. CD80 and CD86 were upregulated after 16 h of culture compared with freshly isolated DCs (Figs. 5A and 6A), probably reflecting maturation upon culture. Upregulation of both molecules was further increased in the presence of GM-CSF (Fig. 6A), corroborating the previous studies [19, 28]. However, this upregulation of activation molecules in GM-CSF licensed DCs can only explain the augmented ability of splenic DCs to support T-cell proliferation, since thymic DCs did not require licensing to induce efficient T-cell proliferation, and both freshly isolated and cultured thymic and splenic CD8⁺ DCs showed no major difference in the expression of CD80 and CD86 (Figs. 5A and 6A). GM-CSF had no effect on the expression of MHC-I (H-2Kb), and the expression level of this molecule was similar between thymic and splenic DCs (Fig. 6A). Furthermore, GM-CSF had no effect on the status of CD103, indicating that, rather than representing an activation/maturation molecule, CD103 may be expressed on a distinct subpopulation of thymic CD8⁺ DCs. We found that both thymic CD8⁺ DC subpopulations, CD103⁻ and CD103⁺, can support cross-presentation of T cells, independent of GM-CSF (Fig. 6B), indicating that at least in the thymus, CD103 is not directly associated with cross-presentation. Interestingly, it was shown in a parallel study with DCs produced in Flt3 ligand-stimulated BM cultures, that acquisition of cross-presentation capacity is promoted by GM-CSF, and that although this coincided with expression of CD103, the expression of this molecule was dissociated from the capacity to cross-present [29]. Taken together, these results indicate that, as opposed to splenic CD8⁺ DCs, thymic CD8⁺ DCs possess an intrinsic cross-presentation property, and that upregulation of costimulatory molecules or expression of CD103 is not required for efficient cross-priming of naïve T cells.

**Discussion**

In this study, we compared the ex vivo cross-priming capabilities of CD8⁺ DCs isolated from steady-state thymus and spleen. Our data show that cross-priming of naïve OVA-specific CD8⁺ T cells by splenic CD8⁺ DCs is not an inherent but a regulated property, requiring licensing factors such as GM-CSF. Surprisingly, however, cross-priming appears to be a “built-in” property of the thymic CD8⁺ DC subset, as these cells were fully able to cross-present soluble OVA to naïve CD8⁺ T cells in the absence of licensing factor.

Figure 6. Thymic CD8⁺ CD103⁺ and CD8⁺ CD103⁻ DCs both can efficiently cross-prime in the absence of licensing factor. (A) Thymic and splenic cells were cultured in the absence (shaded histograms) or the presence (open histograms) of GM-CSF. After 16–18 h, cells labeled and gated as in Fig. 1A were stained with CD103, CD80, CD86, and MHC-I (H2Kb) antibodies or isotype control (dotted histograms). (B) Sorted thymic CD8⁺ CD103⁺ and CD8⁺ CD103⁻ DCs were loaded with soluble OVA and cocultured with CFSE-labeled OT-I cells in the absence (medium) or the presence of GM-CSF. All results are representative of one out of two independent experiments.
licensing. The defect of splenic CD8⁺ DCs in cross-priming in the absence of GM-CSF did not correlate with antigen uptake since splenic and thymic DCs were equally able to internalize soluble OVA (Fig. 2F). Previous studies have shown that most DCs in peripheral tissues have a resting phenotype, and that although these cells can efficiently capture antigens, they are unable to process and present them efficiently to T cells [30]. Indeed using an Ab that recognizes OVA peptide in the context of MHC-I, we observed a signal above the background only in thymic but not in splenic CD8⁺ DCs, indicating that under steady-state conditions, only the thymic CD8⁺ DCs can process and cross-present antigen.

GM-CSF has been shown to enhance the expression of the costimulatory molecules CD80 and CD86 in DCs (Fig. 6A and [19, 28]). While the upregulation of costimulatory molecules by GM-CSF may contribute to enhance the cross-priming capacity of splenic CD8⁺ DCs, it cannot explain the superior cross-priming capacity of thymic CD8⁺ DCs in the absence of licensing, since freshly isolated and cultured thymic DCs did not present major differences in overall DC maturation/activation when compared with splenic DCs (Figs. 5A and 6A).

Several endocytosis receptors of the C-type lectin receptor family have been implicated in introducing exogenous antigens directly into the organelles in which cross-presentation occurs [31]. Among them, CD205 and MR could provide a mechanistic explanation for the efficiency and potential differences in cross-presentation of different sub-populations of CD8⁺ DCs. However, we did not find any divergence in the expression of these molecules in CD8⁺ DCs isolated from spleen and thymus (Fig. 5B). The major phenotypical difference between thymic and splenic CD8⁺ DCs we observed was regarding the surface expression of CD103. Nevertheless, cross-priming efficiency, at least in thymus, did not correlate with the CD103 status, as both CD103⁺CD8⁺ and CD103⁻CD8⁺ DCs isolated from thymus were able to efficiently cross-prime naïve T cells (Fig. 6B). However, we cannot rule out the possibility that CD103⁺CD8⁺ splenic DCs can efficiently cross-prime in the absence of GM-CSF. While the CD103 status of thymic DCs has not previously been assessed, the proportion of CD103⁺ cells among the CD8⁺ splenic DCs differs widely between our data and previous reports. We found only intermediate expression of this molecule in approximately 10–20% of the splenic CD8⁺ DCs, in contrast to the previous reports in which 50–70% of splenic CD8⁺ DCs were shown to coexpress CD103⁺ [12, 26]. Accordingly, it has been recently shown in two independent studies that splenic CD8⁺ DCs are heterogeneous regarding their ability to cross-present antigens [11, 12]. Since IL-3, TGFβ, and GM-CSF promote CD103 expression [29], variability of these cytokines could be the source of the differences concerning CD103 expression/cross-presentation by splenic DCs from different laboratories. Furthermore, GM-CSF is commonly added to in vitro cross-presentation assays, incorrectly leading to the conclusion that splenic CD8⁺ DCs are intrinsically capable of antigen cross-presentation. We also observed that the thymic CD8⁺ DCs were more viable than the splenic CD8⁺ DCs, and that GM-CSF had an effect on DC survival (Fig. 4B). Although we did not assess the presence of pro/anti-apoptotic factors, it is possible that such factors are differentially expressed in the two DC populations, and that cytokines such as GM-CSF provide critical survival signaling to splenic CD8⁺ DCs to render an efficient T-cell response. The difference in viability (rather than costimulatory molecules on the DC surface) may also explain the lower efficiency in T-cell priming by peptide-loaded splenic CD8⁺ DCs compared with thymic CD8⁺ DCs (Fig. 2E). However, it is unlikely that the observed differences are solely due to reduced viability of splenic DCs in the absence of licensing because when a much smaller number of thymic CD8⁺ DCs than splenic DCs was used, the thymic subpopulation efficiently induced T-cell proliferation. In addition, K⁺-OVA₂₅⁷₋₂₆₄ complexes were detected on viable thymic CD8⁺ DCs but not on viable splenic CD8⁺ DCs (Fig. 4A).

Effector immune responses require that DCs encounter antigens in the presence of infectious agents or “danger signals,” which activates TLR, for example. As such “danger signals” are provided, in general, only at the primary site of infection, it is unclear how migratory DCs or other antigen-presenting cells can transmit the antigen and the costimulatory signals obtained from the contact with the infectious agent. It is tempting to speculate that the effect of licensing factors on the cross-priming ability of secondary lymphoid organ DCs could replace the activation patterns provided by pathogens when tissue resident DCs acquire antigen transmitted by infected migratory DCs. In this context, antigen-receiving tissue resident DCs may obtain costimulatory signals from local cells/cytokines in the absence of a direct contact with the infectious agent. In accordance with this idea, there is evidence that GM-CSF can trigger IL-6 production by DCs, thereby enhancing survival of auto-reactive T cells [32]. Furthermore, GM-CSF has been shown to be an inflammatory cytokine, and production of GM-CSF by CNS infiltrating CD4⁺ T cells promotes induction of experimental autoimmune encephalomyelitis (EAE) by immunogenic T cells. Moreover, transfer of OVA-loaded splenic DCs that have been previously cultured with GM-CSF induced stronger expansion of CD4⁺ and CD8⁺ T cells in vivo than freshly isolated DCs [28]. The necessity for DC licensing in the periphery can be viewed as a safety strategy to avoid autoimmunity due to cytotoxic effector responses against self-antigens. In this context, DC licensing through either CD40 or GM-CSF activation could be only provided when different T cells specific for the same auto-antigen are primed, synergistically providing the factors necessary to activate DCs and induce efficient T-cell priming. This situation may reflect the requirements for proficient cross-presentation also by human DCs. It was recently demonstrated that BDCAC₃⁻ DNGR-1⁻ (also known as CLEC9A) DCs found in human spleen are the equivalents of the mouse CD8⁺ DCs and that these cells were activated more efficiently in the presence of T-cell-derived stimuli [33]. On the other hand, in the thymus this strategy may not be necessary, as thymocytes that bind self-peptide–MHC complexes with high affinity are excluded from the mature T-cell pool [34]. Our data document that thymic DCs have a distinct superior cross-priming ability and, as opposed to splenic DCs, licensing is not required for inducing naïve antigen-specific T-cell proliferation. This may
ensure that even low-antigen doses provided by thymic epithelial cells or other sources are sufficient for the deletion of autoreactive T cells by thymic DCs and may explain the reason why in the presence of small antigen amounts in steady-state circumstances, DCs are not able to delete autoreactive T cells in the periphery [14]. The cellular and molecular mechanisms responsible for these differences have to be further investigated.

Materials and methods

Mice

C57BL/6 (CD45.1), Balb/c, and OT-I (CD45.2) mice were maintained and bred in the animal facility of the Institute of Virology, University of Zurich (Zurich, Switzerland). OT-I mice have transgenic Vα2/VJβ TCRs specific for OVA257–264/H2-Kb. β2m/RAG knockout mice were provided by E. Palmer (University Hospital Basel, Basel, Switzerland). All animal experiments were performed using mice between 4–12 wk of age and were approved by the ethical committee of the Canton Zurich (Permit No. 24/2008).

DC isolation, flow cytometry, and sorting

DCs were isolated from pooled mouse C57BL/6 thymuses and spleens as described in detail elsewhere [16]. Briefly, organs were chopped and digested with collagenase A and DNase I (Roche) for 30 min at 37°C, followed by 15 min treatment with EDTA. Single-cell suspensions were prepared, and light-density cells were collected by a density centrifugation procedure with OptiPrep (Sigma-Aldrich). The DC-enriched preparations were stained and subsequently sorted with a FACSAria (Becton Dickinson). Sorting was performed in phosphate-buffered saline (PBS, pH 7.3) and resulted in purity of >97%. FACS analysis was performed with a FACSCalibur (Becton Dickinson) and the data analyzed with FlowJo software (Tree Star).

Antibodies

The following mAbs were purchased from BD Pharmingen: CD4 (L3T4), CD45RA (14.8), CD8a (53–6.7), CD11b (M1/70), CD172a (P84). The following mAbs were purchased from BioLegend: CD3 (145-2C11), CD11c (N418), CD19 (6D5), CD103 (2E7), Gr-1 (RB6-8C5), NK1.1 (PK136), CD40 (3/23), BioLegend: CD3 (145-2C11), CD11c (N418), CD19 (6D5), CD172a (P84). The following mAbs were purchased from BD Pharmingen: CD4 (L3T4), CD45RA (14.8), CD8a (53–6.7), CD11b (M1/70), CD172a (P84). The following mAbs were purchased from E. Palmer (University Hospital Basel, Basel, Switzerland). All animal experiments were performed using mice between 4–12 wk of age and were approved by the ethical committee of the Canton Zurich (Permit No. 24/2008).

Analysis of in vitro antigen presentation to naïve T cells

Unless otherwise stated, sorted DCs were loaded with 0.1 mg/mL of soluble OVA (Grade V, Sigma-Aldrich), or with OVA257–264 peptide (SIINFEKL; NeoMPS) in complete medium (RPMI 1640 complemented with 10% FBS, 100 units/ml penicillin, 100 µg/mL streptomycin, 10 mM Hepes). After incubation for 1 h at 37°C in a 95% air/5% CO2 atmosphere, the cells were washed three times. Unless otherwise stated, 2 × 10^5 DCs per well were plated in U-bottom 96-well plates (Becton Dickinson) in complete medium at 37°C in a 95% air/5% CO2 atmosphere. For some experiments, 200 U/mL of recombinant mouse GM-CSF (premium grade, Miltenyi Biotec) or 2 µg/mL of anti-CD40 Ab (FGK45.5, Miltenyi Biotec) were added. For the experiment of GM-CSF neutralization, blocking anti-mouse GM-CSF (MP1-22E9, BioLegend) or isotype control (RTK2758, BioLegend) were used at a ratio of GM-CSF:Ab of 1:2000. OT-I cells were isolated from spleen and lymph nodes by negative selection using a CD8-T-cell isolation kit (Miltenyi Biotec), and were labeled with CFSE (Alexis Biochemicals) as described elsewhere [35]. Briefly, 2 × 10^4 CFSE-labeled T cells were cocultured with DCs. The percentage of proliferating T cells (CD45.2+ CFSE<sup>low</sup>) was determined by FACS analysis after 3 days of culture. For the experiments using cell-associated OVA, DCs and T cells were cocultured with the indicated amounts of β2m/RAG knockout apoptotic splenocytes coated with OVA. For this, splenocytes were washed twice in RPMI 1640 medium and resuspended in 1 mL of hypertoninc medium (0.5 M sucrose, 10% w/v polyethylene glycol 1000, and 10 mM Hepes in RPMI 1640, pH 7.2) containing 10 mg/mL OVA. After incubation for 10 min at 37°C, 13 mL of pre-warmed hypotonic medium (40% H2O, 60% RPMI 1640) was added, and the cells were incubated for 2 min at 37°C and then washed twice with ice-cold PBS.

Analysis of cross-presentation

Sorted DCs (10^5) were incubated for 20 h at 37°C in a 95% air/5% CO2 atmosphere in complete medium containing 10 mg/mL of soluble OVA (Grade V, Sigma-Aldrich), BSA (PAA), OVA257–264 peptide (NeoMPS) or MOG<sub>35–55</sub> peptide (ProSpec). Cells were stained with K<sup>B</sup>-OVA257–264 (25-D1.16, eBioscience) Ab and cross-presentation of viable cells was determined by FACS analysis.

Internalization of soluble fluorochrome-conjugated OVA

Sorted DCs were incubated in complete medium containing 20–80 µg/mL Alexa Fluor 488-conjugated OVA (Invitrogen) for 45 min at 37°C or on ice (80 µg/mL Alexa Fluor 488-conjugated OVA). Cells were washed and analyzed by flow cytometry.
BM-DCs

BM-DCs were prepared from tibia and femur. Cells were seeded at $1 \times 10^6$ cells/mL in complete medium supplemented with homemade GM-CSF at optimal titration. At day 8 of culture, the cells were harvested from the supernatant.

Statistical analysis

Data were analyzed using the Student’s t-test (GraphPad prism version 4.00 software). A p-value $<0.05$ was considered significant.

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References


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Abbreviations: BM-DC: BM-derived DC, cDC: conventional DC, DN: double negative, MR: mannose receptor.

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