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Research Article

Immunology

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Specialized role of migratory dendritic cells in peripheral tolerance induction

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Harnessing DCs for immunotherapies in vivo requires the elucidation of the physiological role of distinct DC populations. Migratory DCs traffic from peripheral tissues to draining lymph nodes charged with tissue self antigens. We hypothesized that these DC populations have a specialized role in the maintenance of peripheral tolerance, specifically, to generate suppressive Foxp3⁺ Tregs. To examine the differential capacity of migratory DCs versus blood-derived lymphoid-resident DCs for Treg generation in vivo, we targeted a self antigen, myelin oligodendrocyte glycoprotein, using antibodies against cell surface receptors differentially expressed in these DC populations. Using this approach together with mouse models that lack specific DC populations, we found that migratory DCs have a superior ability to generate Tregs in vivo, which in turn drastically improve the outcome of experimental autoimmune encephalomyelitis. These results provide a rationale for the development of novel therapies targeting migratory DCs for the treatment of autoimmune diseases.

Introduction

Tregs actively suppress pathogenic self-reactive CD4⁺ T cells and, therefore, represent an important avenue for the treatment of autoimmune diseases (1). The peripheral Treg pool comprises naturally arising Tregs (nTregs), which originate in the thymus, and induced Tregs (iTregs), which are generated in the periphery from naive CD4⁺ T cells (1, 2). iTreg conversion is triggered by DCs and requires antigen presentation in the presence of TGF- β (2, 3). Therefore, in vivo manipulation of DCs for the generation of antigen-specific iTregs is a potential approach to prevent, halt, or reverse autoimmune disorders.

The DC lineage is heterogeneous and can be classified on the basis of phenotype and origin. Lymphoid tissues, i.e., spleen and tissue-draining LNs, contain lymphoid-resident DCs that arise from blood-borne precursors (4) and can be loosely categorized as CD8⁺ and CD8⁻ DCs, expressing DEC205 (DEC) and DCIR2, respectively (5, 6). These lymphoid-resident DCs rapidly take up antigens from the lymph and bloodstream for presentation to T cells (7). A second group of DCs are migratory DCs, which traffic from peripheral tissues to the draining LN charged with tissue antigens (8). The nature of migratory DCs depends on the site of LN drainage. In skin draining LNs (sLNs), migratory DCs include epidermal Langerhans cells (LCs) and dermal DCs, which consist of two main subsets, CD103⁺ and CD11b⁺ DCs (9, 10).

The general consensus is that DCs control the induction of adaptive immune responses against pathogens, while maintaining tolerance to self antigens. However, it is becoming more apparent that not all DCs have the same physiological functions in vivo. Lymphoid-resident DCs and migratory DCs have distinct roles in the induction of immune responses under inflammatory

conditions (11–14). Yet the contribution of steady-state DC populations to the induction of peripheral tolerance, specifically to the generation of antigen-specific Foxp3⁺ Tregs in vivo, has been, until now, poorly defined. Understanding the functional specialization of DC populations is critically important for the rational design of novel suppressive therapies and the generation of DC-targeted vaccines, particularly in the skin, which is the most attractive site for vaccination (15).

In this study, we used an antigen-targeting approach to address in vivo the ability of skin migratory versus lymphoid-resident DCs to promote the development of antigen-specific Tregs. Taking advantage of the differential expression of 4 surface receptors, i.e., DEC, Langerin, DCIR2, and triggering receptor expressed on myeloid cells-like 4 (Trem14), we delivered a self antigen, myelin oligodendrocyte glycoprotein (MOG), to skin migratory or lymphoid-resident DCs using anti-receptor–antigen (α -receptor–antigen) fusion mAbs. By applying this strategy to mice lacking specific DC populations, we found that skin Langerin⁺ migratory DCs have a unique ability to promote the generation of Tregs in vivo. We also show that delivery of a self antigen to migratory DCs is a promising therapeutic strategy to induce suppressive, disease-modulating Tregs.

Results

Strategy to target a self antigen to skin migratory or lymphoid-resident DCs in vivo. We previously reported that Trem14, an Ig superfamily member receptor, is abundantly expressed on splenic CD8⁺ lymphoid-resident DCs (16, 17), whereas expression of DCIR2 is restricted to splenic CD8⁻ lymphoid-resident DCs (18, 19). To further characterize the expression of these receptors in skin migratory DCs (Figure 1A) and compare it with the expression of DEC and Langerin, we performed microarray analysis (Figure 1B) and flow cytometry (Figure 1C) of sLN DC subsets. Skin migratory DCs lacked *Trem14* and *Clec4a4* (DCIR2) gene expression, whereas migratory CD103⁺ DCs and LCs co-expressed *Ly75* (DEC) and

Authorship note: Christopher Fiorese, Lori Zbytnuik, and Ashira Lubkin contributed equally to this work.

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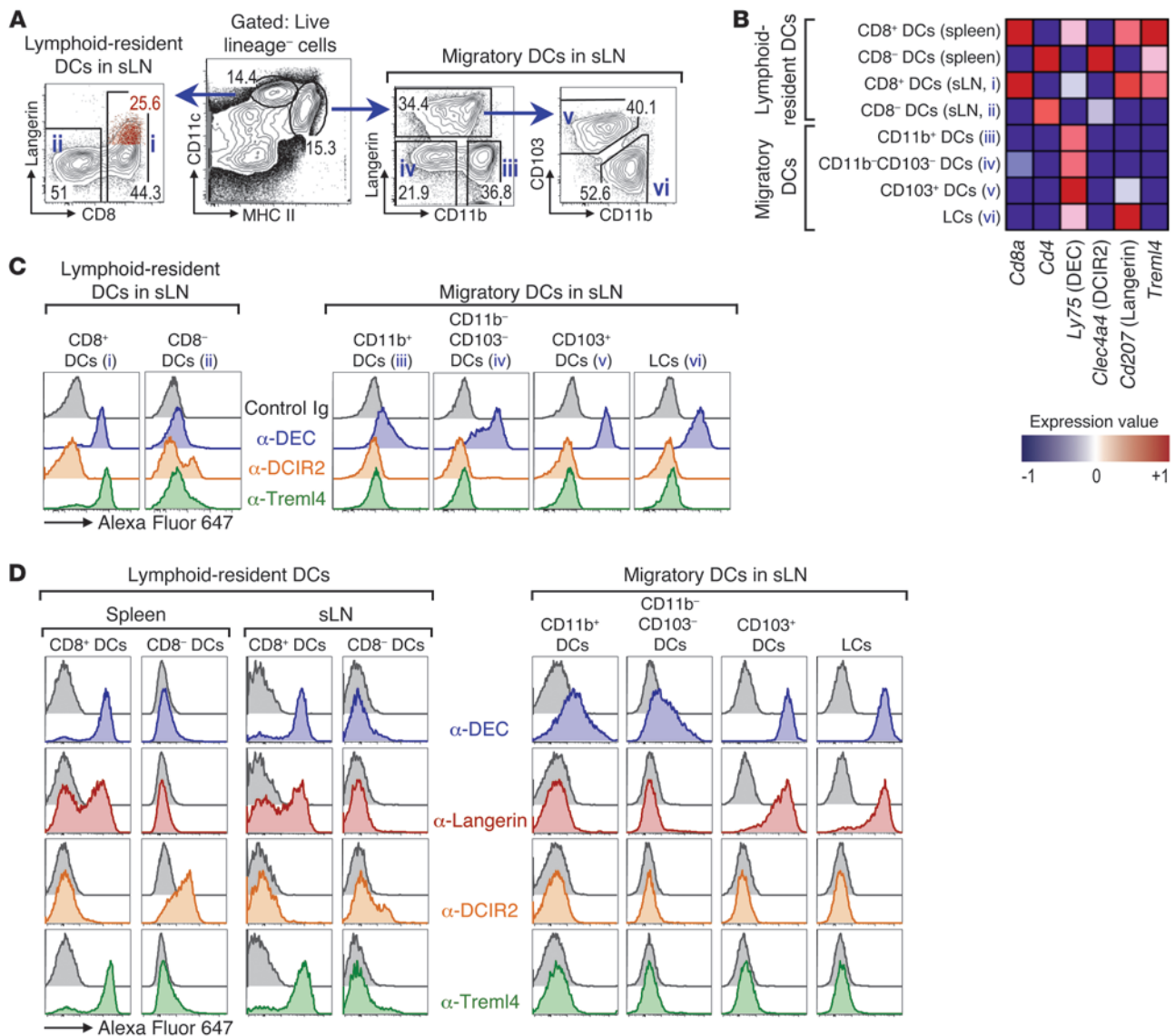


Figure 1

α-DEC and α-Langerin, but not α-DCIR2 and α-Trem14 mAbs, target skin migratory DCs in vivo. (A) Gating strategy for DC subsets in sLN (see also Supplemental Figure 1). Live lineage⁻ cells (CD19⁻Ter119⁻DX5⁻CD3ε⁻) were examined for CD11c and MHC II (center panel). CD11c^{hi}MHC II^{int} DCs were stained for the expression of CD8 to identify CD8⁺ (i) and CD8⁻ (ii) DC subsets (left panel). ~25% of CD8⁺ DCs in B6 mice expressed Langerin (overlaid red dot plot). CD11c^{int/hi} MHC II^{hi} migratory DCs were further gated into CD11b^{hi} DCs (iii), CD11b⁻ DCs (iv), Langerin⁺CD103⁺ DCs (v), and Langerin⁺ CD103⁻ LCs (vi). (B) Microarray analysis of *Ly75* (DEC), *Clec4a4* (DCIR2), *Cd207* (Langerin), and *Trem14* by distinct migratory and lymphoid-resident DC subsets sorted from spleen or sLN. Lymphoid-resident DCs were sorted based on CD8 and CD4 expression. Heat map depicts normalized values averaged from 3 replicates. Red and blue represent high and low relative expression, respectively. (C) Protein expression of DEC, DCIR2, and Trem14 by distinct DC subsets analyzed by FACS. (D) Langerin-EGFP mice were inoculated s.c. via footpad with 10 μg Alexa Fluor 647–labeled α-receptor mAbs or control Ig mAb (GL117, gray histograms). Uptake of labeled mAb by distinct DC populations in the spleen and sLN was evaluated 18–24 hours later by FACS. One experiment representative of 2–3 is shown.

Cd207 (Langerin) (Figure 1B). Consistent with the gene expression, α-Trem14 and α-DCIR2 mAbs failed to stain migratory DCs but labeled CD8⁺ and CD8⁻ lymphoid-resident DCs, respectively (Figure 1C). On the other hand, α-DEC mAbs labeled all subsets of migratory DCs and CD8⁺ lymphoid-resident DCs (Figure 1C). α-Langerin mAb labeled migratory CD103⁺ DCs and LCs (Figure 1A) and poorly labeled CD8⁺ DCs (Figure 1A, overlaid red dot plot in population i) as previously reported (20).

Next we asked whether different α-receptor mAbs selectively targeted distinct DC subset(s) in vivo. Eighteen to 24 hours after s.c. inoculation, α-DEC and α-Trem14 mAbs were captured by most CD8⁺ lymphoid-resident DCs in spleen and sLN, whereas α-DCIR2 was taken up by a fraction of CD8⁻ DCs (Figure 1D). α-Langerin mAb was captured by ~50% of CD8⁺ DCs (Figure 1D), which corresponds to Langerin expression in this mouse strain (21). As expected, neither α-Trem14 nor α-DCIR2 mAbs were taken

**Table 1**

Summary of the uptake of Alexa Fluor 647 mAbs by DC subsets 18–24 hours after s.c. inoculation

α -Receptor mAb	Lymphoid-resident DCs		Migratory DCs in sLN			
	CD8 ⁺ DCs	CD8 ⁻ DCs	CD11b ⁺ DCs	CD11b ⁻ CD103 ⁻ DCs	CD103 ⁺ DCs	LCs
α -DEC	90% ^A	–	40%	40%	99%	99%
α -Langerin	50%	–	–	–	90%	90%
α -DCIR2	–	20%/70% ^B	–	–	–	–
α -Trem14	90%	10%	–	–	–	–

^AAll values are the percentage of Alexa Fluor 647–positive cells relative to control Ig mAb 18–24 hours after s.c. inoculation. ^B20%/70% represents values in sLN and spleen, respectively.

up by migratory DCs, whereas both α -Langerin and α -DEC mAbs strongly labeled migratory CD103⁺ DCs and LCs (Figure 1D). α -DEC was also captured, to some extent, by migratory CD11b⁺ and CD11b⁻CD103⁻ DCs in vivo (Figure 1D).

Overall, these data indicate that α -DEC and α -Langerin mAbs target skin migratory CD103⁺ DCs and LCs, whereas CD8⁺ lymphoid-resident DCs capture α -DEC, α -Trem14, and variable amounts of α -Langerin mAbs (Table 1). Thus, inoculation of each α -receptor mAb can be used as a tool to target antigen to specific DC subset(s).

DEC- and Langerin-expressing DCs expand and induce de novo antigen-specific Foxp3⁺ T cells. To deliver a self antigen to distinct DC subsets in vivo, we engineered each α -receptor mAb to express aa 29–59 of MOG (MOGp, Supplemental Figure 2), containing a disease-causing epitope in EAE, the mouse model for MS (22). MOGp delivered to DCs using each α -receptor mAb, but not a control Ig-MOGp mAb without receptor affinity, was efficiently processed for MHC II presentation, as shown by the proliferation of MOG-specific CD4⁺ T cells (Figure 2A).

To compare the ability of distinct DC subsets to generate de novo Foxp3⁺ T cells, we evaluated the differentiation of adoptively transferred naive MOG-specific Foxp3-EGFP⁻ T cells into Foxp3-EGFP⁺ T cells 7 days after inoculation with α -receptor-MOGp mAbs (Figure 2B and Supplemental Figure 3A). Strikingly, α -DEC and α -Langerin mAbs consistently led to greater frequencies and absolute numbers of MOG-specific Foxp3⁺ T cells compared with α -DCIR2 and α -Trem14 mAbs (Figure 2B). Interestingly, α -DEC-induced Foxp3⁺ T cells trafficked to both spleen and sLN, while α -Langerin-induced Foxp3⁺ T cells remained mostly in the sLN (Figure 2B), even though α -DEC and α -Langerin mAbs generated similar amounts of Foxp3⁺ T cells (total number in spleen plus sLN).

MOG-specific Foxp3⁺ T cells were not generated with control Ig-MOGp mAb (Figure 2B), with unconjugated α -Langerin mAb (Supplemental Figure 4A), or after inoculation of mAbs in DEC-deficient or Langerin-deficient mice (Supplemental Figure 4B), indicating that the response was dependent on the delivery of MOG to DEC⁺ or Langerin⁺ DCs. Generation of MOG-specific Foxp3⁺ T cells by α -DEC or α -Langerin mAbs was ~100-fold greater than the same dose of soluble MOG35-55 peptide (MOG35-55p, Supplemental Figure 4A) and required steady-state DCs, as shown by the abolition of Foxp3⁺ T cell generation in the presence of DC-maturation stimuli (Supplemental Figure 4C) (23).

We further evaluated the ability of distinct DC subsets to expand transferred nTregs in vivo. Since MOG-specific Foxp3⁺ nTregs proliferated minimally in response to control Ig-, α -DEC-, or α -Langerin-MOGp (Supplemental Figure 3B), we co-trans-

ferred MOG-specific naive T cells as a source for IL-2 (24, 25), along with MOG-specific Foxp3-EGFP⁺ nTregs (Figure 2C). As with iTregs, α -DEC and α -Langerin were more efficient at stimulating the proliferation of Foxp3⁺ nTregs in vivo than α -DCIR2 and α -Trem14 mAbs (Figure 2C).

We concluded that DCs expressing DEC and Langerin promote de novo induction and expansion of antigen-specific Foxp3⁺ T cells more potently

than Trem14⁺ or DCIR2⁺ DCs. Since α -receptor-MOGp mAbs have a combined action on nTregs and iTregs, for the rest of the study we opted to transfer total MOG-specific CD4⁺ T cells.

α -DEC and α -Langerin more potently generate Foxp3⁺ T cells than α -DCIR2 and α -Trem14 mAbs. At all time points tested, MOGp delivered using α -DEC and α -Langerin mAbs resulted in increased frequencies and total number of MOG-specific Foxp3⁺ T cells compared with α -DCIR2 and α -Trem14, with a peak between days 7 and 14 (Supplemental Figure 5A). After α -receptor-MOGp mAb inoculation in steady state, MOG-specific CD4⁺ T cells lacking Foxp3 expression peaked on days 4–7, but rapidly declined thereafter (Supplemental Figure 5B), probably by deletion/cell death (26, 27). As previously described (28), DC targeting in the steady state did not prime effector CD4⁺ T cells producing cytokines (Supplemental Figure 5C).

Given that Foxp3⁺ T cell conversion in vivo may depend on the dose of antigen inoculated (23, 29), we tested α -receptor mAbs at 0.3, 3, or 30 μ g. Three micrograms was the most effective dose in generating Foxp3⁺ T cells, and α -DCIR2 and α -Trem14 were less efficient than α -Langerin or α -DEC mAbs at all doses tested (Figure 3A). In addition, α -DCIR2 and α -Trem14 mAbs conjugated with OVA were ineffective compared with α -DEC and α -Langerin at driving OVA-specific Treg induction in vivo (data not shown).

Since high numbers of lymphoid-resident DCs expressing DCIR2 and Trem14 are localized in the spleen, we injected α -receptor-MOGp mAbs systemically to facilitate antigen delivery to these DC subsets. Intraperitoneal and i.v. inoculation of α -DCIR2 and α -Trem14 mAbs failed to increase the total number or frequency of MOG-specific Foxp3⁺ T cells (Figure 3B). Similar results were obtained upon i.m. administration of α -receptor-MOGp mAbs (Figure 3B).

We concluded that the reduced ability of α -DCIR2 and α -Trem14 mAbs to generate Foxp3⁺ T cells was independent of mAb dose, route of administration, time course, or type of antigen.

Skin Langerin⁺ migratory DCs control the differentiation of Foxp3⁺ T cells after inoculation of α -DEC and α -Langerin mAbs. α -DEC and α -Langerin, but not α -DCIR2 and α -Trem14, mAbs were captured by migratory DCs expressing Langerin, i.e., CD103⁺ DCs and LCs (Table 1); thus, we hypothesized that these subsets generate Foxp3⁺ T cells more efficiently than lymphoid-resident DCs in vivo.

To rule out a role of CD8⁺ lymphoid-resident DCs capturing α -DEC mAbs or α -Langerin mAbs, we used mouse models lacking specific DC populations. Inoculation of α -Langerin-MOGp mAb in splenectomized mice yielded similar numbers of MOG-specific Foxp3⁺ T cells compared with sham controls (Figure 4A), suggest-

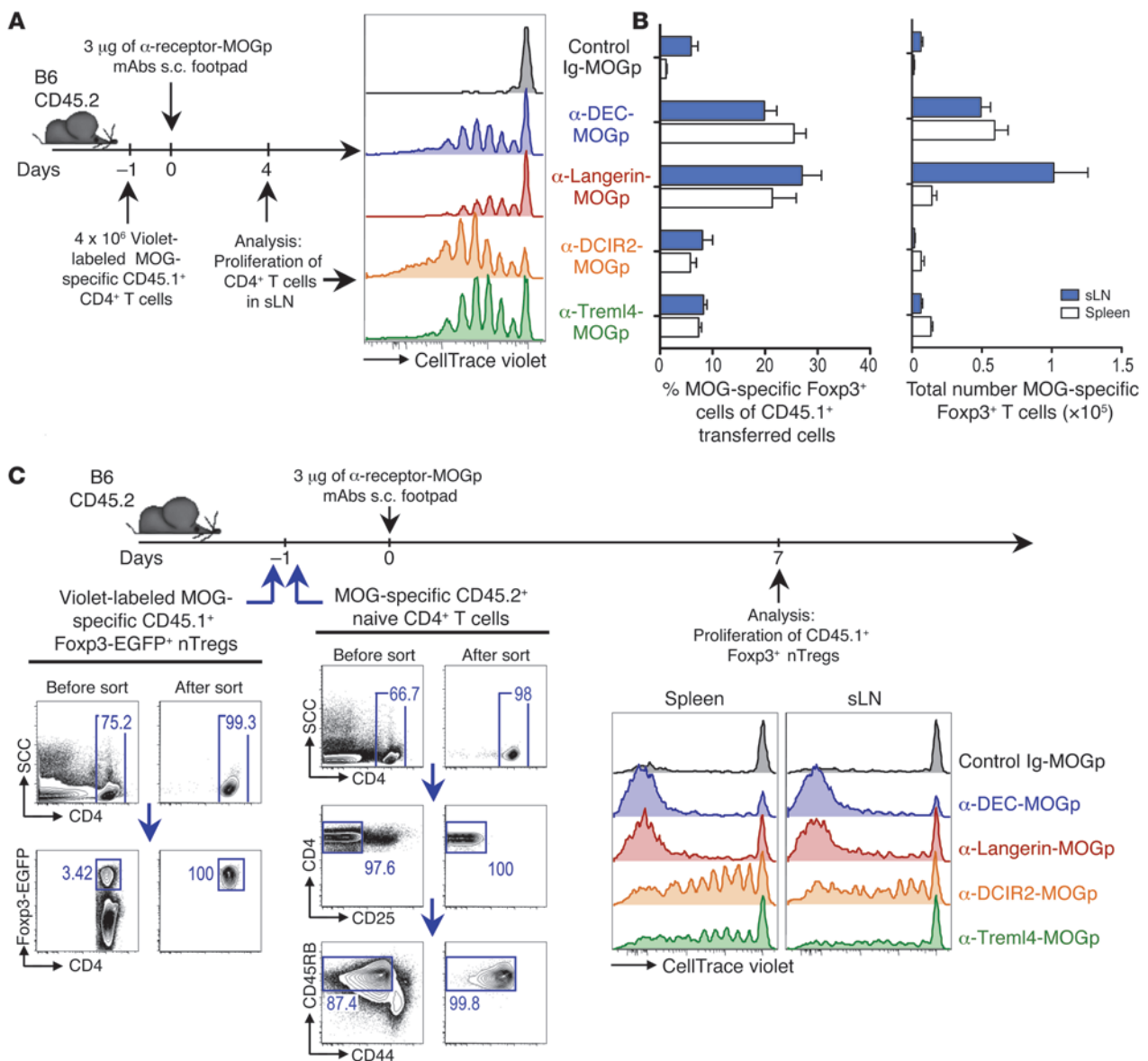


Figure 2

Targeting MOGp to DEC⁺ and Langerin⁺ DCs expands and induces de novo Foxp3⁺ T cells in vivo. **(A)** Experimental design (left) to assess the proliferation of MOG-specific CD4⁺ T cell in sLN 4 days after s.c. inoculation of α-receptor-MOGp mAbs. Histograms (right) are gated on MOG-specific donor T cells (Vβ11⁺, CD45.1⁺), and are representative of 2–3 experiments. **(B)** De novo induction of Foxp3⁺ T cells by α-receptor-MOGp mAbs. 4 × 10⁶ naive CD4⁺ T cells (CD25⁻, Foxp3-EGFP⁻, CD44^{lo}, CD45RB^{hi}) FACS sorted from MOG-specific Foxp3-EGFP reporter mice (see Supplemental Figure 3A for sorting strategy) were transferred 1 day before the inoculation of 3 μg α-receptor-MOGp mAbs s.c. footpad. Seven days later, the frequency (left) and total number (right) of induced MOG-specific Foxp3-EGFP⁺ T cells were analyzed in the sLN or spleen. Bars are the mean ± SEM of 4–8 mice in 2–4 experiments. **(C)** Expansion of nTregs by α-receptor-MOGp mAbs. Recipient mice were co-transferred with 1 × 10⁶ Violet-labeled MOG-specific CD45.1 Foxp3-EGFP⁺ nTregs and 4 × 10⁶ MOG-specific CD45.2 naive T cells (left panels show sorting strategy) 1 day before inoculation of 3 μg α-receptor-MOGp mAbs s.c. via footpad. Histograms (right) show the proliferation of Violet-labeled MOG-specific Foxp3⁺ nTregs in spleen and sLN and are representative of 2 experiments.

ing that the spleen, which lacks migratory DCs, is dispensable for this process. Interestingly, injection of α-DEC-MOGp mAb into splenectomized mice led to increased accumulation of MOG-specific Foxp3⁺ T cells in sLN, with total numbers similar to that induced by α-Langerin (Figure 4A), suggesting that Foxp3⁺ T cell induction with α-DEC-MOGp mAb occurs in sLN, but some transgenic T cells circulate to the spleen when this organ is present.

We further evaluated the role of skin migratory DCs using *Ccr7*^{-/-} mice. CCR7 is required for migratory DCs, but not lymphoid-resident DCs, to enter the LN in steady state (30). Consequently, skin migratory DCs are absent in sLN of *Ccr7*^{-/-} mice, whereas lymphoid-resident DCs are still present in substantial numbers in the sLN and spleen (40%–70% and 90% respectively; Supplemental Figure 6A). Generation of MOG-specific Foxp3⁺ T cells was completely

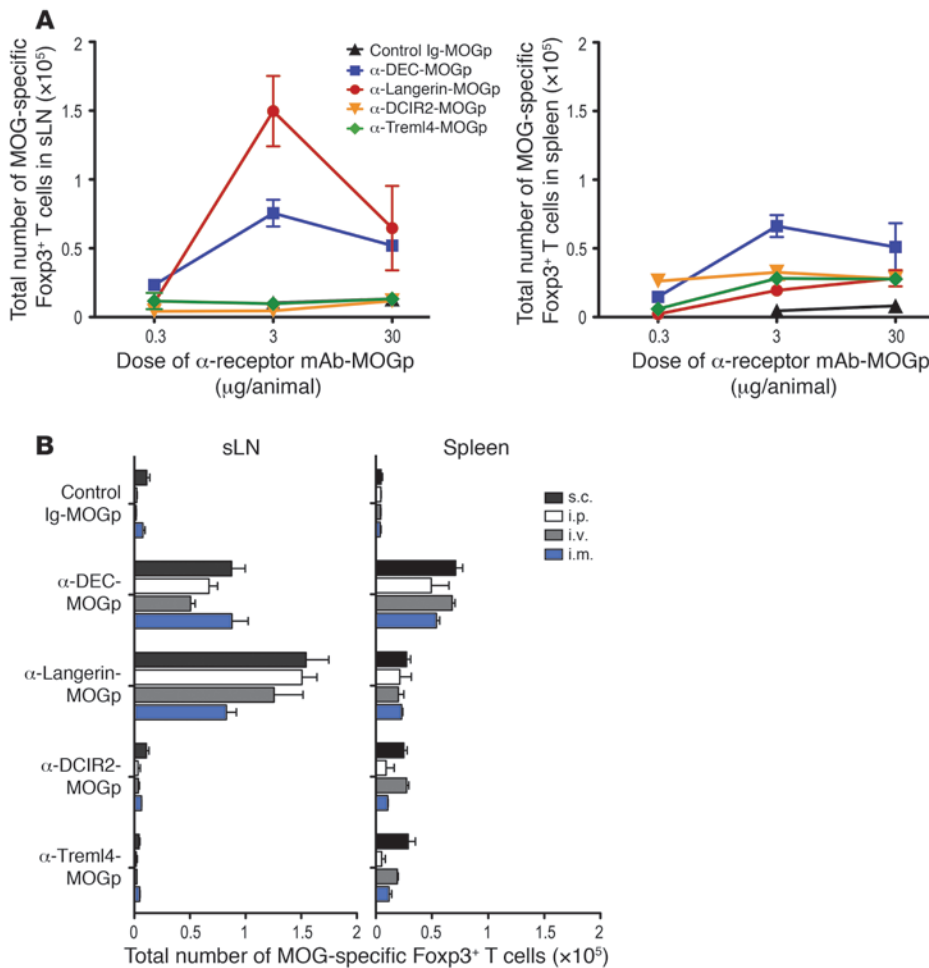


Figure 3

Antigen delivery with α-DEC and α-Langerin mAbs is more efficient than α-DCIR2 and α-Trem14 for the generation of MOG-specific Foxp3⁺ T cells. **(A)** Dose response of MOG-specific Foxp3⁺ T cell generation in sLN (left) and spleen (right). Recipient mice transferred with 4 × 10⁶ MOG-specific CD4⁺ T cells 1 day before s.c. inoculation of α-receptor-MOGp mAbs were analyzed 7 days later. Mean ± SEM of 3–5 experiments, with a total of 6–10 mice per group. **(B)** As in **A**, but comparison of different inoculation routes: s.c. via footpad, i.p., i.v., and i.m. Mean ± SEM of 4–8 mice per group in 2–4 experiments.

abolished in *Ccr7*^{-/-} mice injected with α-DEC- or α-Langerin-MOGp mAbs or high-dose untargeted MOG35-55p (Figure 4B). The lack of Foxp3⁺ T cell generation in *Ccr7*^{-/-} mice was not due to lymphoid-tissue structure disruption, as MOG-specific CD4⁺ T cells proliferated in response to untargeted MOG35-55p (Figure 4C), indicating that other DCs were able to present the antigen but were unable to induce Foxp3 expression.

Finally, we evaluated the role of skin Langerin⁺ migratory DCs in the generation of Foxp3⁺ T cells. Diphtheria toxin (DT) treatment of Langerin-DTR mice resulted in the complete depletion of skin Langerin⁺ migratory DCs (Supplemental Figure 6B) and abolishment of Foxp3⁺ T cell generation by α-Langerin-MOGp mAb, which served as a positive control (Figure 4D). MOG-specific Foxp3⁺ T cell generation triggered by α-DEC-MOGp mAb or high-dose untargeted MOG35-55p was also abrogated in DT-treated Langerin-DTR mice (Figure 4D).

Together, these results provide strong evidence that skin Langerin⁺ migratory DCs, and not CD8⁺ lymphoid-resident DCs, are responsible for the *in vivo* generation of MOG-specific Foxp3⁺ T cells after DC targeting with α-DEC and α-Langerin, or after inoculation of a high dose of untargeted peptide.

Both LCs and dermal CD103⁺ migratory DCs promote Foxp3⁺ T cell generation. Given that α-Langerin and α-DEC mAbs target both LCs and dermal CD103⁺ migratory DCs (Table 1), we next determined their specific role in Foxp3⁺ T cell generation. To limit the delivery

of antigen to each DC subset, we prepared bone marrow chimeras (31) wherein only radioresistant LCs or radiosensitive dermal DCs expressed DEC (Figure 5A). Selective targeting of α-DEC-MOGp mAb to either radioresistant or radiosensitive cells led to efficient generation of Foxp3⁺ T cells (Figure 5B). This result indicates that both subsets of migratory DCs, i.e., LCs and dermal CD103⁺ DCs, are able to generate Foxp3⁺ T cells.

Lung Langerin⁺ migratory DCs are also able to drive the differentiation of Foxp3⁺ T cells. The above data suggest that skin Langerin⁺ migratory DCs potentially induce the differentiation of antigen-specific Foxp3⁺ T cells *in vivo*. Migratory DCs coexpressing Langerin and CD103 are also found in the lung (32, 33). However, s.c. inoculation of α-Langerin-MOGp mAb failed to accumulate MOG-specific Foxp3⁺ T cells in lung draining mediastinal LNs (Supplemental Figure 7). Thus, to facilitate antigen delivery to lung DCs, we inoculated α-receptor-MOGp mAbs intranasally. α-Langerin- and α-DEC-MOGp mAbs administered *i.n.* potentially induced MOG-specific Foxp3⁺ T cells at high frequencies and high absolute numbers, whereas α-DCIR2 did not significantly generate Foxp3⁺ T cells compared with control Ig-MOGp (Figure 6, A and B). Strikingly, and in contrast to s.c. injection, *i.n.* inoculation of α-Trem14-MOGp mAb also led to the induction of MOG-specific Foxp3⁺ T cells (Figure 6, A and B). In all cases, MOG-specific T cells were found mainly in mediastinal LN, and very few cells were recovered from lung, mesenteric LN, spleen, and sLN (Figure 6B).

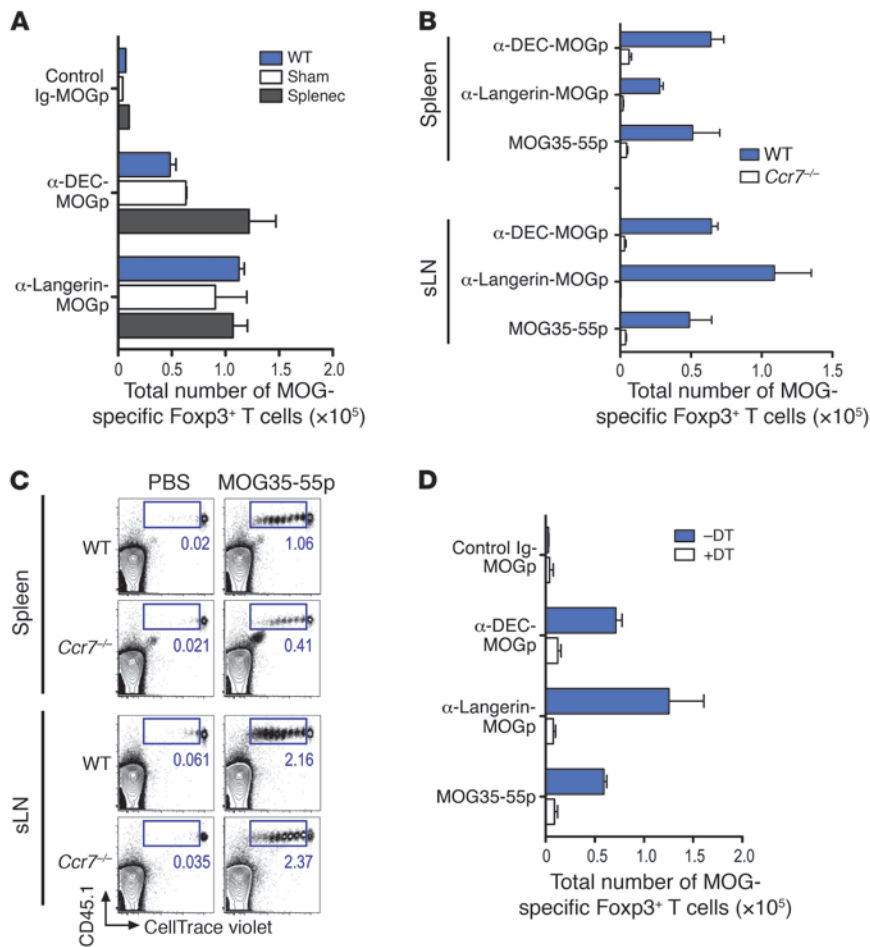


Figure 4

Skin Langerin⁺ migratory DCs mediate Foxp3⁺ T cell generation after α-DEC and α-Langerin targeting. **(A)** Generation of MOG-specific Foxp3⁺ T cells in sLN of B6 WT, sham, or splenectomized (Splenec) mice. Mice receiving transfer of 4 × 10⁶ MOG-specific CD4⁺ T cells 1 day before s.c. inoculation of 3 μg α-Langerin- or α-DEC-MOGp were analyzed 7 days later. Mean ± SEM of 2 experiments with 4 mice per group. **(B)** As in **A**, but comparing MOG-specific Foxp3⁺ T cell generation in sLN and spleen of B6 WT and *Ccr7*^{-/-} mice. Mice inoculated with untargeted MOG35-55p received 300 μg. Mean ± SEM of 4–8 mice in 2–4 experiments. **(C)** Four-day proliferation of Violet-labeled MOG-specific CD45.1⁺CD4⁺ T cells (4 × 10⁶) transferred into CD45.2 WT or *Ccr7*^{-/-} recipient mice 1 day before s.c. inoculation of untargeted MOG35-55p (300 μg). Gates represent the percentage of donor CD45.1⁺ T cells undergoing one or more divisions. Plots are gated on CD3^ε⁺CD4⁺ T cells and are representative of 2 experiments. **(D)** Generation of MOG-specific Foxp3⁺ T cells in sLN of mice depleted of Langerin⁺ cells. As in **B**, but Langerin-DTR mice were treated with or without 500 ng DT i.v. the day of T cell transfer (day -1), followed by 250 ng DT i.p. on days 1, 3, and 5. Mean ± SEM of 4–6 mice per group in 2–3 experiments.

Generation of MOG-specific Foxp3⁺ T cells by α-Trem14-MOGp mAb prompted us to evaluate the expression of this receptor in lung DC subsets (Figure 6, C and D). In contrast to the skin, lung CD103⁺ migratory DCs also expressed *Trem14* transcripts (Figure 6C) along with *Cd207* (Langerin) and *Ly75* (DEC), which was confirmed at the protein level (Figure 6D). Expression of *Clec4a4* (DCIR2) was not detected in any subset of lung migratory DCs (Figure 6, C and D). Together these results show that targeting lung CD103⁺ migratory DCs with α-Langerin-, α-DEC-, or α-Trem14-MOGp mAbs generates MOG-specific Foxp3⁺ T cells.

Overall, similar to skin Langerin⁺ migratory DCs, lung Langerin⁺ migratory DCs are able to promote antigen-specific Foxp3⁺ T cells in vivo.

Foxp3⁺ T cells induced by α-DEC- and α-Langerin-MOGp mAbs are bona fide Tregs capable of preventing EAE. Foxp3⁺ T cells induced by α-DEC- and α-Langerin-MOGp mAbs (iTregs) showed considerable expression of suppressive molecules such as GITR, CTLA4, and ICOS but low levels of the IL-7Rα chain (CD127) (Supplemental Figure 8A). Induced Foxp3⁺ T cells also expressed CD5; however, in contrast to a previous report (34), we did not find differences in the expression of CD5 between Tregs and CD4⁺ Foxp3⁻ T cells (Supplemental Figure 8A). Importantly, MOG-specific Foxp3⁺ T cells generated in vivo with either α-DEC- or α-Langerin-MOGp were able to suppress the in vitro proliferation of MOG-specific CD4⁺ responder T cells (Supplemental Figure 8, B–D). Moreover, MOG-specific Foxp3⁺ T cells were able to

suppress T cells of other specificities only when provided with their cognate antigen (Supplemental Figure 8, E–F).

We also evaluated the in vivo suppressive function of α-DEC- and α-Langerin-induced Foxp3⁺ Tregs. C57BL/6 (B6) mice that had received transfer of MOG-specific CD4⁺ T cells and were immunized with α-receptor-MOGp mAbs were challenged 14 days later for EAE induction (Figure 7A). Pre-treatment of mice with α-DEC- or α-Langerin conjugated with MOGp, but not OVA, reduced EAE incidence, delayed onset, and decreased clinical scores compared with control Ig-MOGp-inoculated mice (Figure 7A, Supplemental Figure 9A and Supplemental Table 1). In contrast, mice vaccinated with α-DCIR2- and α-Trem14-MOGp mAbs showed minimal protection, which not significantly different from control Ig-MOGp (Figure 7A and Supplemental Table 1), consistent with their limited ability to generate Foxp3⁺ T cells.

To further confirm that induced Foxp3⁺ Tregs were responsible for EAE prevention, we crossed MOG transgenic mice with Foxp3-DTR mice (35). We depleted α-Langerin-induced Tregs expressing Foxp3-DTR using DT (Figure 7B) before EAE induction. DT treatment led to the abolishment of EAE protection (Figure 7C), indicating that Foxp3⁺ Tregs induced by α-Langerin-MOGp mAbs were responsible for preventing disease onset and progression in vivo. At the dose used, DT treatment itself did not contribute to EAE susceptibility (Supplemental Figure 9B). Finally, treatment with α-Langerin- or α-DEC-MOGp mAbs 10 days after EAE induction, just prior to clinical onset, did not alter the

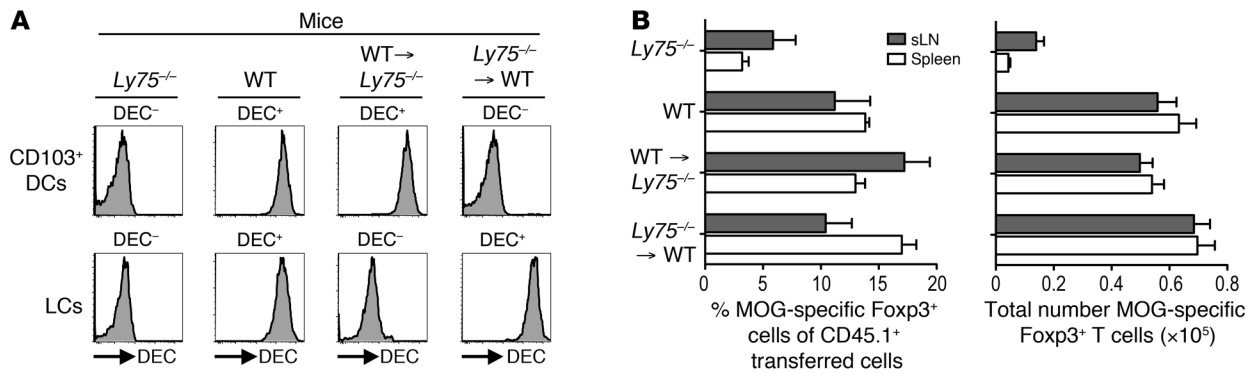


Figure 5 LCs and dermal CD103⁺ migratory DCs are able to generate MOG-specific Foxp3⁺ T cells. (A) Lethally irradiated DEC-deficient mice (*Ly75^{-/-}*; CD45.2) and WT (CD45.1) mice were injected with bone marrow cells from WT (CD45.1) or DEC-deficient (*Ly75^{-/-}*; CD45.2) mice, respectively, and reconstituted for 12 weeks. The expression of DEC in radiosensitive CD103⁺ DCs and radioresistant LCs was evaluated by FACS using the congenic markers CD45.1 and CD45.2 (gating strategy as in Supplemental Figure 1A). *Ly75^{-/-}* and WT mice were used as controls. (B) Frequency (left) and total number (right) of Foxp3⁺ T cells in sLN and spleen from chimeric mice 7 days after s.c. inoculation with 3 μg α-DEC-MOGp mAb, given 1 day after transfer of 4 × 10⁶ MOG-specific CD4⁺ T cells. Mean ± SEM of 4–12 mice in 2–4 experiments.

course of the disease, suggesting that the generation of disease-protective Tregs by migratory DCs has an optimal therapeutic window, which probably depends on the inflammatory context (Supplemental Figure 9C).

Amelioration of EAE in α-Langerin-MOGp-inoculated mice was associated with reduced infiltration of CD4⁺ T cells into the CNS, whereas EAE progression in control Ig-MOGp-treated mice was associated with high infiltration of CD4⁺ T cells, including IFN-γ⁺ and IL-17A⁺ T cells (Supplemental Figure 10, A–C). Notably, these elevated numbers of IFN-γ⁺ and IL-17A⁺ T cells were the result of increased leukocyte infiltration and not due to a change in their frequency among the CD4⁺ T cells (Supplemental Figure 10D). Consistent with the limited infiltration of CD4⁺ T cells, very few Tregs were detected in the CNS of α-Langerin-MOGp-inoculated mice, while they were present in the control group (Supplemental Figure 10C), as previously reported for EAE (36). Together, these results suggest that EAE protection in our system is probably occurring in the periphery rather than the CNS.

In summary, our findings demonstrate that α-DEC- and α-Langerin-MOGp vaccination is a highly effective strategy for inducing suppressive, disease-modulating Foxp3⁺ Tregs.

Discussion

DCs play a pivotal role in regulating the balance between immunity and tolerance. Advances in DC biology and the development of technologies for manipulating the function of these cells are revealing their broad therapeutic potential for the treatment of immune disorders, including autoimmune diseases. However, DCs are heterogeneous, composed of distinct subsets with specialized functions. Consequently, the outcome of immune responses is determined, in part, by the subset of DCs involved. Here we show that, in the steady state, Langerin⁺ migratory DCs induce antigen-specific Foxp3⁺ Tregs more potently than lymphoid-resident DCs in vivo. We further demonstrate that targeting a self antigen to Langerin⁺ migratory DCs, but not lymphoid-resident DCs, provides a very effective strategy for modulating autoimmune disease.

Antigen delivery to DCs using α-receptor mAbs is an attractive strategy for vaccination and therapy, and to date, many receptors

have been harnessed as putative targets (37, 38). We previously established that priming of Th1 CD4⁺ and CD8⁺ T cells is comparable whether antigens are delivered to poly(IC)-matured DCs through DEC, Langerin, Clec9A, or Trem14 (17, 19). In contrast, delivery of antigen to steady-state DCs using α-DEC mAbs can lead to tolerance through deletion (26, 27) and/or the generation of Tregs (23, 39, 40), but it was suggested that CD8⁺ lymphoid-resident DCs mediated Treg conversion (41).

Capitalizing on the differential expression of surface receptors by DC subsets, we provide the first evidence that lymphoid-resident CD8⁺ and CD8⁻ DCs are inefficient for Treg generation in vivo, which was corroborated in several ways. First, the spleen, which lacks migratory DCs and harbors ~10 times more lymphoid-resident DCs than LN, was dispensable for the generation of Tregs, suggesting that Foxp3⁺ T cell conversion occurs in LN. Second, abolition of Treg induction in *Ccr7^{-/-}* mice indicates that migratory DCs are essential for this process. Third, abrogation of Treg induction in DT-treated Langerin-DTR mice, which conserve ~40%–60% functional CD8⁺ lymphoid-resident DCs (42), suggests that Langerin⁺ migratory DCs are critical for Treg conversion. Finally, in vivo experiments in bone marrow chimeric mice demonstrated that LCs and CD103⁺ migratory DCs potently and sufficiently generated Tregs to an equivalent degree. Of note, it has been described that Langerin⁺CD103⁺ migratory DCs are necessary for the generation of pathogenic Th1/Th17 cells after immunization with MOG35-55p plus CFA (43). Together with our results, this would suggest that Langerin⁺CD103⁺ migratory DCs could promote either Foxp3⁺ Tregs in steady state or effector Th1/Th17 cells under inflammatory conditions.

Importantly, we demonstrated that the Treg-inducing ability of Langerin⁺ migratory DCs is not restricted to skin DCs and is not determined by any particular receptor, as delivery of MOGp via α-Trem14 mAb to lung Langerin⁺ migratory DCs also generated antigen-specific Tregs. Strikingly, Tregs induced by α-DEC mAb trafficked to the spleen, whereas those induced by α-Langerin remained mostly in the sLN, suggesting that α-Langerin mAbs may be effective for treating cutaneous dysregulated immune responses.

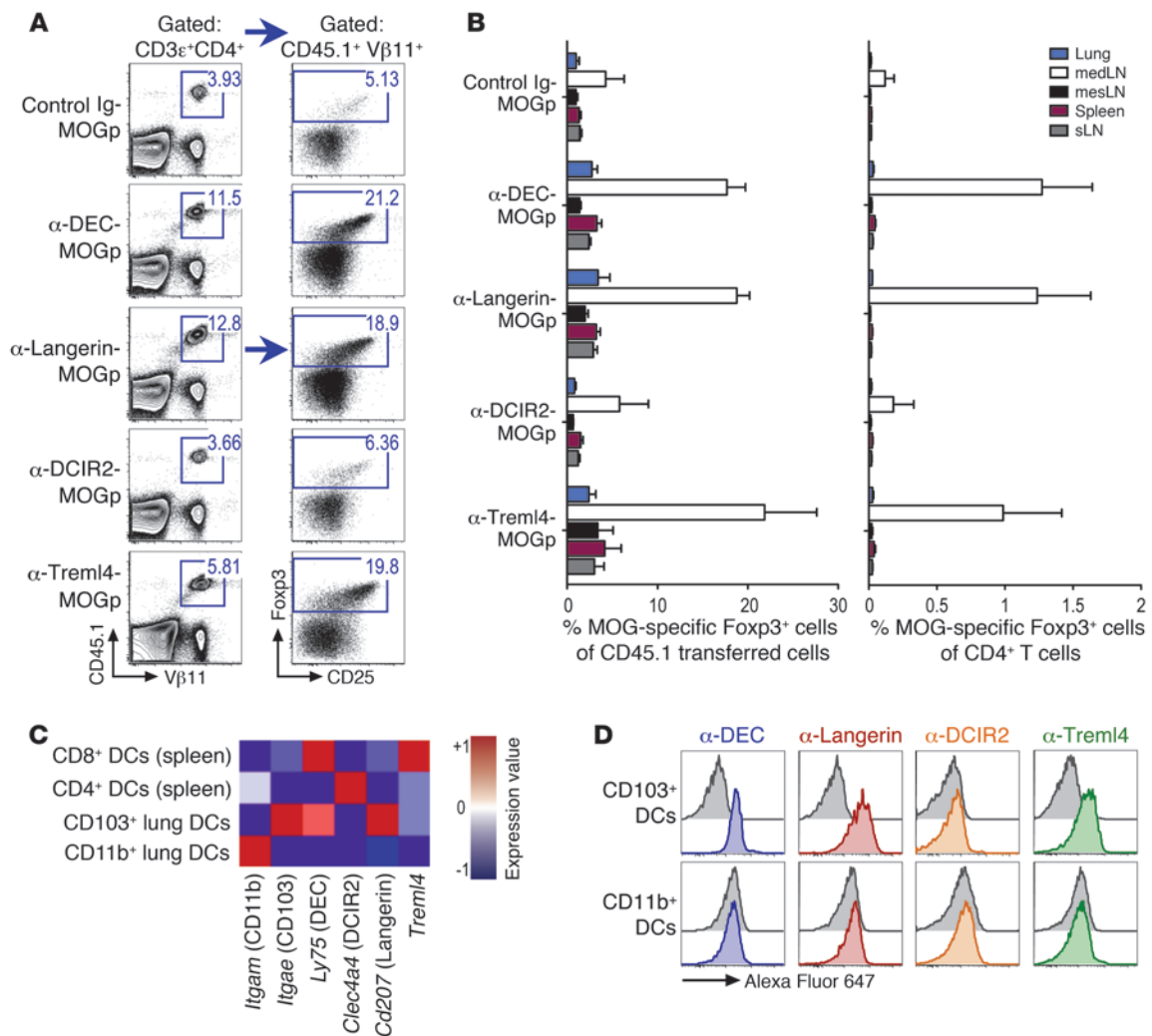


Figure 6

Lung CD103⁺ migratory DCs generate MOG-specific Fopx3⁺ T cells after i.n. inoculation of α-DEC-, α-Langerin-, and α-Trem14-MOGp mAbs. **(A)** Fopx3 expression in MOG-specific T cells from mediastinal LN of CD45.2 B6 recipient mice 7 days after i.n. inoculation of 1 μg α-receptor-MOGp mAbs, given 1 day after transfer of 4 × 10⁶ MOG-specific CD45.1⁺ T cells. **(B)** As in **A**: Percentage of Fopx3⁺ T cells from CD45.1 transferred cells (left) or from total CD4⁺ T cells (right) within lung, mediastinal LN (medLN), mesenteric LN (mesLN), spleen, and sLN. Mean ± SEM of 3 experiments with 6 mice per group. **(C)** Microarray analysis of *Ly75* (DEC), *Clec4a4* (DCIR2), *Cd207* (Langerin), and *Trem14* by distinct lung DCs). Heat map depicts normalized values averaged from 3 replicates. Red and blue represent high and low relative expression, respectively. **(D)** Histograms show protein levels of DEC, Langerin, DCIR2, and Trem14 in CD103⁺ and CD11b⁺ DCs as evaluated by FACS. GL117 control mAb (gray histograms). One experiment of 2 is shown.

Our results reveal for the first time in vivo that migratory DCs are superior to lymphoid-resident DCs at inducing antigen-specific Tregs. Vitali et al. recently reported that migratory DCs are more efficient than lymphoid-resident DCs at generating Tregs; however, this comparison was performed in vitro (44). Other in vivo reports have suggested that dermal Langerin⁺ DCs present keratinocyte-associated OVA to OT-II CD4⁺ T cells, inducing some Fopx3⁺ T cells (45). In contrast to the study by Azukizawa et al., where only migratory DCs had access to keratinocyte-expressing antigens, in our targeting system, antigen was accessible to both migratory DCs and lymphoid-resident DCs, strongly establishing the superior ability of Langerin⁺ migratory DCs to induce Tregs in vivo. Our targeting strategy also revealed the potential of LCs to induce Tregs in vivo, which is consistent with recent reports

(46, 47). The role of other subsets of migratory DCs, e.g., CD11b⁺ migratory DCs, in Treg conversion was not assessed and cannot be excluded. Skin CD11b⁺ migratory DCs produce retinoic acid (RA) and have been implicated in Fopx3⁺ T cell generation in vitro (44, 48). The fact that CD11b⁺ DCs remain present in DT-treated Langerin-DTR mice and are able to capture α-DEC mAbs (42) suggests that migratory CD11b⁺ DCs are not sufficient to induce antigen-specific Tregs in vivo. However, RA produced by CD11b⁺ migratory DCs might act in a paracrine fashion to increase the numbers of Tregs induced by Langerin⁺ migratory DCs.

The properties that distinguish skin Langerin⁺ migratory DCs from lymphoid-resident DCs in their capacity to induce Tregs remain unclear. LCs produce TGF-β, IL-10, and indoleamine 2,3-dioxygenase (IDO) and promote regulatory responses in mice

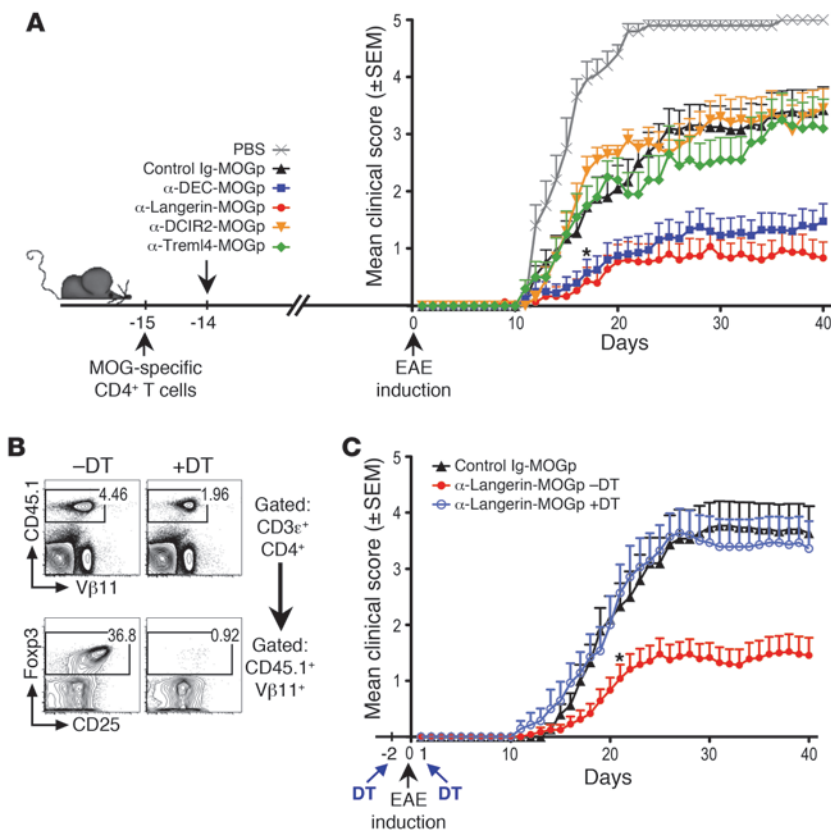


Figure 7

α -Langerin- and α -DEC-generated Foxp3⁺ T cells are functional Tregs that prevent the development and progression of EAE. **(A)** One day after transfer of 4×10^6 MOG-specific CD4⁺ T cells (day -15), B6 mice were inoculated s.c. with 3 μ g α -receptor mAbs (day -14). EAE was induced on day 0, and disease was monitored for 40 days. Mean clinical score \pm SEM is plotted over time (10–20 mice in 2–4 experiments). α -DEC and α -Langerin mAbs were statistically significant (2-way ANOVA and Bonferroni's post hoc testing) from control Ig, α -DCIR2, and α -Trem14 mAbs (starting on day 17, * $P < 0.05$). Further information can be found in Supplemental Table 1. **(B)** Recipient B6 mice transferred with 8×10^6 MOG-specific Foxp3-DTR CD4⁺ T cells (day -15) 1 day before s.c. inoculation of 3 μ g α -Langerin-MOGp (day -14) were inoculated i.v. (day -2) and i.p. (day 1) with 250 ng DT. FACS plots (representative of 3 experiments) show depletion of MOG-specific Foxp3⁺ T cells in sLN 1 day after the last DT inoculation. **(C)** As in **B**, but on day 0 mice were challenged for EAE induction. Data are shown as the mean clinical score \pm SEM of 15–20 mice per group in 3–4 experiments. The α -Langerin-MOGp -DT group was statistically significant (2-way ANOVA and Bonferroni's post hoc testing) from the α -Langerin-MOGp +DT and the control Ig-MOGp groups (starting on day 21, * $P < 0.05$).

and humans (46, 47, 49–51). In the gut, TGF- β and RA have been shown to control the ability of CD103⁺ migratory DCs to induce Tregs in vitro (52). In addition, activation of β -catenin in intestinal DCs controls the generation of anti-inflammatory mediators and Tregs (53). Our approach of targeting antigens to functionally specialized DCs provides a feasible means of studying DC-mediated mechanisms of tolerance in vivo.

Our work establishes the superior ability of Langerin⁺ migratory DCs to induce disease-specific suppressive T cells. Prophylactic targeting of MOGp to Langerin⁺ DCs significantly decreased the incidence and severity of EAE, although the course of the disease was not altered by targeting migratory DCs 10 days after EAE induction. Yet it was recently reported that DCs are indispensable for induction of Tregs after disease onset (54), suggesting that our self antigen DC-targeting approach has therapeutic potential if the right timing for intervention is established. Further studies will be required in order to pave the way for the rational design of novel DC-targeting treatments for MS and other autoimmune disorders.

Methods

Mice. B6, *Ccr7*^{-/-}, and splenectomized and sham-operated mice were obtained from The Jackson Laboratory. 2D2 mice specific for MOG35-55 (The Jackson Laboratory) (55) were bred in house onto CD45.1, Foxp3-EGFP reporter mice (The Jackson Laboratory) (56), and Foxp3-DTR mice (provided by A. Rudensky, Memorial Sloan-Kettering Cancer Center, New York, New York, USA) (35). *Ly75*^{-/-} (DEC-deficient) mice were obtained from M. Nussenzweig (The Rockefeller University) (57) and Langerin-EGFP/DTR mice from B. Malissen (Université de la Méditerranée, Marseille, France) (21). Langerin-DTR mice (provided by B.E. Clausen, Eras-

mus University Medical Center, Rotterdam, Netherlands) (58) were bred in house as homozygotes to obtain Langerin-deficient mice.

Production of α -receptor-MOGp mAbs. DNA for MOG29-59 (MOGp) was cloned in frame into the COOH terminus of the heavy chain of α -Langerin (19), α -DCIR2 (18), and α -Trem14 (17), as previously described for α -DEC, and a control Ig without receptor affinity (34). mAbs were expressed by transient transfection (calcium phosphate) in 293T cells in serum-free DMEM supplemented with Nutridoma SP (Roche), purified on protein G columns (GE Healthcare), and characterized by SDS-PAGE and Western blotting using α -mouse IgG1-HRP (Southern Biotech) or biotin-conjugated α -MOG (R&D Systems), followed by HRP-Sva (Jackson ImmunoResearch Laboratories Inc.) (Supplemental Figure 2B). mAb binding was verified on receptor-transfected CHO cells by FACS using PE α -mouse IgG (Jackson ImmunoResearch Laboratories Inc.) (Supplemental Figure 2C).

Reagents. Fluorochrome-labeled mAbs (see Supplemental Methods). Other reagents were Cytotfix/Cytoperm solution (BD Biosciences), Live/Dead Fixable Aqua or Blue (Life Technologies), and DAPI (Sigma-Aldrich). The Foxp3 staining set was from eBiosciences.

Microarray and data analysis. RNA was prepared from purified cell populations from Langerin-EGFP mice using TRIzol (Life Technologies) as described previously (59). Populations were sorted (Aria II; BD) using the ImmGen Standardized Sorting protocol (<http://www.ImmGen.org>) at the Mount Sinai Flow Cytometry Shared Resource Facility. RNA was amplified and hybridized on the Affymetrix Mouse Gene 1.0 ST array according to the manufacturer's procedures. All datasets were deposited in the National Center for Biotechnology Information/GEO database under accession number GSE15907. Data generation, pre-processing, normalization, and quality control were as described previously (59).

Purification of MOG-specific CD4⁺ T cells. CD4⁺ T cells were enriched (>90%) from spleen and LN of 2D2 mice by negative selection using α -F4/80,



α -MHC II (TIB120), α -B220 (RA3-6B2), α -CD8 (2.43), and α -NK1.1 (PK136) mAbs (produced in house), followed by α -rat Dynabeads (Life Technologies). Naive T cells were FACS purified (Aria II or III; BD) from 2D2 Foxp3-EGFP mice by gating on live CD4⁺ Foxp3-EGFP-CD25⁻ CD45RB^{hi}CD44^{lo}. nTregs were FACS purified from 2D2 Foxp3-EGFP mice by gating on live CD4⁺ Foxp3-EGFP⁺. FACS-sorted preparations were usually of >99% purity. In some cases, 2×10^7 /ml CD4⁺ T cells were labeled with 5 μ M CellTrace Violet (Life Technologies) for 10 minutes at 37°C.

Adoptive transfer of MOG-specific CD4⁺ T cells. 4×10^6 MOG-specific CD45.1⁺CD4⁺ T cells from 2D2 or 2D2 Foxp3-EGFP mice, or 8×10^6 from 2D2 Foxp3-DTR mice were transferred into B6 CD45.2 recipient mice. Recipient mice were inoculated 1 day later with different doses (0.3–30 μ g) of α -receptor–MOGp mAbs, control Ig-MOGp, unconjugated α -Langerin mAbs, or soluble MOG35-55p (3–300 μ g). Inoculations were performed s.c. in footpad (50 μ l/hind pad), i.p., i.v., i.m., or i.n. (25 μ l/nostri). α -Receptor mAbs were injected alone or with 25 μ g α -CD40 mAb (IC10; produced in house), 50 μ g poly(I:C) (InvivoGen), or 20 μ g glucopyranosyl lipid A (GLA; Immune Design). Langerin-DTR mice were inoculated i.v. with 500 ng DT (Sigma-Aldrich) on the day of T cell transfer, followed by 250 ng i.p. every 2 days thereafter to maintain DC ablation.

Preparation of cell suspensions. DCs were obtained by digesting spleen or sLN with 400 U/ml Collagenase D (Roche) and 50 μ g/ml DNase I (Roche) for 25 minutes at 37°C. 10 μ M EDTA (Life Technologies) was added for the last 5 minutes. DC enrichment was performed with CD11c microbeads (Miltenyi Biotec). For analysis of Foxp3 expression, spleen and LNs were force passed through a 70- μ m cell strainer to obtain a homogenous cell suspension, followed by rbc lysis (BioWhittaker). Lungs of perfused mice were cut in small pieces and digested with 0.13 U/ml Liberase TM (Roche) and 50 μ g/ml DNase I for 30 minutes at 37°C, with 5 μ M EDTA added for the last 5 minutes of incubation. Lung leukocytes were enriched by Percoll (GE Healthcare) gradient (67.5%–45%) centrifugation (800 *g* for 15 minutes).

Bone marrow chimeras. 3×10^6 to 5×10^6 bone marrow cells recovered from tibias and femurs were injected i.v. into lethally irradiated (11.5 Gy in 2 doses, 3 hours apart) recipients. Mice were maintained for 1 month on antibiotic-supplemented food (Sulfa-Trim; TestDiet) and water (1.1 mg/ml neomycin; Sigma-Aldrich). Hematopoietic engraftment was analyzed at week 8 using CD45.1/CD45.2 congenic markers (>98% blood B cells, granulocytes, and monocytes were of donor origin). Mice were used at weeks 12–14.

EAE induction. B6 mice were immunized s.c. (into the flanks) with 100 μ g MOG35-55p (synthesized by H. Zebroski, The Rockefeller University) in complete Freund's adjuvant (CFA; Difco Laboratories) emulsion containing 500 μ g of *Mycobacterium tuberculosis* H37 RA (Difco). Animals received 200 ng pertussis toxin i.v. (List Biological Laboratories) on days 0 and 2 after immunization. In some experiments, MOG-specific Foxp3-DTR⁺ cells were depleted by inoculation of 250 ng DT administered 2 days before (i.v.) and 1 day after (i.p.) EAE immunization. Animals were monitored daily for symptoms and scored in a blinded manner as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; and 5, dead (22). Mice were euthanized and scored 5 if they reached a score ≥ 4 for 2 consecutive days.

Statistics. Statistical analysis was performed with Prism software (GraphPad). Statistical significance between 2 groups was determined by a 2-tailed Student's *t* test. EAE scores were evaluated by 2-way ANOVA and Bonferroni's post hoc analysis. *P* values 0.05 or less were considered statistically significant.

Study approval. All animal studies were approved by the Animal Care and Use Committee of The Rockefeller University.

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