IN THIS ISSUE, PSYCHOLOGY
Correction for the “In This Issue” summary entitled “Universal displays of pride and shame,” which appeared in issue 33, August 19, 2008, of Proc Natl Acad Sci USA (105:11587–11588).

The authors note that the figure is copyrighted by Bob Willingham and is reprinted with permission. The online version has been corrected. The figure and its corrected legend appear below.

Blind athletes (Right) show pride in victory like sighted athletes (Left). [Reproduced with permission (Copyright 2004, Bob Willingham).]

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PERSPECTIVE

The authors note that a reference was inadvertently omitted from their article. On page 4607, right column, in Conclusion: How Do New Gene Clusters Form?, line 17, the reference callout “(109–111)” should instead read “(109–111, 113).” The added reference appears below.


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DEVELOPMENTAL BIOLOGY

The authors note that due to a printer’s error, the affiliation information for some authors appeared incorrectly. The correct affiliation for V. Havlicek and U. Besenfelder is “Reproduction Centre-Wieselburg, University of Veterinary Medicine, 1210 Vienna, Austria”; and the correct affiliation for H. Lehrhach and J. Adjaye is “Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany.”

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APPLIED BIOLOGICAL SCIENCES

The authors note that the author name Christian Steinkulher should have appeared as Christian Steinkuhler. The author line has been corrected online. The corrected author line appears below.

Claudia Colussi, Chiara Mozzetta, Aymone Gurtner, Barbara Illi, Jessica Rosati, Stefania Straino, Gianluca Ragone, Mario Pescatori, Germana Zaccagnini, Annalisia Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinkuhler, Emilio Clementi, Carmela Dell’Aversana, Lucia Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano

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IMMUNOLOGY

The authors note that due to a printer’s error, in the Abstract, beginning on line 6, “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirp+ and CD8hiSirp+, which have different origins. We found that the CD8Sirp+ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus” should instead read: “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirp+ and CD8hiSirp+, which have different origins. We found that the CD8Sirp+ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.”

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Dendritic cells in the thymus contribute to T-regulatory cell induction

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Central tolerance is established through negative selection of self-reactive thymocytes and the induction of T-regulatory cells (Tregs). The role of thymic dendritic cells (TDCs) in these processes has not been clearly determined. In this study, we demonstrate that dendritic cells (DCs) in the thymus contribute to Treg generation in vivo. TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirpα+cDCs (CD8loSirpα+ and CD8loSirpα−) and CD8hiSirpα−cDCs (CD8hiSirpα−), which have different origins. We found that the CD8hiSirpα−cDCs represent a conventional DC subset that originates from the bone marrow and migrates into the thymus. Moreover, we show that the CD8hiSirpα−cDCs demonstrate a superior capacity to induce Tregs in vitro. Finally, using a thymic transplantation system, we demonstrated that the DCs in the periphery can migrate into the thymus, where they efficiently induce Treg generation and negative selection.

Tolerance to self-antigens is established in the thymus. Developing thymocytes undergo stringent selection to eliminate self-reactivity (1). Developing T cells that recognize self-peptide with a sufficiently high affinity can encounter two fates: (i) deletion through negative selection or (ii) differentiation into T-regulatory cells (Tregs). Tregs express the transcription factor Foxp3 (2–4) and can suppress self-reactive T cells that have escaped negative selection (5, 6). During mouse ontogeny, Tregs appear in the thymus 3 days after birth (7). Deficiency in Treg development or function results in multiorgan autoimmunity (6).

A role for thymic dendritic cells (TDCs) in negative selection (8–12) and for thymic epithelial cells (TECs) in negative selection and Treg induction has been demonstrated (9, 13–16). The role of dendritic cells (DCs) in Treg generation in the thymus is unclear, however. Given the importance of DCs in the generation of thymic-derived Tregs (5, 14, 15, 19, 25, 26), these systems enabled us to discern the contribution of DCs to Treg induction.

Because some DCs are radioresistant, it was important to establish whether TDCs in the chimeras were all of donor origin (27, 28). Staining the TDC-enriched light density cell fraction for donor-derived DCs and CD86 is essential for the induction of thymic-derived Tregs (5, 14, 15). CD8hiBM chimeras, the host epithelial cells can still present antigen via MHCI, whereas the BM-derived cells, including TDCs, cannot. In the second system, irradiated CD45.1 recipients were reconstituted with B6 BM lacking CD80 and CD86 (27) or WT BM for controls. Because expression of MHCI and costimulatory molecules CD80 and CD86 is essential for the induction of thymic-derived Tregs (5, 14, 15, 19, 25, 26), these systems enabled us to discern the contribution of DCs to Treg induction.

To assess the effect on thymocyte development in mice lacking MHCI on DCs, the proportion and total numbers of the individual donor-derived thymocyte populations were determined (Fig. 1 C–E). Total thymic cellularity was comparable between the MHCI+/− and WT BM chimeras (supporting information SI Table S1), and the numbers of CD4−CD8− double-negative, CD4+CD8− double-positive, and CD8+CD4− (CD8− hereafter) T-cell populations were impaired in antigen presentation (MHCI class II [MHCIii−] or T-cell activation (B7−)). Using an in vitro culture system, we established that the Sirpα+ TDCs played the major role in Treg induction when compared with other DC subtypes. This functional capacity of the Sirpα+ TDCs correlates with a unique set of properties, particularly their maturity, their chemokine production, and their migratory origin. These findings suggest that a subset of TDCs migrating from the periphery makes a specialized contribution to Treg induction in the thymus.

Results

TDCs Contribute to Treg Induction and Negative Selection In Vivo. To dissect the contribution of DCs from that of mTECs in the induction of Tregs, two different in vivo systems were used. In the first, irradiated C57BL/6 (B6) WT CD45.1 recipients were reconstituted with BM from MHCIii− or B6 WT (CD45.2) mice. In MHCIii− BM chimeras, the host epithelial cells can still present antigen via MHCI, whereas the BM-derived cells, including TDCs, cannot. In the second system, irradiated CD45.1 recipients were reconstituted with B7− BM (lacking CD80 and CD86) or WT BM for controls.

Because expression of MHCI and costimulatory molecules CD80 and CD86 is essential for the induction of thymic-derived Tregs (5, 14, 15, 19, 25, 26), these systems enabled us to discern the contribution of DCs to Treg induction.

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The authors declare no conflict of interest.

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The induction of thymic TRs and in the negative selection of BLA-positive, CD4+ WT or B7−/− BM reconstitution. Total thymic cellularity did not differ between the two groups (Table S1). There was a 20% increase in the number of CD4+CD8− (CD4+ hereafter) thymocytes in the MHCI−/− BM chimeras, however, suggesting that there was incomplete negative selection (Fig. 1D). Syngeneic mixed leukocyte reaction assays confirmed that the CD4+ thymocytes contained auto-reactive T cells (data not shown). Concomitant with this increase was a statistically significant 30% decrease in the number of CD4+CD25+Foxp3+ Tregs (P = 0.008) (Fig. 1E).

To test the function of the Tregs in the WT and MHCI−/− chimeras, they were sorted and used in an in vitro Treg suppression assay. The Tregs from both groups were functional (Fig. 1F).

In the second BM chimeric system, B7−/− mice were used. Initially, it was established that B7−/− mice have a deficiency in the proportion of Tregs that equated to a 94% decrease (Fig. S1A). To establish if this was attributable to cells in the hematopoietic or epithelial cell compartment, four cohorts of chimeras were set up. CD45.1 WT or CD45.1 B7−/− mice were reconstituted with CD45.2 WT or B7−/− BM and analyzed for TR development 8 weeks after reconstitution. Total thymic cellularity did not differ between the four cohorts (data not shown). There was a 50% decrease in the number of thymic Tregs in the B7−/− to WT chimeric mice, however (Fig. 1G; Fig. S1B).

Overall, these results suggest a nonredundant role for TDCs in the induction of thymic Tregs and in the negative selection of self-reactive CD4+ thymocytes.

DCs Induce Antigen-Specific Treg and Negative Selection In Vivo. Treg induction and negative selection of self-reactive thymocytes require self-peptide presentation on MHCI in an antigen-presenting cell (14, 15). To address Treg induction by DCs in an antigen-specific system, Rag2−/− OTII T-cell receptor (TCR) transgenic (tg) mice (which lack OVA-specific Tregs because of the absence of the OVA antigen) were crossed with CD11cOVA tg mice (membrane-bound OVA expressed under the CD11c promoter). In these Rag2−/− OTII/CD11cOVA (Rag2−/−/OVA) double-tg mice, OVA is expressed on CD11c+ TDCs and can influence the development of CD4+ T cells that express the OVA-specific TCR (29). To follow development of newly formed thymocytes from the double-tg BM cells, irradiated WT CD45.1 recipients were reconstituted with the BM of CD45.2 Rag2−/−/OVA/OVA mice or Rag2−/− OTII mice for controls. Thymocytes were analyzed by flow cytometry 6 weeks later. Total cellularity of the Rag2−/−/OVA BM chimeric thymuses was reduced compared with controls (Fig. 2A). The presentation of OVA by DCs in Rag2−/−/OVA BM chimeric mice led to the deletion of the majority of OTII+CD4+ cells, as seen by a >90% reduction in the total number of CD45.2+CD4+Vα2+ OTII-Tregs compared with controls (Fig. 2B). Furthermore, there was a clear induction of OTII Tregs in the thymus of Rag2−/−/OVA BM chimeras (mean 15 ± 2% of OTII+CD4+ cells) compared with the controls (0.1% of OTII+CD4+ thymocytes). This represented a greater than 150-fold increase in Treg numbers in the thymus of Rag2−/−/OVA BM chimeras compared with controls (Fig. 2B and D).

Overall, these results demonstrate that DCs are capable of Treg induction and negative selection in an antigen-specific manner.

![Fig. 2](https://www.pnas.org/cgi/doi/10.1073/pnas.0810268105)
Sirpα+ TcDCs Are More Mature in Surface Phenotype Than the Sirpα− TcDCs. Because TDCs were involved in T₉ generation and negative selection, we investigated the contribution of the TDC subtypes to these processes. We compared TDCs for expression of MHCI, costimulatory molecules, because these are important in T₉ induction and negative selection (19, 30–34). We then compared the TDCs with their splenic DC (SDC) equivalents. TDCs and SDCs were segregated into pDCs and cDCs, which could be further segregated as Sirpα+ CD11c+ and Sirpα− CD11c− cDCs (21) (Fig. 3A). Strikingly, the Sirpα+ TcDCs expressed higher levels of MHCI and CD86 and slightly increased levels of the activation marker CD69 compared with Sirpα− TcDCs (Fig. 3B). These differences were not observed between the DC subsets in the spleen, where both cDC subsets expressed comparable levels of these markers (refs. 35, 36; Fig. 3B). Nor was there a difference in expression of these molecules in thymic versus splenic pDCs (Fig. 3C). Thus, in the steady state, Sirpα+ TcDCs are phenotypically more “mature” than other TDC subtypes.

Sirpα+ TcDCs Are More Efficient at Inducing Functional T₉s In Vitro. To compare the capacity of each TcDC subset to induce T₉s, sorted TcDC subsets were cocultured with syngeneic CD4+ CD8− CD25− thymocytes (which contain T₉ precursors) for 5 days. To maintain T-cell survival, an optimal level of IL-7 was added (37). The number of T₉s that developed in these cultures was enumerated. The Sirpα+ TcDCs were the most efficient at inducing T₉s, as shown by the higher number of CD4+ CD25+ Foxp3+ T cells in the cultures (Fig. 3D and E). In the cultures containing Sirpα− TcDCs, there was also some level of T-cell activation, as evidenced by a population of CD4+ CD25+ Foxp3− (Fig. 3D and data not shown). This T-cell activation was accompanied by T-cell proliferation and a total number of T cells within the cultures (Fig. S2A). Given that the proportion of T₉s induced in Sirpα− TcDC cocultures (14 ± 5%) was also significantly higher compared with Sirpα− TcDC cocultures (9 ± 1%) (Fig. 3D), it was clear that the increased number of T₉s could not be attributable solely to a higher absolute number of T cells generated. Furthermore, we determined that the T₉ induction observed was attributable to de novo generation and not to proliferation of preexisting CD4+ CD25− Foxp3− T cells within the starting population of thymocytes by using Foxp3-GFP mice to gate out CD4+ CD25− Foxp3− cells (Fig. S2B).

T₉ generation in vitro was thymus specific. When TcDCs were cultured with splenic CD4+ CD25− naïve T cells rather than thymic CD4+ CD25− T cells, no T₉ induction was observed (Fig. S2C), even in the presence of T-cell activation and proliferation (Fig. S2D). Conversely, when SDCs were cocultured with thymic CD4+ CD25− T cells, few T₉s were generated (data not shown).

To test the function of in vitro–derived T₉s, T₉s were sorted as CD4+ CD25+ CD62L+ cells and used in a T₉ suppression assay. CD62L was included as a marker to exclude activated T cells. In vitro–derived T₉s were able to suppress T-cell proliferation (Fig. 3F).

BM-derived cells that express MHCI within the thymus include B cells and macrophages. To exclude the possibility of T₉ induction by those cells, the same coculture method was used. We demonstrated that the T₉ induction capacities of both of these cell types were negligible (Fig. S2E).

Sirpα+ TcDCs Produce Chemokines and Attract CD4+ Thymocytes. The chemokine-mediated migration of developing thymocytes through the thymus ensures their interaction with the appropriate thymic stromal cells. We examined chemokine production as a factor that may explain the effectiveness of the Sirpα+ TcDCs in inducing T₉s. The expression of the genes encoding six chemokines known to be involved in thymocyte differentiation was examined by real-time (RT) PCR, comparing the TDC and SDC subsets, macrophages, and thymic mTECs.

The mTECs expressed significantly higher levels of CCL19, CCL21, and CCL25, higher than the DC subsets (Fig. S3A). In contrast, CCL17 and CCL22 were expressed at very high levels only by the Sirpα+ TcDCs (Fig. S3A). The expression of CCL22 by the Sirpα+ TcDCs was confirmed at the protein level by intracellular chemokine staining (Fig. S3B).

CCL17 and CCL22 both bind to CCR4. Using RT-PCR, we found that the CD4+ thymocytes expressed the highest levels of CCR4 (Fig. S3C), a finding consistent with other studies (38). To test whether the DC-expressed chemokines were chemotactic for CD4+ thymocytes, migration assays were performed. Sorted TDC and SDC subsets were cultured alone for 3 h. The supernatants were then used as a source of chemotactins for CD4+ thymocytes, seeded in transwells, and incubated for 2 h. The supernatants from the Sirpα+ TcDC cultures showed the greatest capacity to attract CD4+ thymocytes (Fig. S3D). Thus, the Sirpα+ TcDCs, through their chemokine production, have a special capacity to attract newly formed CD4+ T cells.

CD11c+ Sirpα− CD11b− cDCs Are Found in Blood and Migrate into the Thymus. A number of observations have led to the suggestion that the TcDC subsets have different developmental origins, with a major proportion of the TcDCs being derived from an early intrathymic precursor (24, 39). To test the origin of each TcDC, the earliest intrathymic precursors (Lineage− “Thy-1+c-kit”) that have DC po-
normal TcDC subsets is shown (Fig. S4). Among the cDCs, 4% were mature CD8<sup>+</sup> cDCs (Fig. 4B), correlating with DCs found circulating in the blood.

Impact of Migrating DCs on T-Cell Development. To determine the impact of circulating DCs on thymic T-cell selection, day 1 neonatal thymic lobes from CD45.1/OTII tg mice were grafted into the kidney capsule of recipient CD45.2 WT or CD45.2 CD11cOVA tg mice. This system allows recipient DCs to migrate into the grafted thymic lobes via the blood. Therefore, the effects of peripheral derived CD45.2 CD11cOVA migrating DCs on OTII T-cell development in the grafted lobes could be assessed. The kinetics of DC migration were determined. At day 7, before the recipient BM progenitors had contributed to the TDC population, the DCs entering the thymic lobes were predominantly the Sirp<sub>α</sub> cDCs (80% ± 5%; data not shown). We therefore waited a further 3–5 days to see the effects of these incoming DCs on T-cell development. Thymic lobes were removed 10–12 days after transplantation, and the phenotype of the incoming CD45.2<sup>+</sup> DCs and the resident CD45.1<sup>+</sup> OTII T cells was studied.

At day 10, DCs in the grafted thymic lobes were analyzed for DC markers to assess the phenotype of the host-derived CD45.2<sup>+</sup> migrating DCs. Of these CD11c<sup>+</sup> cells, 54 ± 6% were mature MHCII<sup>hi</sup>CD8<sup>-</sup> Sirp<sub>α</sub> cDCs, 4 ± 1% were mature CD8<sup>-</sup>Sirp<sub>α</sub>-cDCs, and the remaining were MHCII<sup>lo</sup>CD8<sup>-</sup> cDCs, which were formed by resident BM progenitors that had seeded the thymic grafts.

Thymocyte populations were analyzed by flow cytometry. The number of CD45.1<sup>+</sup>OTII CD4<sup>+</sup>Vα2<sup>+</sup>Vβ<sup>+</sup> T cells was reduced in lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4D), whereas the number of CD45.1<sup>+</sup>Vα2<sup>+</sup>Vβ<sup>-</sup> CD4<sup>-</sup> was similar in both groups (data not shown), suggesting that antigen-specific negative selection of OTII<sup>+</sup> T cells was occurring. In addition, a more than twofold increase in the number of CD4<sup>+</sup>Foxp<sup>+</sup> T cells was seen in the lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4E). Together, these results indicate that DCs migrating into the thymus from the periphery can induce negative selection and antigen-specific T<sub>R</sub> development.

Discussion

The present study demonstrates a role for mouse TDCs in T<sub>R</sub> differentiation as well as negative selection. In the absence of a MHCII-expressing hemopoietic compartment, we found a 30% reduction in the total number of polyclonal T<sub>R</sub>s and an increase in the number of self-reactive CD4<sup>+</sup> T cells in the thymus. This demonstrates that in addition to mTECs (16, 40), BM-derived cells make a significant contribution to T<sub>R</sub> generation and negative selection of CD4<sup>+</sup> T cells in a steady-state mouse. In addition, a 50% reduction in T<sub>R</sub> numbers was observed when the hemopoietic compartment lacked expression of CD80 and CD86. Although

Fig. 4. Sirp<sub>α</sub>-TcDCs originate from peripheral blood and can migrate into the thymus. (A) DC generation from purified Lineage<sup>−</sup>Thy-1<sup>−</sup>c-kit<sup>−</sup> intrathymic precursors (CD45.2) was analyzed 2 weeks after precursor transfer. The intrathymic precursor-derived cDCs were mainly CD8<sup>+</sup>Sirp<sub>α</sub> (Right). A representative contour plot of the normal TcDC subsets is shown (Left) for comparison. (B) Blood cells (2 × 10<sup>6</sup>) from CD45.1 mice were transferred i.v. into nonirradiated CD45.2 recipients. The phenotype of donor-derived cells in the thymus of recipients was determined 3 days later by gating for CD45.1<sup>+</sup>CD11c<sup>+</sup>CD45RA<sup>−</sup> cDCs. Expression of Sirp<sub>α</sub>, CD11b, CD8, and MHCII was determined on this population. (C–E) Thymic lobes from OTII tg CD45.2<sup>+</sup> mice crossed to CD45.1<sup>−</sup> WT mice were grafted under the kidney capsule of CD45.2<sup>−</sup>CD11cOVA tg or WT recipients. (C) The phenotype of recipient-derived CD45.2<sup>−</sup>CD45.1<sup>−</sup> DCs in the grafted thymic lobes from WT and CD11cOVA tg mice was determined. The recipient CD45.2<sup>−</sup>CD45.1<sup>−</sup>CD45RA<sup>−</sup> cDCs were gated for, and the expression of CD8 and Sirp<sub>α</sub> was determined. The level of expression of MHCII was determined on Sirp<sub>α</sub>− and Sirp<sub>α</sub><sup>+</sup> cDCs. (D) The total number of CD45.1<sup>−</sup>CD4<sup>+</sup>Vα2<sup>+</sup>Vβ<sup>+</sup> cells (OTII) was calculated in OTII lobes grafted into WT or CD11cOVA tg recipients. Data are the mean of three independent experiments (error bars, ±SD) (n = 11–21). ∗, P < 0.05. (E) CD45.1<sup>−</sup>CD4<sup>+</sup>Vα2<sup>+</sup>Vβ<sup>-</sup> cDCs in the OTII lobes from WT and CD11cOVA tg recipients (as in D) were further analyzed for CD25 and Foxp3 expression. The total number of CD45.1<sup>−</sup>CD4<sup>+</sup>Vα2<sup>+</sup>Vβ<sup>-</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>R</sub>s was calculated. Data are the mean of three independent experiments (error bars, ±SD) (n = 11–21). ∗, P < 0.05.
these BM chimeras indicated that a BM-derived cell was important for T<sub>R</sub> induction, the in vitro coculture system indicated that only TDCs, and not B cells and macrophages, were efficient in inducing T<sub>R</sub>s. Thus, taken together, it appears that TDCs are the major hemopoietic cells that contribute significantly to T<sub>R</sub> generation and negative selection of both negativereactive CD4<sup>+</sup> T cells in vivo. Previous studies have discouraged a nonredundant role for DCS in T<sub>R</sub> induction (41, 42).

The irradiation protocol used (850–900 rad), which may not be sufficient to completely ablate host-derived cells, coupled with the later time point for analysis (8–10 weeks), may have contributed to these results, however. Mice with reduced thymic cellularity and a later time point for analysis (8–10 weeks), may have contributed to the massive accumulation of autoreactive CD4<sup>+</sup> T cells in the thymus has masked the changes in T<sub>R</sub> numbers.

Apart from the issue of their quantitative contribution to the total T<sub>R</sub> population, our results now demonstrate that TDCs can induce Ag-specific T<sub>R</sub>. Three types of DCS are found in the mouse thymus (pDCs, Sirp<sup>+</sup> CD8<sup>+</sup> TcDCs, and Sirp<sup>+</sup> CD8<sup>+</sup> TcDCs), and these have counterparts in the human thymus (43). We show that the minor Sirp<sup>+</sup> TcDC subset is much more efficient than the other DCS at polytional T<sub>R</sub> induction in vitro.

Why would the Sirp<sup>+</sup> TcDCs be more efficient in T<sub>R</sub> generation? First, the Sirp<sup>+</sup> TcDCs are more mature, in terms of expression of MHCI and costimulatory molecule, than the other TDCs, consistent with the phenotype of migratory DCS. This may enable them to interact more efficiently with the CD4<sup>+</sup> thymocytes. Second, the Sirp<sup>+</sup> cDCs are more efficient at presentation of antigens on MHCI than the CD8<sup>+</sup> cDCs (44–46). Finally, we show that the Sirp<sup>+</sup> TcDCs express high levels of CCL17 and CCL22, and this may facilitate an interaction with the CD4<sup>+</sup> thymocytes expressing high levels of CCR4. In a cell migration assay, CD4<sup>+</sup> thymocytes preferentially migrated toward supernatants from the Sirp<sup>+</sup> TcDCs. This provides a mechanism by which rare antigen-specific thymocytes can encounter their cognate antigen with a higher frequency.

Previous studies showed that migratory DC can induce negative selection of T cells specific for a peripherally expressed antigen (10). We now add to this picture by demonstrating that Sirp<sup>+</sup> cDCs or their immediate precursors present in blood migrate into the thymus, thus facilitating both negative selection and T<sub>R</sub> development.

Would this process be detrimental to the host during a viral infection? Viral antigens in the periphery ferried to the thymus may induce T<sub>R</sub>s, which could induce tolerance to the virus and potentially jeopardize a memory response. It is possible that thymic homing receptors or lymphoid egress receptors are down-regulated in DCS that have been activated by virus infection. Indeed, activated T cells down-regulate the egress receptor sphingosine 1-phosphate receptor-1, leading to retention of T cells in the lymphoid tissues (47). Whether this also occurs in activated DCS would be an interesting question to address in future studies.

In summary, we demonstrate that thymic DCs contribute to T<sub>R</sub> induction in vitro. More significantly, we show that peripheral DCS can migrate into the thymus, where they induce the development of T<sub>R</sub>s and the deletion of self-reactive CD4<sup>+</sup> thymocytes. Based on these observations, we propose a mechanism by which central tolerance to peripherally expressed antigens is induced by migrating DCS, a mechanism additional to the ectopic expression of peripheral antigens by mTECs.

**Materials and Methods**

**Mice.** All mice were bred under specific pathogen-free conditions. B<sup>7<sub>-/-</sub></sup> mice were purchased from The Jackson Laboratory and maintained in the University Laboratory Animal Research Facility at the University of Michigan. All other mice were obtained from The Walter and Eliza Hall Institute animal breeding facility. C57BL/6 (B6) mice 6–8 weeks of age were used for isolation of DCS and thymocytes. B<sup>6</sup> CD45.1 mice 10 weeks of age were used as BM recipients. The mouse strains used included OTII Tg (CD4<sup>+</sup> T cells expressing the TCR specific for MHCI-restricted Ova peptide) (48) on a B6, CD45.1, or Rag2<sup>-/-</sup> background; IA<sub>A</sub><sup>-/-</sup> (MHCI<sup>-/-</sup>) (49); B<sup>7<sub>-/-</sub></sup> (50); and CD11c<sup>+</sup>ova tg mice that express membrane-bound ovalbumin (amino acids 323–339) under control of the CD11c promoter (29, 51).

**BM Chimeras.** CD45.1 recipient mice were lethally irradiated with two doses of 5.5 Gy (3 h apart) and then received 5 × 10<sup>6</sup> CD45.2 donor BM cells i.v. from B6 or MHCI<sup>-/-</sup> mice or from Rag2<sup>-/-</sup> OTII/CD11c<sup>+</sup>ova double-tg mice. For B<sup>7<sub>-/-</sub></sup> chimeras, CD45.1 recipient mice were lethally irradiated with 8.0 Gy of total body irradiation. A total of 5 × 10<sup>6</sup> T-cell-depleted B6 WT or B<sup>7<sub>-/-</sub></sup> donor BM cells were injected i.v. into the recipients the next day. Chimeras were analyzed by flow cytometry 6–8 weeks after reconstitution.

**Antibodies.** Details can be found in SI Experimental Procedures.

**Isolation of DCS.** Details can be found in SI Experimental Procedures.

**Isolation of Thymocytes.** Details can be found in SI Experimental Procedures.

**Carboxyfluorescein Succinimidyl Ester Labeling.** Details can be found in SI Experimental Procedures.

**Isolation of Thymic B Cells, Macrophages, and mTECs.** Details can be found in SI Experimental Procedures.

**T<sub>R</sub> Suppression Assay.** Details can be found in Supplementary Experimental Procedures.

**Generation of T<sub>R</sub>s in Vitro.** In vitro T<sub>R</sub> induction assays were performed in triplicate in a round-bottom 96-well plate with 1 × 10<sup>5</sup> sorted thymic or splenic DC subsets from CD45.2 mice and 2 × 10<sup>4</sup> sorted CD4<sup>+</sup> CD25<sup>+</sup> thymocytes from CD45.1 mice, cultured together with an optimal concentration of IL-7 for 5 days. T<sub>R</sub>s were assessed by staining for CD45.1 (A201.1), CD4, CD25, and Foxp3. When GFP-Foxp3<sup>+</sup> mice were used as the CD4<sup>+</sup> thymocyte source, thymocytes were sorted as CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>-</sup> (excluding all Foxp3<sup>+</sup>) cells. IL-7 used was from the culture supernatant of J558 cell transfected with the murine IL-7 cDNA in the B MG neo vector (55). To determine the optimal concentration of the IL-7 supernatant, the supernatant was titrated on IL-7–dependent pro-B cells (37).

**Quantitative PCR.** Quantitative PCR was performed for chemokine gene expression by DC subsets as previously described (56). Further details can be found in Supplementary Experimental Procedures.

**Cell Migration Assay.** Sorted TDC and SDC subsets (5 × 10<sup>5</sup> in 600 μl) were cultured in a 24-well plate for 3 h. The supernatant was removed and placed in the base of transwell chambers (5.0-μm pore, Viz, COSTAR). Sorted CD4<sup>+</sup> CD25<sup>-</sup> thymocytes (2 × 10<sup>5</sup>) were placed in the top of the chamber and allowed to migrate for 2 h at 37 °C. The number of cells that had migrated was enumerated using fixed numbers of beads as a calibration standard.

**In Vivo DC Migration Assay.** Details can be found in Supplementary Experimental Procedures.

**Staining Blood DCS.** Details can be found in Supplementary Experimental Procedures.

**Thymic Grafting.** Thymic lobes from 1-day-old donor mice were grafted under the kidney capsule of anesthetized 8-week-old recipient mice using a procedure described elsewhere (57). At specified times postgrafting, grafted thymic lobes were recovered and processed individually. Thymic lobes were digested in collagenase/DNase and analyzed by flow cytometry.

**Statistical Analysis.** Statistical significance was assessed by the two-tailed unpaired Student’s t test. Differences with P values less than 0.05 were considered significant.

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