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).

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

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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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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, CD8hiSirpalo/lo (CD8hiSirpalo) and CD8loSirpalo/hi (CD8loSirpalo), which have different origins. We found that the CD8hiSirpalo 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, CD8hiSirpalo/lo (CD8hiSirpalo) and CD8loSirpalo/hi (CD8loSirpalo), which have different origins. We found that the CD8hiSirpalo DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.”

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Claudia Colussi, Chiara Mozzetta, Ayumone 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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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 in vivo, TDCs not only play a role in negative selection but in the induction of Treg. TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirpα+/+ (CD8loSirpα+tDCs) and CD8hiSirpα− (CD8hiSirpα−), which have different origins. We found that the CD8loSirpα+tDCs represent a conventional DC subset that originates from the blood and migrates into the thymus. Moreover, we show that the CD8loSirpα+tDCs demonstrate a superior capacity to induce Tregs in vitro. Finally, using a thymic transplantation system, we demonstrate that the DCs in the periphery can migrate into the thymus, where they efficiently induce Treg generation and negative selection.

tolerance | migratory dendritic cells | tolerance

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 through negative selection or (5) 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 TDCs in these processes has not been clearly determined. In this study, we demonstrate that the TDCs in the periphery can migrate into the thymus and function in regulating T-cell tolerance.

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 MHCII−/− or B6 WT (CD45.2) mice. In MHCII−/−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.

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 6 weeks after BM reconstitution demonstrated that >98% of DCs were of donor origin (MHCII−/−BM chimeras), indicating effective elimination of host DCs (Fig. L4). The TDCs from the MHCII−/−BM chimeras did not express MHCI (Fig. 1B). Furthermore, both cDC subsets were observed in similar proportions and number in WT and MHCII−/−chimeras (data not shown).

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 MHCII−/−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 popula-


The authors declare no conflict of interest.

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tions were not significantly different 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 T_Rs in the WT and MHCI^+/- BM chimeras, they were sorted and used in an in vitro T_R induction assay. The T_Rs 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 T_Rs that equated to a 94% decrease (Fig. S1A). To establish if this was attributable to cells in the hemopoietic 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 T_R 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 T_Rs 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 T_Rs and in the negative selection of self-reactive CD4^+ thymocytes.

**DCs Induce Antigen-Specific T_Rs and Negative Selection In Vivo.** T_R induction and negative selection of self-reactive thymocytes require self-peptide presentation on MHCI via an antigen-presenting cell (14, 15). To address T_R induction by DCs in an antigen-specific system, Rag^2^-/- OTII T-cell receptor (TCR) transgenic (tg) mice (which lack OVA-specific T_Rs because of the absence of the OVA antigen) were crossed with CD11cOVA tg mice (membrane-bound OVA expressed under the CD11c promoter). In these Rag^2^-/-OTII/CD11cOVA (Rag^2^-/-O/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 Rag^2^-/-O/OVA mice or Rag^2^-/-OTII mice for controls. Thymocytes were analyzed by flow cytometry 6 weeks later. Total cellularity of the Rag^2^-/-O/OVA BM chimeric thymuses was reduced compared with controls (Fig. 2A). The presentation of OVA by DCs in Rag^2^-/-O/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 CD4^+CD4^+V_o^+ OTII thymocytes compared with controls (Fig. 2B and C). Furthermore, there was a clear induction of OTII^+ T_Rs in the thymus of Rag^2^-/-O/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 T_R numbers in the thymus of Rag^2^-/-O/OVA BM chimeras compared with controls (Fig. 2B and D).

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

![Fig. 1.](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 T<sub>R</sub> generation and negative selection, we investigated the contribution of the TDC subtypes to these processes. We compared TDCs for expression of MHCI and costimulatory molecules, because these are important in T<sub>R</sub> 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<sup>lo</sup> and Sirpα⁻ CD11c<sup>+</sup> cDCs (21) (Fig. 3A). Strikingly, the Sirpα⁺ TcDCs expressed higher levels of MHCI and CD86 and slightly increased levels of the activation marker CD69 and the costimulatory molecules CD40 and CD80 compared with Sirpα⁻ TcDCs (Fig. 3B). This difference was 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 T<sub>R</sub> In Vitro. To compare the capacity of each TcDC subset to induce T<sub>R</sub><sub>8</sub>, sorted TcDC subsets were cocultured with syngeneic CD4<sup>+</sup> CD8<sup>-</sup> CD25<sup>-</sup> thymocytes (which contain T<sub>R</sub> precursors) for 5 days. To maintain T-cell survival, an optimal level of IL-7 was added (37). The number of T<sub>R</sub><sub>8</sub> that developed in these cultures was enumerated. The Sirpα⁺ TcDCs were the most efficient at inducing T<sub>R</sub><sub>8</sub>, as shown by the higher number of CD4<sup>+</sup> CD25<sup>-</sup>Foxp3<sup>+</sup> T cells in the cultures (Fig. 3D and E). In the cultures containing Sirpα⁺ TDCs, there was also some level of T-cell activation, as evidenced by a population of CD4<sup>+</sup> CD25<sup>+</sup>Foxp3<sup>-</sup> (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 T<sub>R</sub><sub>8</sub> 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 T<sub>R</sub><sub>8</sub> could not be attributed solely to a higher absolute number of T cells generated. Furthermore, we determined that the T<sub>R</sub> induction observed was attributable to de novo generation and not to proliferation of preexisting CD4<sup>+</sup> CD25<sup>-</sup>Foxp3<sup>-</sup> T cells within the starting population of thymocytes by using Foxp3-GFP mice to gate out CD4<sup>+</sup> CD25<sup>-</sup>Foxp3<sup>+</sup> cells (Fig. S2B).

T<sub>R</sub> generation in vitro was thymus specific. When TDCs were cultured with splenic CD4<sup>+</sup> CD25<sup>-</sup> naïve T cells rather than thymic CD4<sup>+</sup> CD25<sup>-</sup> T cells, no T<sub>R</sub> 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<sup>+</sup> CD25<sup>-</sup> T cells, few T<sub>R</sub><sub>8</sub> were generated (data not shown).

To test the function of in vitro–derived T<sub>R</sub> in vitro, T<sub>R</sub><sub>8</sub> were sorted as CD4<sup>+</sup> CD25<sup>-</sup> CD62L<sup>+</sup> cells and used in a T<sub>R</sub> suppression assay. CD62L was included as a marker to exclude activated T cells. In vitro–derived T<sub>R</sub><sub>8</sub> 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 T<sub>R</sub> induction by those cells, the same coculture method was used. We demonstrated that the T<sub>R</sub> induction capacities of both of these cell types were negligible (Fig. S2E).

Sirpα⁺ TcDCs Produce Chemokines and Attract CD4<sup>+</sup> 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 T<sub>R</sub>. 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> thymocytes, seeded in transwells, and incubated for 2 h. The supernatants from the Sirpα⁺ TcDC cultures showed the greatest capacity to attract CD4<sup>+</sup> thymocytes (Fig. S3D). Thus, the Sirpα⁺ TcDCs, through their chemokine production, have a special capacity to attract newly formed CD4<sup>+</sup> T<sub>R</sub> 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 TDC 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<sup>lo</sup>CD5<sup>-</sup>”) that have DC po-
ential were transferred intrathymically into sublethally irradiated CD45.1 recipient mice. DC generation was analyzed 2 weeks after transfer. The cDCs that developed from the intrathymic precursors were mainly CD8⁺Sirpα⁺ (Fig. 4A).

In contrast, the CD8⁻CD11b⁺ TDC subset has been shown to migrate in parabiotic mice from the circulation into the thymus of the conjoined mouse (24). To determine whether the Sirpα⁺ TcDCs correspond to this population, the CD11c⁺ DCs within mouse blood were characterized. Total peripheral blood mononuclear cells were enriched for DCs. The preparation was then stained with DCs, 25% expressing high levels of MHCII, indicating that immature and mature DCs were present in mouse blood.

To determine whether these blood DCs migrate to the thymus, white blood cells from CD45.1 mice were transferred i.v. into nonirradiated CD45.2 recipients and the phenotype of donor-derived cells in the recipient thymus was determined 3 days later. Donor-derived cells made up 0.1% of total cells in the recipient thymus, and of these, 10% were CD11c⁺CD45RA⁻ cDCs. These cDCs were all Sirpα⁺CD11b⁺CD8⁺MHCII⁺ (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 under the kidney capsule of recipient CD45.2 WT or CD45.2 CD11cOVA tg mice. This system allows recipient DCs to migrate into the grafted thymus 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 thymus were predominantly the Sirpα⁺ 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⁺ DCs and the resident CD45.1⁺ OTII T cells was studied.

At day 10, DCs in the grafted thymus lobes were analyzed for DC markers to assess the phenotype of the host-derived CD45.2⁺ migrating DCs. Of these donor-derived cells, 54 ± 6% were mature MHCII⁺ CD8⁻Sirpα⁺ cDCs, 4 ± 1% were mature CD8⁺Sirpα⁻ cDCs, and the remaining were MHCII⁺CD8⁻Sirpα⁻ cDCs (Fig. 4C). The latter two populations represented newly formed cells derived from recipient BM progenitors that had seeded the thymic grafts.

Thymocyte populations were analyzed by flow cytometry. The number of CD45.1⁺ OTII⁺ CD4⁺ CD8⁻Vjβ5⁺ T cells was reduced in lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4D), whereas the number of CD45.1⁺ CD4⁺ CD8⁻Vjβ5⁺ T cells was similar in both groups (data not shown), suggesting that antigen-specific negative selection of OTII T cells was occurring. In addition, more than twofold increase in the number of OTII⁺ Fopx3⁺ 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 TIR development.

Discussion

The present study demonstrates a role for mouse TDCs in TIR 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 TIR and an increase in the number of self-reactive CD4 T cells in the thymus. This demonstrates that in addition to mTECs (16, 40), BM-derived cells make a significant contribution to TIR generation and negative selection of CD4 T cells in a steady-state mouse. In addition, a 50% reduction in TIR numbers was observed when the hemopoietic compartment lacked expression of CD80 and CD86. Although...
Second, the Sirp found that the Sirp profound increase in CD4 these results, however. Mice with reduced thymic cellularity and a later time point for analysis (8–10 weeks), may have contributed to this increase. TRs and the deletion of self-reactive CD4 cells can migrate into the thymus, where they induce the development of masked the changes in TR numbers.

All mice were bred under specific pathogen-free conditions. B7 materials and Methods. Aeral antigens by mTECs. Tolerance to peripherally expressed antigens is induced by migrating rare antigen-specific thymocytes can encounter their cognate an-

In VivoDC Migration Assay. Details can be found in Supplementary Experimental Procedures.

Quantitative PCR. Quantitative PCR was performed for chemokine gene expres-

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 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. B6 CD45.1 mice 10 weeks of age were used as BM recipients. The mouse strains used included OTII tg (CD4+ T cells expressing the TCR specific for MHCII-restricted Ova peptide) (48) on a B6, CD45.1, or Rag2−/− background; IA/IA (MHCII−/−) (49); B7−/− (50); and CD11cOVA 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 × 10^6 CD45.2 donor BM cells i.v. from B6 or MHCIId−/− mice or from Rag2−/− OTII/CD11cOVA double-tg mice. For B7−/− chimeras, CD45.1 recipient mice were lethally irradiated with 8.0 Gy of total body irradiation. A total of 5 × 10^6 T-cell depleted B6 WT or B7−/− 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.

Thymic Chimeras. B6 CD45.1 mice 10 weeks of age were used as BM recipients. The mouse strains used included OTII tg (CD4+ T cells expressing the TCR specific for MHCII-restricted Ova peptide) (48) on a B6, CD45.1, or Rag2−/− background; IA/IA (MHCII−/−) (49); B7−/− (50); and CD11cOVA tg mice that express membrane-bound ovalbumin (amino acids 323–339) under control of the CD11c promoter (29, 51).
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