Regulatory T cells mediate specific suppression by depleting peptide–MHC class II from dendritic cells

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Regulatory T cells (Treg cells) can activate multiple suppressive mechanisms in vitro after activation via the T cell antigen receptor, resulting in antigen-independent suppression. However, it remains unclear whether similar pathways operate in vivo. Here we found that antigen-specific Treg cells activated by dendritic cells (DCs) pulsed with two antigens suppressed conventional naive T cells (T naive cells) specific for both cognate antigens and non-cognate antigens in vitro but suppressed only T naive cells specific for cognate antigen in vivo. Antigen-specific Treg cells formed strong interactions with DCs, resulting in selective inhibition of the binding of T naive cells to cognate antigen yet allowing bystander T naive cell access. Strong binding resulted in the removal of the complex of cognate peptide and major histocompatibility complex class II (pMHCII) from the DC surface, reducing the capacity of DCs to present antigen. The enhanced binding of Treg cells to DCs, coupled with their capacity to deplete pMHCII, represents a novel pathway for Treg cell–mediated suppression and may be a mechanism by which Treg cells maintain immune homeostasis.

Forkhead box protein P3–positive (Foxp3+) regulatory T cells (Treg cells) are critical for the maintenance of immune homeostasis. One of the major unresolved issues regarding their function is whether they can mediate antigen-specific suppression. Several early in vivo studies of Treg cells suggested a role for antigen specificity, in that CD4+ T cells from mice lacking the target organ were poor suppressors of disease in those organs1–13. Although these studies indicated the importance of antigen-mediated priming of Treg cells, they did not examine whether antigen recognition by Treg cells had any further role in suppression in vivo. Several mechanisms have been proposed for the Treg cell–mediated suppression that can target both effector T cell function and antigen presentation. These include the following: production of tolerogenic molecules8–11; consumption of interleukin 2 (IL-2)12; cytotoxic T lymphocyte–associated protein 4 (CTLA-4)-mediated inhibition of the binding of T naive cells to cognate antigen yet allowing bystander T naive cell access. Strong binding resulted in the removal of the complex of cognate peptide and major histocompatibility complex class II (pMHCII) from the DC surface, reducing the capacity of DCs to present antigen. The enhanced binding of Treg cells to DCs, coupled with their capacity to deplete pMHCII, represents a novel pathway for Treg cell–mediated suppression and may be a mechanism by which Treg cells maintain immune homeostasis.

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T<sub>reg</sub> cells and T<sub>naive</sub> cells, T<sub>reg</sub> cells that recognized the same antigen that T<sub>naive</sub> cells recognized selectively excluded T<sub>naive</sub> cells. However, T<sub>reg</sub> cell pretreatment of double-pulsed DCs in vitro disabled the capacity of DCs to activate T<sub>naive</sub> cells specific for the antigen recognized by T<sub>reg</sub> cells but not the response of T<sub>naive</sub> cells specific for an unrelated antigen expressed on the same DC surface. These findings suggest that T<sub>reg</sub> cells use suppressor mechanisms, in addition to preventing access of T<sub>naive</sub> cells to antigen expressed on the DC surface. We demonstrated that antigen-specific T<sub>reg</sub> cells removed complexes of peptide and major histocompatibility complex class II (pMHCII) from the DC surface and thereby decreased the capacity of DCs to present antigen. Most importantly, the removal of pMHCII complexes was antigen specific, since T<sub>reg</sub> cells captured only the pMHCII complexes that they recognize but not any unrelated antigen expressed on the same DC.

Thus, we describe a novel pathway for antigen-specific T<sub>reg</sub> cell-mediated suppression. It first requires a strong interaction of the antigen-specific T<sub>reg</sub> cells with DCs presenting their cognate antigen; secondarily, it requires removal of the cognate pMHCII from the DC surface in a TCR-specific fashion.

**Results**

**Antigen-specific T<sub>reg</sub> cells mediate antigen-specific suppression in vivo.** To determine if antigen-specific T<sub>reg</sub> cells exhibit bystander suppression, we generated antigen-specific T<sub>reg</sub> cells using naive CD4<sup>+</sup>Foxp3<sup>−</sup> T cells from OT-II transgenic mice. OT-II T<sub>reg</sub> cells markedly suppressed the proliferation of OT-II T cells stimulated with ovalbumin (OVA)323–339-pulsed DCs in vitro but had only a minimal effect on the proliferation of SMARTA T cells stimulated with DC pulsed with lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP)61–80 (Supplementary Fig. 1a,b). Similarly, SMARTA T<sub>reg</sub> cells completely suppressed the response of SMARTA T cells to GP61–80-pulsed DCs but had no effect on the response of OT-II T cells to OVA-pulsed DCs (Supplementary Fig. 1c,d). The failure of OT-II T<sub>reg</sub> cells to suppress the response of SMARTA T cells could be secondary to the requirement that T<sub>reg</sub> cells be restimulated in culture to exert their suppressive function. To clarify this point, we cocultured OT-II T cells and SMARTA T cells in the presence of OT-II T<sub>reg</sub> cells and a mixture of DCs pulsed with OVA323–339 and DCs pulsed with GP61–80 or with DCs simultaneously pulsed with OVA323–339 and GP61–80. Under both of these activation conditions, antigen-specific T<sub>reg</sub> cells suppressed the proliferation of OT-II T cells and SMARTA T cells (Supplementary Fig. 1e,f). This result is similar to previous observations<sup>16</sup> with antigen-specific T<sub>reg</sub> cells isolated from TCR-transgenic mice. One difference between studies is that the T<sub>reg</sub> cells required restimulation with their cognate antigen even though they were previously activated in culture.

To evaluate whether activated antigen-specific T<sub>reg</sub> cells could also suppress the response to unrelated antigens in vivo, we developed a model in which peptide-pulsed DCs, antigen-specific T cells and antigen-specific T<sub>reg</sub> cells are transferred intravenously into immunocompetent syngeneic recipients. To determine whether antigen-specific T<sub>reg</sub> cells activated with their cognate antigen could suppress the responses of a second antigen-specific T cell population when stimulated with both their cognate antigen and the unrelated antigen, we first cotransferred OT-II T cells, SMARTA T cells, OT-II T<sub>reg</sub> cells and separate populations of DCs pulsed with OVA323–339 or GP61–80. OT-II T<sub>reg</sub> cells completely suppressed the response of OT-II T cells but failed to suppress the response of SMARTA T cells. Similarly, when SMARTA T<sub>reg</sub> cells were transferred with OT-II T cells and SMARTA T cells and separate populations of pulsed DCs, they completely suppressed the proliferation of SMARTA T cells but had no effect on the proliferation of OT-II T cells (Fig. 1a). To determine whether antigen-specific T<sub>reg</sub> cells activated with their cognate antigen could suppress the responses of a second antigen-specific T cell population in the same environment when stimulated with DCs pulsed with both peptides, we cotransferred OT-II T cells, SMARTA T cells, OT-II T<sub>reg</sub> cells, and DCs simultaneously pulsed with OVA323–339 and GP61–80. OT-II T<sub>reg</sub> cells completely suppressed the response of OT-II T cells and only slightly suppressed the response of SMARTA T cells. Likewise, when SMARTA T<sub>reg</sub> cells were transferred with OT-II T cells and SMARTA T cells and double-pulsed DCs, they completely suppressed the proliferation of SMARTA T cells but had no effect on the proliferation of OT-II T cells (Fig. 1b). Similar to T<sub>reg</sub> cells, antigen-specific T<sub>reg</sub> cells from TCR-transgenic mice that were expanded in vitro via plate-bound antibody to CD3 (anti-CD3), anti-CD8 and IL-2 or in vivo via IL-2–anti-IL-2 complex treatment also displayed antigen-specific suppression when adoptively transferred in vivo (Supplementary Fig. 2a–c).

The ability of antigen-specific T<sub>reg</sub> cells to suppress their responses to their cognate antigen expressed on DCs, but not to suppress responses to a second antigen expressed on the same DC population, strongly suggested that T<sub>reg</sub> cell suppressor mechanisms involving the downregulation of costimulatory function on DCs or suppressor cytokines played little to no role in T<sub>reg</sub> cell suppression in vivo. To test this possibility, we made use of TCR-transgenic strains specific for pigeon cytochrome C (PCC) on a Rag2<sup>−/−</sup> background that were either deficient in CTLA-4 or IL-10. Because these mice lacked a normal TCR repertoire, they were phenotypically normal and exhibited no signs of T cell activation or autoimmune disease. PCC-specific T<sub>reg</sub> cells generated from either Il10<sup>−/−</sup> mice or Ctila4<sup>−/−</sup> mice were as suppressive as PCC-specific T<sub>reg</sub> cells generated from 5CC7 TCR–transgenic Rag2<sup>−/−</sup> donors (Fig. 1c,d), strongly suggesting that neither IL-10 nor CTLA-4 played a major component in T<sub>reg</sub> cell suppressor function in vivo.

Antigen-specific T<sub>reg</sub> cells engage in intense and unique interactions with antigen-pulsed DCs. As an initial approach to determine the mechanism of antigen-specific suppression in vivo, we characterized the interactions of freshly isolated 5CC7 T<sub>naive</sub> cells, 5CC7 activated T cells (T<sub>activated</sub> cells), and 5CC7 T<sub>reg</sub> cells with moth cytochrome C (MCC)66–105-pulsed DCs using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) after 2 h of coculture in vitro. We quantified T cells per DC for a total of 40 DCs using SEM images and found a significantly larger number of T<sub>reg</sub> cells bound per DC (Fig. 2a,b). Tiled SEM images of the T cell–DC binding sites revealed an average of three to nine membrane-fusion nanodomains per T cell–DC couple. Membrane-fusion nanodomains have been reported to contain clusters of TCR and co-receptors<sup>18–21</sup>. Length measurements of nanodomains visible in random, representative micrographs showed that T<sub>reg</sub> cells displayed significantly wider nanodomains than the nanodomains formed by T<sub>naive</sub> cells or T<sub>activated</sub> cells, suggesting a more mature contact (Fig. 2c,d).

TEM images of T cell–DC couples demonstrated that after binding to DCs, T<sub>reg</sub> cells exhibited a morphology distinct from that of DC-bound T<sub>activated</sub> cells, with uropods at the rear and finger-like projections at the leading edge. T<sub>reg</sub> cell–DC contact sites contained filopodia that were not observed at the T<sub>activated</sub> cell–DC interaction sites (Fig. 2e). We also detected by live confocal microscopy that OT-II T<sub>reg</sub> cells, but not OT-II T<sub>activated</sub> cells, displayed prominent amoeboid movements with highly dynamic filopodial protrusions and contractions at the DC binding site, which increased the volume of interaction with the antigen-pulsed DCs (Fig. 2f and Supplementary Videos 1 and 2a,b).

We then characterized the real-time interactions of T<sub>reg</sub> cells and T<sub>activated</sub> cells with DCs in vivo by cotransferring OT-II T<sub>reg</sub> cells or OT-II T<sub>activated</sub> cells with antigen-pulsed DCs into wild-type mice (Fig. 3a). DCs that were pulsed with OVA323–339 were directed to popliteal lymph nodes by injection into the footpad to facilitate interactions with either OT-II T<sub>reg</sub> cells or OT-II T<sub>activated</sub> cells that were simultaneously injected intravenously. We visualized the lymphatic
Fig. 1 | Antigen-specific iTreg cells suppress Tnaive cells with identical antigen specificity regardless of CTLA-4 expression or IL-10 production. a, b, C57BL/6 DCs were loaded with 3 μM OVA223–239 (DCOVA), LCMV GP61–80 (DCGP) or both peptides (DCOVA-GP). Carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve OT-II cells (1 × 10⁶) and SMARTA cells (1 × 10⁶) were transferred intravenously into CD45.1+ mice with DCs (5 × 10⁵) and iTreg cells (2 × 10⁵). The histograms demonstrate the day 3 post-transfer proliferation status of Thy1.1+ OT-II cells and Thy1.1+ SMARTA cells after cotransfer with 1:1 mixture of DCOVA and DCGP (a) or DCOVA-GP (b). The graphs show the number of CFSElo proliferating cells. c, d, B10.A DCs were pulsed with 3 μM PCCs6–104, CD45.1+ CFSE-labeled 5CC7 Tnaive cells (1 × 10⁵) were transferred intravenously into B10.A mice together with DCs (5 × 10⁵) and wild-type, Il10−/− or Ctla4−/− 5CC7 iTreg cells (2 × 10⁵). The histograms demonstrate the day 3 post-transfer proliferation status; the graphs show the number of CFSElo 5CC7 T cells. The bars indicate the mean of n = 3 mice (one mouse per symbol); the data are representative of two (c, d) or three (a, b) independent experiments. P values were calculated using a one-way ANOVA with Dunnett’s multiple-comparisons test.
**Fig. 2 | Antigen-specific iTreg cells have unique binding morphology and stoichiometry.**

**a–d.** OVA323–339-pulsed splenic DCs (4 × 10⁴) were cocultured 1:1 with OT-II T naïve cells, OT-II T activated cells or OT-II iTreg cells for 3 h and were visualized by SEM. **a,** Representative images of T cell–DC clusters (scale bar, 5 µm). **b,** Images were collected from a total of 40 DCs; the graph shows the number of T cells bound per DC. Dashed lines mark the mean of n = 40 DCs (one cell per symbol) obtained from n = 2 biological replicates per experiment. Data are representative of two independent experiments. **c,** SEM images of the DC–T cell binding sites that reveal membrane-fusion domains (nanodomains; scale bar, 300 nm). **d,** Biological replicates (n = 2) were screened to collect images of 9–11 fusion sites per group. The bars demonstrate individual measurements pooled from the replicates; the data represent three independent experiments. **e,** OVA323–339-pulsed DCs (2 × 10⁵) were cocultured 1:1 with OT-II T naïve cells, OT-II T activated cells or OT-II iTreg cells for 3 h and were imaged with TEM. The yellow rectangles and arrows mark the uropods and filopodia, respectively. Scale bars for images: upper, 2 µm; lower, 500 nm. Images are representative of three independent experiments with similar results. **f,** OVA323–339-pulsed DCs (2 × 10⁵) were cocultured 1:1 with OT-II T activated cells or OT-II iTreg cells for 3 h and were imaged for real-time interactions (red, CD4; blue, CD11c). The graph shows the 3D volume of the T cell–DC contact site that was derived from time-dependent colocalization analysis. Dashed lines mark the mean of individual data points (symbols) pooled from n = 2 biological replicates. The data are representative of five independent experiments with similar results. P values were calculated using a Kruskal–Wallis test (b), one-way ANOVA (d) or two-sided Welch’s t-test (f).
nodes by intravital two-photon microscopy at 18–20 h post-transfer and found that OT-II iTreg cells formed larger clusters around the DCs, whereas OT-II T naive cells were located more remotely (Fig. 3b). OT-II iTreg cells engaged in more intense interactions with DCs, as quantified by the greater volume of contact and longer contact durations, recapitulating our in vitro observations in vivo (Fig. 3c and Supplementary Video 3a,b).

One possible explanation for antigen-specific suppression in vivo (Fig. 1a,b) is that the greater avidity of iTreg cells for antigen-pulsed DCs inhibits the access of antigen-specific T naive cells. To address whether antigen-specific competition exists in vivo, we transferred OT-II T naive cells with OT-II iTreg cells or with OT-II T activation cells. The dynamic movement and localization of OT-II T naive cells were visualized by intravital two-photon microscopy 18–20 h post-transfer. Intravital microscopy showed that in the presence of cognate antigen, OT-II T naive cells exhibited slower movement and enhanced clustering around DCs. The presence of OT-II T activation cells resulted in some degree of increased mobility and reduced clustering around the DC of the cotransferred OT-II T naive cells. In contrast, in the presence of OT-II iTreg cells, OT-II T naive cells exhibited significantly greater mobility, as reflected by larger increases in average T cell track velocities and longer distances to the closest DC than in the presence of OT-II T activation cells (Fig. 3d,e and Supplementary Video 4a–d). In parallel studies of T cell proliferation in vivo at 72 h after transfer, only modest inhibition of proliferation of OT-II T naive cells was seen in the presence of OT-II T activation cells, while profound inhibition of the proliferation of OT-II T naive cells was observed in the presence of OT-II iTreg cells (Fig. 3f,g). Taken together, these microscopic studies demonstrate that the profound inhibition of T cell proliferation by antigen-specific iTreg cells might be consistent with intense contact between Treg cells and DCs.

Visualization of T reg cell–mediated antigen-specific suppression in vivo. To further analyze the mechanism of suppression mediated by antigen-specific iTreg cells, we adoptively transferred DCs pulsed with both OVA323–339 and GP61–80 into wild-type mice via the footpad. We then cotransferred OT-II iTreg cells or SMARTA iTreg cells with a mixture of OT-II T naive cells and SMARTA T naive cells intravenously to visualize how real-time interactions between DCs and antigen-specific T naive cells and bystander T naive cells are regulated by iTreg cells. We extracted the lymph nodes 18–20 h post-transfer and sectioned them in ice-cold PBS to preserve the microarchitecture of the lymph node and the viability of cells. After warming the sections to 37 °C to restore cell movement, we tracked the cells for up to 12 h. We found that the presence of OT-II iTreg cells led to the selective exclusion of SMARTA T naive cells, from DC contact. In contrast, bystander CD4+ T cells maintained their interaction with DCs (Fig. 4a and Supplementary Video 5). Regardless of the antigen dose, antigen-specific T naive cells moved at higher speeds and interacted for shorter times than did bystander T naive cells, indicating that the T reg cell–mediated disruption of stable contacts was antigen specific (Fig. 4b–d).

To visualize the morphological changes that occur in vivo during T cell activation, we transferred OVA323–339 and GP61–80 double-pulsed DCs, OT-II T naive cells and SMARTA T naive cells with OT-II or SMARTA iTreg cells into normal recipients. We then performed confocal microscopy of whole popliteal lymph node sections 24 h post-transfer. In the absence of Treg cells, OT-II T cells were enlarged, as a morphological sign of activation. The volume of the OT-II T cell blasts was significantly decreased in the presence of OT-II iTreg cells but not in the presence of SMARTA iTreg cells, indicating that the inhibition of T cell blasting is antigen specific (Fig. 4e,f). Furthermore, the OT-II T cells retained the spherical morphology of unstimulated T cells only in the presence of OT-II iTreg cells (Fig. 4f), but not in the presence of SMARTA iTreg cells. These findings confirm that at the morphological level, antigen-specific iTreg cells do not mediate bystander inhibition in vivo.

Treg cells acquire pMHC II complexes by capturing DC membrane fragments. The data presented in the previous section are compatible with a model in which the major inhibitory function of antigen-specific iTreg cells is to prevent the access of T naive cells to pMHC II on the DC surface in a competitive fashion. If this were the case, removal of iTreg cells from cocultures in vitro would abolish their suppressive effects. To test this, we precultured iTreg cells with DCs pulsed with both OVA323–339 and GP61–80. We then depleted the iTreg cells from the DCs and evaluated the capacity of the treated DCs to stimulate TCR-transgenic T naive cells. DCs preincubated with OT-II iTreg cells failed to activate OT-II T cells but retained the capacity to activate SMARTA T cells. Similarly, DCs preincubated with SMARTA iTreg cells failed to activate SMARTA T cells but stimulated OT-II T cells as efficiently as control DCs did (Fig. 5a,b). Furthermore, when we added fresh peptide to DCs preincubated with iTreg cells, the antigen-presentation capacity of DCs was restored (Fig. 5c,d). These results strongly suggest that antigen-specific iTreg cells can decrease the capacity of DCs to present their cognate antigen but leave intact the presentation of antigens not recognized by their TCR.

We hypothesized that the intense interaction of iTreg cells with DCs might result in the removal of pMHCII complexes from the DC surface. To evaluate whether iTreg cells acquire DC-derived
membranes by a process of trogocytosis, we labeled DC membranes with the lipophilic dye PKH26 and pulsed them with MCC<sub>86–103</sub>. DCs were then cultured with 5CC7 T<sub>naive</sub> cells, T<sub>activated</sub> cells or iT<sub>reg</sub> cells for 18h. 5CC7 iT<sub>reg</sub> cells acquired a greater amount of the DC membrane than did T<sub>naive</sub> cells or T<sub>activated</sub> cells, as measured by the increase in their PKH26 fluorescence intensity (Fig. 6a). We then tested whether iT<sub>reg</sub> cells acquire multiple membrane antigens from the DC surface. Indeed, when we stained antigen-specific iT<sub>reg</sub> cells, we could easily detect surface molecules involved in the immune synapse, such as MHCII, CD86, ICOSL (inducible costimulatory ligand) and PD-L2 (programmed death-ligand 2) (Supplementary Fig. 3a). It is very likely that these antigens were derived from the surface of the DCs, since they could not be detected on the surface of iT<sub>reg</sub> cells either before the coculture or after polyclonal activation with plate-bound anti-CD3 and anti-CD28 in the absence of DCs (Supplementary Fig. 3b).
Fig. 4 | Antigen-specific iTreg cells inhibit the stable contact of T cells and DCs in a TCR-restricted manner. a–d, Splenic DCs from CD11c-YFP mice were double-pulsed with OVA\textsubscript{223–235} and GP\textsubscript{61–80} at a dose of either 0.5 μM or 5 μM ex vivo. DCs (2 × 10\textsuperscript{6}) were adoptively transferred into C57BL/6 mice via the footpad. The mice then received OT-II DsRed T\textsubscript{naive} cells (1.2 × 10\textsuperscript{6}), e670-labeled SMARTA T\textsubscript{naive} cells (1.2 × 10\textsuperscript{6}) and e450-labeled OT-II or SMARTA iTreg cells (4.8 × 10\textsuperscript{5}) intravenously. Live popliteal lymph node sections were imaged 18 h post-transfer. a, The time series demonstrate the movement and interactions of SMARTA iTreg cells, OT-II DsRed T\textsubscript{naive} cells and SMARTA T\textsubscript{naive} cells at 5-min intervals. The yellow circle represents the contact with the DC. The yellow arrow shows the OT-II T\textsubscript{naive} cells that had sustained interaction with the DC (scale bar, 20 μm). b–d, The graphs show the average track speed (b, c) and colocalization duration (d) of OT-II cells and SMARTA cells; dashed lines mark the mean of the cells (one per symbol: OT-II, red; SMARTA, blue) tracked in one recipient. The data are representative of n = 3 independent experiments with similar results. e–f, DCs (2 × 10\textsuperscript{6}) double-pulsed with 5 μM OVA\textsubscript{223–235} and GP\textsubscript{61–80} were adoptively transferred via the footpad. OT-II DsRed T\textsubscript{naive} cells (7 × 10\textsuperscript{5}) were transferred intravenously together with e450-labeled OT-II or SMARTA iTreg cells (1.4 × 10\textsuperscript{5}). Popliteal lymph node sections were imaged 18–20 h post-transfer. Representative demonstration (e) and graphs (f) of 3D surface area, volume and sphericity of OT-II T\textsubscript{naive} cells. Dashed lines mark the mean of the cells (one per symbol) from one recipient; the data are representative of n = 3 independent experiments with similar results. P values were calculated using a one-way ANOVA with Tukey’s correction (b–d, volume) or the Kruskal–Wallis test (f, sphericity).
Fig. 5 | Antigen-specific iTreg cells selectively inhibit presentation of cognate antigen. 

a, b, DCs (4 × 10^6) were double-pulsed with 3 μM OVA_{323-339} and 3 μM GP_{61-80} and were cultured with CFSE-labeled OT-II iTreg cells (1.5 × 10^6; Rx-OT-II) or SMARTA iTreg cells (1.5 × 10^6; Rx-SM) or alone (Rx-control) for 18 h; live CFSE−CD3ε−DCs were isolated by flow-cytometry sorting. 

b, c, Sorted DCs (5 × 10^3) were cocultured with a 1:1 mixture of CellTrace Violet (CTV)-labeled CD45.1+ OT-II T naive cells (5 × 10^4) and SMARTA T naive cells (5 × 10^4) for 3 d. 

a, Flow cytometry plots demonstrate the proliferation status and CD25 expression of T naive cells. 

b, The graphs show the number of CFSElow T cells. The bars indicate the means of n = 4 biological replicates (one per symbol); the data are representative of two independent experiments. 

c, d, Sorted DCs (5 × 10^3) were pulsed with either OVA_{323-339} or GP_{61-80}, and were cultured with CTV-labeled CD45.1+ OT-II or SMARTA T naive cells (5 × 10^4) for 3 d. 

c, Flow cytometry plots demonstrate the proliferation status and CD25 expression of T naive cells. 

d, The graphs show the number of CFSElow T cells. The bars indicate the mean of n = 3 biological replicates (one per symbol); the data are representative of two independent experiments. 
P values were calculated using a one-way ANOVA with Dunnett’s test.
To visualize the membrane patches containing total MHCII acquired by antigen-specific iTreg cells, we fixed cocultures of OVA323–339-pulsed DCs with OT-II T naive cells, T activated cells or iTreg cells at 3 h after coculture and imaged them using confocal microscopy. To distinguish between the isolated membrane patches separated from the DCs and the actual DC–T cell contact sites, we performed three-dimensional (3D) reconstruction of DCs and used it to mask other channels to eliminate the signal coming from DCs.
Using masked channels, we created 3D surfaces for the T cells and T\textsubscript{reg} cells and quantitated the mean intensity of the acquired MHCIIs (Fig. 6b). The MHCIIs acquired by iT\textsubscript{reg} cells had greater intensity than that in T\textsubscript{activated} cells (Fig. 6c). Together these findings suggest that antigen-specific T\textsubscript{reg} cells have greater trogocytic capacity than that of T\textsubscript{activated} cells.

**Antigen-specific iT\textsubscript{reg} cells downregulate antigen presentation by DCs by removing pMHCIIs from the DC surface.** Although confocal microscopy was sensitive enough to detect the total MHCIIs signal, its sensitivity was not adequate to visualize the acquisition of antigen-specific pMHCIIs. We then used TEM to visualize and quantify the acquisition of antigen-specific pMHCIIs early during T cell–DC interaction. We made use of a monoclonal antibody (mAb), D4, that detects complexes of MCC\textsubscript{88-103} and the MHCII molecule I-E\textsuperscript{k}. We cultured MCC\textsubscript{88-103}-pulsed DCs with 5CC7 T\textsubscript{naive} cells, T\textsubscript{activated} cells or iT\textsubscript{reg} cells for 3 h and stained with biotinylated mAb D4, followed by streptavidin-conjugated quantum dots, and imaged the cells by TEM. Only iT\textsubscript{reg} cells had intense DC contacts where they engulfed parts of the DC membrane (Fig. 6d). More importantly, we observed transfer of quantum dots only to iT\textsubscript{reg} cells, not to T\textsubscript{naive} cells or T\textsubscript{activated} cells (Fig. 6e). The quantum dots were detected both on the T\textsubscript{reg} cell surface and within the endosomes (Fig. 6f).

To assay whether a similar process of uptake of DC membranes by iT\textsubscript{reg} cells occurred in vivo, we adoptively transferred MCC\textsubscript{88-103}-pulsed DCs via the footpad and simultaneously transferred 5CC7 T\textsubscript{naive} cells, T\textsubscript{activated} cells or iT\textsubscript{reg} cells into wild-type B10.A mice. Internalized MCC\textsubscript{88-103}–I-E\textsuperscript{k} complexes were found in 5CC7 iT\textsubscript{reg} cells on day 3 post-transfer in the draining popliteal lymph node, whereas no complexes could be detected in 5CC7 T\textsubscript{naive} cells or T\textsubscript{activated} cells, indicating that antigen-specific iT\textsubscript{reg} cells indeed captured and retained cognate pMHCIIs (Fig. 7a).

To further delineate the parameters that governed iT\textsubscript{reg} cell–mediated uptake of pMHCIIs complexes, we cocultured 5CC7 T\textsubscript{naive} cells, T\textsubscript{activated} cells or iT\textsubscript{reg} cells for 18 h with freshly isolated splenic DCs that had been pulsed with MCC\textsubscript{88-103} peptide at different loading doses and used flow cytometry to measure the amount of MCC\textsubscript{88-103}–I-E\textsuperscript{k} complexes acquired by T cells. We detected significantly larger amounts of pMHCIIs complexes both on the surface and in the intracellular compartment of iT\textsubscript{reg} cells than in T\textsubscript{naive} cells or T\textsubscript{activated} cells at any peptide concentration (Fig. 7b,c). pMHCIIs complexes on 5CC7 iT\textsubscript{reg} cells were detectable by flow cytometry as early as 3 h post coculture, with greater accumulation over time, whereas they became detectable on the surface of T\textsubscript{naive} cells only after 6 h. No pMHCIIs complexes were detected on the surface of polyclonal iT\textsubscript{reg} cells, substantiating the role of TCR–pMHCIIs interactions for the acquisition of pMHCIIs complexes by T\textsubscript{reg} cells (Supplementary Fig. 4a,b). The MCC\textsubscript{88-103}–I-E\textsuperscript{k} level detected on the DC surface was reduced to a greater extent in the presence of SCC7 iT\textsubscript{reg} cells than with T\textsubscript{naive} cells or T\textsubscript{activated} cells (Fig. 7d). This result is consistent with iT\textsubscript{reg} cell–mediated depletion of pMHCIIs from the DC surface. We observed similar results with T\textsubscript{reg} cells isolated from TCR-transgenic mice on a Rag2\textsuperscript{-/-} background. Both antigen-specific iT\textsubscript{reg} cells and T\textsubscript{reg} cells (probably iT\textsubscript{reg} cells) were able to reduce the DC surface pMHCIIs to the same degree (Fig. 7e,f).

The interaction of CTLA-4 on the surface of T\textsubscript{reg} cells with the costimulatory molecule CD80 or CD86 on the surface of DCs has been previously shown to result in the capture of these molecules by the T\textsubscript{reg} cell through a process of transendocytosis\textsuperscript{41}. To determine if CTLA-4-mediated transendocytosis plays a role in the uptake of MCC\textsubscript{88-103}–I-E\textsuperscript{k} or the removal of MCC\textsubscript{88-103}–I-E\textsuperscript{k} from the DC surface, we again generated MCC\textsubscript{88-103}-specific iT\textsubscript{reg} cells from CTLA-4-deficient 5CC7 TCR-transgenic mice and cultured them with MCC\textsubscript{88-103}-pulsed DCs. The uptake of the MCC\textsubscript{88-103}–I-E\textsuperscript{k} complex by wild-type iT\textsubscript{reg} cells and Cldn4\textsuperscript{-/-} iT\textsubscript{reg} cells was identical, indicating that CTLA-4 did not play a role in the uptake of pMHCIIs complexes (Supplementary Fig. 5). These findings indicate that antigen-specific T\textsubscript{reg} cells capture a critical amount of pMHCIIs from DCs, thus decreasing the presentation of cognate antigen without the involvement of CTLA-4-mediated mechanisms.

**Acquisition of pMHCIIs complexes by T\textsubscript{reg} cells is antigen specific.** As T\textsubscript{reg} cells manifested their suppressive effect in an antigen-specific manner in vivo, it was important to determine whether the acquisition of pMHCIIs complexes was limited to cognate pMHCIIs. To test this, we pulsed DCs from B10.A mice with equimolar amounts of MCC\textsubscript{88-103}–peptide and hen egg white lysozyme (HEL)\textsubscript{46-61}–peptide and cocultured the pulsed DCs for 18 h with 5CC7 and 3A9 (HEL\textsubscript{46-61}-specific) iT\textsubscript{reg} cells or T\textsubscript{reg} cells. 5CC7 T\textsubscript{reg} cells acquired MCC\textsubscript{88-103}–I-E\textsuperscript{k} complexes but not HEL\textsubscript{46-61}–I-A\textsuperscript{k} complexes (detected with mAb AW3.18\textsuperscript{b}). Similarly, HEL\textsubscript{46-61}–I-A\textsuperscript{k} complexes, but not MCC\textsubscript{88-103}–I-E\textsuperscript{k} complexes, were captured by 3A9 T\textsubscript{reg} cells, even though both 5CC7 T\textsubscript{reg} cells and 3A9 T\textsubscript{reg} cells were capable of interacting with the same DC (Fig. 8a and Supplementary Fig. 6). Furthermore, DC levels of pMHCIIs were depleted in an antigen-specific manner, while the level of the non-cognate complex...
remained unchanged (Fig. 8b,c and Supplementary Fig. 6). Next we confirmed the functional consequence of antigen-restricted pMH-CII removal in this particular two-antigen system as in Fig. 5 by preculturing the iTreg cells with double-pulsed DCs and evaluating the capacity of the treated DCs to stimulate naive TCR-transgenic T cells. We observed that DCs precultured with 5CC7 or 3A9 iTreg cells failed to activate T naive cells specific for the cognate antigen recognized by the antigen-specific iTreg cells yet maintained the capacity to activate T naive cells specific for the non-cognate antigen (Supplementary Fig. 7). When antigen-specific Treg cells were transferred in vivo with double-pulsed DCs, they acquired only their cognate complexes (Fig. 8d,e and Supplementary Fig. 6).
Furthermore, double-pulsed DCs were depleted in cognate pMHCIICII, while the amount of non-cognate complex was unchanged (Fig. 8f and Supplementary Fig. 6). Together our data confirm that antigen-specific Treg cells can execute suppressor function by acquiring pMHCIICII complexes from double-pulsed DCs in an antigen-specific manner (Supplementary Fig. 8).
The molecular basis for the strong binding of T<sub>reg</sub> cells to antigen-pulsed DCs and their subsequent ability to remove membrane complexes from the DC surface remains unknown. Recent studies using phosphoproteomics have identified a set of proteins differentially expressed in T<sub>reg</sub> cells that are linked to the cytoskeletal machinery and that may confer unique properties onto the T<sub>reg</sub> cell immune synapse<sup>44</sup>. The integrin LFA-1 (lymphocyte function–associated antigen-1) has been shown to promote stable interactions of T<sub>reg</sub> cells with DCs<sup>31</sup>. However, blocking LFA-1–intercellular adhesion molecule 1 interactions inhibits the binding of both non-T<sub>reg</sub> cells and T<sub>reg</sub> cells with DCs and has not allowed us to specifically block T<sub>reg</sub> cell interactions. A second candidate molecule on T<sub>reg</sub> cells is CTLA-4, whose binding to CD80 and CD86 may also be involved in T<sub>reg</sub> cell–DC adhesion, in addition to signal transduction<sup>35</sup>. However, CTLA-4 appeared to play no role in our model system either in mediating suppression or in capturing antigen from the DC surface. Integrins other than LFA-1 could also play a role in T<sub>reg</sub> cell–DC interactions. Integrin α<sub>β</sub> has been shown to colocalize in the synapse and potentially augment T<sub>reg</sub> cell–DC binding<sup>4</sup>. Lastly, neuropilin 1 has been proposed<sup>41</sup> to increase the binding of T<sub>reg</sub> cells to DCs and enhance their sensitivity to antigenic signals.

One argument raised against the existence of additional pathways of T<sub>reg</sub> cell–mediated suppression is that such pathways would have been discovered in studies of genetic diseases that result in serious autoimmune manifestations, similar to mice with deficiencies in Foxp3, CTLA-4 or the cytokine TGF-β. Genetic defects in the model proposed in this study involving physiological interactions of the TCR with pMHCII would lead to a failure to mount any type of immune response and would not be regarded as being unique to T<sub>reg</sub> cells. However, a subpopulation of activated T<sub>reg</sub> cells can be identified in vivo<sup>40–42</sup>, and these activated T<sub>reg</sub> cells are lost in mice with T<sub>reg</sub> cell–specific deletion of the TCR. Deletion of the gene encoding the adaptor SLP-76 (SRC homology 2 domain–containing leukocyte phosphoprotein, 76 kDa) in mature T<sub>reg</sub> cells also results in an enhanced naive phenotype and loss of suppressive function<sup>40</sup>. Since SLP-76 plays a major role in actin polymerization in T<sub>reg</sub> cells<sup>41</sup>, loss of SLP-76 may result in impairment of immune-synapse formation in T<sub>reg</sub> cells and the ability to capture pMHCII complexes. These observations raise the possibility that this subpopulation of activated T<sub>reg</sub> cells in vivo is directly mediating suppression via the interaction of their TCR with complexes of self peptide and MHCII, resulting in constant removal of self peptide–MHCII complexes from the DC surface. Thus, continuous removal of self peptide–MHCII complexes may be the mechanism by which T<sub>reg</sub> cells maintain physiological immune homeostasis.

Online content
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Author contributions

B.A., Y.O., and E.M.S. conceived the project. E.M.S. secured the funding. B.A., M.A., Y.O., J.A.S., A.H.H., and O.K. performed the experiments. B.A., Y.O., M.A., J.K., and D.W.D. analyzed the data. D.D.G. provided technical help. B.A. and E.M.S. wrote the manuscript. M.A., Y.O., and R.M. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Articles

Methods

Animals and reagents. C57BL/6NCrl mice were purchased from Charles River. DαD.T3 and Foxp3-GFP mice were purchased from The Jackson Laboratory. MCC261, specific TCR transgenic 5C7-Foxp3GFP Rag-2−/−, 5C7-Cluda−/− Rag-2−/−, 5C7-Cluda−/− Rag-2+/−, 5C7-Cluda−/− specific TCR transgenic OT-II, OT-II-Rag2−−, CD11c+Foxp3+, B10.A mice were obtained from Taconic Biosciences under the National Institute of Allergy and Infectious Diseases (NIAID) contract. LCMV GP41−specific TCR transgenic SMARTA mice were originally obtained from the La Jolla Institute of Allergy and Immunology. OT-II and T3 mice were generated by a single cross between OT-II and DαD.T3 mice. 5C7-Foxp3-GFP Rag2−− and 3A9-Foxp3-GFP Rag2−− mice were generated by two crosses into a B10.A-Foxp3GFP background. OT-II-Foxp3RFP were purchased from BioLegend unless otherwise stated. Anti-MCC88–103-I-E k (D4) and YN1/1.7.4, Thermo Fisher Scientific), streptavidin-BV650, streptavidin-QDot525 L2-PE (clone TY25), anti-LFA-1-AF647 (clone 10E5), anti-ICAM-1-PE (clone GL-1), anti-CD80-PercpCy5.5 (clone 16–10A1), anti-CD86-PE.Cy7 (clone GL-1), anti-ICOSL-APC (clone HK5.3), anti-PD-L1-BV711 (clone 10F9G2), anti-PD-L2-PE (clone TY25), anti-LFA-1-AF647 (clone 10E5), anti-ICAM-1-PE (clone YN1/1.7.4, Therof Fisher Scientific), streptavidin-BV650, streptavidin-QDot525 (Therof Fisher Scientific), purified anti-CD161/32 (clone 93). Antibodies were purchased from BioLegend unless otherwise stated. Anti-MCC−C261−1-E/1−D (4D) and anti-HEL−A−1−1A (AW3.18) were used to detect pMHCII complexes. Anti-MCC−C261−1-E/1−E-AF477, anti-MCC−C261−1-E/biotin, anti-MCC−C261−1−E PE, anti-Hel−A−1−E-AF477 conjugations were performed by Columbia Biosciences. Human IL-2 was obtained from the Preclinical Repository of the Biological Resources Branch, National Cancer Institute. Recombinant mouse TGF-β was purchased from PeproTech. The PCI-261-C261 HEL1-4E, OA2-322, and GP1-105 proteins were purchased from the NIH Research Technologies Branch, NIAID Peptide Core Facility.

**TI**<sub>α</sub> cells and T<sub>eff</sub> cell differentiation and T<sub>αβ</sub> cell proliferation. For T<sub>αβ</sub><sub>eff</sub> cells and T<sub>αβ</sub><sub>eff</sub> cell differentiation, T<sub>αβ</sub><sub>eff</sub> cells were isolated and 24-well sterile tissue culture plates were coated with 1 μg/ml of Foxp3 reporter protein. Alternatively, antigen-specific T<sub>αβ</sub><sub>eff</sub> cells were expanded in vitro. For in vitro proliferation, T<sub>αβ</sub><sub>eff</sub> cells were cultured for 3 d in the presence of plate-bound anti-CD3e (2 μg/ml; BioLegend), anti-CD28 (2 μg/ml; BioLegend), and IL-2 (100 μl/ml). On day 3, cells were split 2:1 and cultured with only IL-2. T<sub>αβ</sub> eff cells were collected on day 5 and FACS-sorted by flow cytometry for the Foxp3 reporter.

Cocultures. To isolate DCs, spleens were removed and flushed with complete RPMI containing Liberase and DNase. Spleens were then fragmented and incubated at 37°C for 30 min. After incubation, red blood cells were lysed with ACK Lysing Buffer (Lonza). DCs were isolated using CD11c Microbeads (Miltenyi Biotec) and a magnetic cell separator (autoMACS). Anti-MHCII-PE and anti-MCC88–103-I-E k conjugates of anti-I-A/I-E, anti-CD4, and anti-MCC88–103-I-E k in Perm/Wash buffer (BD Biosciences) at room temperature for 30 min. For staining, cells were incubated with fluorochrome conjugates of anti-1-A/1-E, anti-CD4, and anti-MCC88–103-I-E k in Perm/Wash buffer for 1–2 h at 4°C in the dark. After a total of six washes using Perm/Wash (xC3) and PBS plus 1% BSA (xC3), cells were resuspended.

Confluent microsurgery. Glass-bottomed 14-mm microwell dishes (MatTek) were coated with 10 μg/ml fibronectin (Sigma-Aldrich) in PBS at room temperature for 1 h and were washed twice with complete RPMI. Freshly isolated DCs were then cultured to complete RPMI containing 2–5 μg/ml peptide and were incubated at 37°C for 1 h. After washing three times with complete RPMI, T<sub>αβ</sub><sub>eff</sub> cells and/or effect T cells were added. Dishes were further incubated at 37°C for 2 h. For live imaging, fluorochrome–conjugated anti-CD4 was directly added into the culture medium for the last 30 min of the culture at 5 μg/ml final concentration. For static imaging, cultures were fixed with Cytofix/Cytoperm solution (BioLegend) for 10 min, followed by blocking of the non-specific interactions using mouse serum (Jackson ImmunolResearch) and Fc block in Perm/Wash buffer (BD Biosciences) at room temperature for 30 min. For staining, cells were incubated with fluorochrome conjugates of anti-1-A/1-E, anti-CD4, and anti-MCC88–103-I-E k in Perm/Wash buffer for 1–2 h at 4°C in the dark. After a total of six washes using Perm/Wash (xC3) and PBS plus 1% BSA (xC3), cells were resuspended.

Intravitreal two-photon laser-scanning microscopy of mouse popliteal lymph nodes. The two-photon laser-scanning microscopy setup included a Leica SP8 inverted microscope equipped with a full range of visible lasers, two hybrid detectors, three photomultiplier detectors, and a motorized stage. Immunostained cells were imaged using 63x objective (Leica Microsystems). Microscope configuration was set up for 3D analysis (x, y, z) of the cellular layer. The following lasers were used: diode laser for 405 nm excitation; argon laser for 488 and 514 nm excitation; diode-pumped solid-state laser for 561 nm; and HeNe laser for 594 and 633 nm excitation. All lasers were tuned to minimal power (between 0.3% and 2%) to prevent photobleaching. z stacking of images of 10–12 μm was collected. Mosaic images of large cell culture areas (1 mm²) were generated by acquiring multiple z stacks using the Tile scan mode and were assembled into tiled images using LAS X, version 4.0 (Leica Microsystems). Images were processed with the Imaris software, version 9.2.1 (Bitplane).

Confocal microscopy of live lymph node section. Confocal imaging of live tissue sections was developed as a technique for visualizing tissue structure and cell segregation in the lymph nodes at close to physiological conditions. Mice were killed using a CO2 chamber (Braintree Scientific), and the lymph nodes were harvested and kept on ice in 1% BSA in PBS. The lymph nodes were trimmed from residual connective tissue and cords under a modular Leica MZ6 stereomicroscope (Leica Microsystems) using surgical tweezers (Miltex), Preheated 2% agarose (Leica Microsystems). The two-photon laser-scanning microscope setup included a Leica SP8 inverted microscope equipped with dual multi-photon lasers, Mai Tai and InSight DS (Spectra-Physics) and a 37°C incubation chamber (NIH, Division of Scientific Equipment and Instrumentation Services). Additionally, the microscope was equipped with an L 250 water-immersion objective, 0.95 NA (Leica Microsystems). Mice were anesthetized with 1.13% isoflurane USP administered via a nose cone mask. Surgery was performed on an anesthetized mouse to expose the popliteal lymph nodes. The mouse was placed on the cover glass bottom stage, and the lymph nodes were kept moistened with warm PBS and complementarily heated with an infrared blanket (Braintree Scientific) over the course of imaging. After imaging, mice were killed by cervical dislocation. The lymph nodes were killed using a CO2 chamber (Braintree Scientific) and the lymph nodes were harvested and kept on ice in 1% BSA in PBS. The lymph nodes were trimmed from residual connective tissue and cords under a modular Leica MZ6 stereomicroscope (Leica Microsystems) using surgical tweezers (Miltex), Preheated 2% agarose (Leica Microsystems). The two-photon laser-scanning microscope setup included a Leica SP8 inverted microscope equipped with dual multi-photon lasers, Mai Tai and InSight DS (Spectra-Physics) and a 37°C incubation chamber (NIH, Division of Scientific Equipment and Instrumentation Services). Additionally, the microscope was equipped with an L 250 water-immersion objective, 0.95 NA (Leica Microsystems). Mice were anesthetized with 1.13% isoflurane USP administered via a nose cone mask. Surgery was performed on an anesthetized mouse to expose the popliteal lymph nodes. The mouse was placed on the cover glass bottom stage, and the lymph nodes were kept moistened with warm PBS and complementarily heated with an infrared blanket (Braintree Scientific) over the course of imaging. After imaging, mice were killed by cervical dislocation while still under anesthesia. The Mai Tai laser was tuned to 890 nm to excite e450 and yellow fluorescent protein (YFP); the InSight DS laser was tuned to 1,150 nm to excite d4Red and e670. For time-lapse imaging, a 2-stack consisting of 10–12 single planes (5 μm each over a total tissue depth of 50–60 μm) was acquired every 15 s for a total observation time between 1 h and 4 h. Post-acquisition image processing was performed with the Leica Application Suite (Leica Microsystems), Imaris and Huygens (Scientific Volume Imaging) software. Cell migration parameters were evaluated with Imaris and Prism (GraphPad Software).

Confluent cell cultures of MC3T3-E1 osteoblasts were grown in 60-mm dishes (MatTek) and were imaged with a Leica SP8 inverted confocal microscope equipped with five-channel confocal microscope equipped with an environmental chamber (NIH, Division of Scientific Equipment and Instrumentation Services) and a motorized
stage. The microscope configuration was set up for four-dimensional analysis $(x, y, z, t)$ of cell segregation and migration through tissue sections. A diode laser for 405 nm, an argon laser for 488 and 514 nm, a diode-pumped solid-state laser for 561 nm, and a HeNe laser for 594 and 633 nm excitation wavelengths were tuned to minimal power (between 0.3% and 2%). $z$-stacks of images of 10–25 μm were collected. Mosaic images of whole lymph nodes were generated by acquiring multiple $z$ stacks using a motorized stage to cover the whole lymph node area and assembled into a tiled image with the LAS X software. For time-lapse analysis of cell migration, tiled $z$ stacks were collected over time (1–4 h). Post-acquisition images were processed with the Imaris software.

**SEM and TEM.** For SEM, samples were processed and imaged essentially as described previously$^{44}$. Briefly, cells were allowed to settle onto fibronectin-coated (10μg ml$^{-1}$) silicon chips for 3 h at 37 °C, then fixed by replacing the buffer with Karnovsky’s fixative (Electron Microscopy Sciences). The samples were postfixed in 1% OsO₄ in 0.1 M sodium phosphate, pH 7.2, using two cycles of 170 W microwave irradiation, with the power on for 2 min, off for 2 min and on for 2 min, in a BioWave model processor (Ted Pella). Following two 1-min water washes at 170 W, the samples were dehydrated in 70%, 100% and 100% ethanol for 1 min each at 250 W, critical-point-dried through CO₂, and lightly sputtered with iridium. Digital images were captured at 2 kV using a model SU 8000 scanning electron microscope (Hitachi High-Technologies).

For immune TEM, fibronectin-coated coverslips with cell cultures were fixed and processed with modifications to procedures described previously$^{45,46}$. Samples were prefixed in 0.075 M sodium phosphate buffer, pH 7.4, containing 0.01 M sodium metaperiodate, 0.075 M lysine, 2% paraformaldehyde and 0.25% glutaraldehyde and were incubated on ice overnight. Subsequent steps were performed at room temperature. Following two 5-min rinses in PBS, pH 7.4, cells were permeabilized for 5 min with freshly prepared PBS containing 0.01% saponin (Sigma-Aldrich). The samples were then probed for 1 h with the same mixture containing a 1:100 dilution biotinylated anti-MCC88–103–I-Ek (D4). After two washes with PBS, samples were labeled for 1 h with a 1.50 dilution of streptavidin conjugated to 525 nm quantum dots in PBS. Following three rinses for 5 min each in PBS, samples were treated for 1 h with fixative containing 1.5% glutaraldehyde and 5% sucrose in 0.1 M sodium phosphate, pH 7.4. Further processing steps using microwave irradiation were conducted as described previously, except that Araldite resin (Structure Probem) was used for embedment$^{47}$. Samples were examined and photographed at 80 kV with an H7500 transmission electron microscope (Hitachi High-Technologies) equipped with an HR-100 CCD camera (Advanced Microscopy Techniques).

**Trogocytosis assay.** DCs were labeled with 4 μM of the lipophilic membrane dye PKH26 (Sigma-Aldrich) as described in Puaux et al.$^{48}$, were loaded with 3 μM peptide and were cocultured with T cells for 18 h. Cell conjugates were dissociated by washing of the cells with MACS buffer containing 2 mM EDTA; cell suspensions were prepared for flow cytometry.

**Flow cytometry.** Cells were washed with MACS buffer followed by FACS buffer and were stained with fluorochrome-conjugated antibodies at 4°C for 30 min in the dark. Cells were barcoded as in Akkaya et al.$^{49}$ wherever indicated. Data acquisition was performed with BD LSRII Fortessa and BD LSRII cytometers (BD Biosciences). Data were analyzed with the FlowJo software, version 10.4.

**Image analysis.** Imaris software was used to analyze the confocal microscopy and intravital two-photon microscopy data. The SEM and TEM data were analyzed with Image J (NIH). The normalized velocity in Fig. 3 was calculated as the mean track speed of naive OT-II-DXRed/average for the mean track speeds of polyclonal CD4$^+$ cells. In Fig. 6b, the YFP (CD11c) signal was used to reconstruct the 3D structure of the DC as a surface object. DCs were then removed as 3D objects from the CD45.1 (Alexa Fluor 647, red) channel. The resulting masked CD45.1 (red) channel (with the DC portion removed) was used to reconstruct the CD45.1$^+$ cells; the MHC class II (blue) intensities of these cells were quantified.

**Statistical analysis.** Statistical significance analyses were performed using Prism version 7.0d. The statistical tests and $P$ values are indicated in the figures and figure legends. Briefly, two-sided Student’s $t$-tests and two-sided Welch’s $t$-tests were used to compare two continuous interval variables with a normal distribution. To compare three or more continuous interval variables, either a one-way or two-way analysis of variance (ANOVA) was used depending on the experimental setup. Non-parametric tests, such as the Kolmogorov–Smirnov and Kruskal–Wallis tests, were selected to determine statistical significance when comparing three or more distribution-free discrete variables as appropriate and are mentioned in the figure legends.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon request.

**References**

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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a
- Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Leica Application Suite X, BD FACS Diva |
|-----------------|----------------------------------------|
| Data analysis   | Imaris 9.2.1 (Bitplane), FlowJo 10.4 (FlowJo, LLC), Prism 7 (GraphPad), ImageJ 1.51m9, Huygens (Scientific Volume Imaging) 18.04 |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon request.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined based on previous studies of similar nature. No statistical methods were used to predetermine sample sizes. |
|-------------|-------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data exclusions have been made                                                                                               |
| Replication  | All experiments were repeated and exact number of repeats were stated in the manuscript. All attempts at replication were successful. |
| Randomization | For in vivo experiments, age-sex matched animals were used. Animals were randomly assigned to experimental groups.               |
| Blinding     | No subjective measurement such as clinical scoring that require blinding was used for the study, therefore blinding was not performed. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ✗   | Unique biological materials |
♭ | Antibodies |
♭ | Eukaryotic cell lines |
♭ | Palaeontology |
♭ | Animals and other organisms |
♭ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ✗   | ChIP-seq |
♭ | Flow cytometry |
♭ | MRI-based neuroimaging |

Antibodies

Antibodies used for flow cytometry and microscopy are: anti-CD4-BV786 (RM4-5, catalog number: 100552, lot:B196242), anti-CD4-BV421 (GK1.5, catalog number: 100443, lot:B199185), anti-CD4-BV605 (RM4-5, catalog number: 100548, lot:B244808), anti-CD4-AF488 (RM4-5, catalog number: 100529, lot:B215457), anti-I-A/I-E-BV421 (M5/114.15.2, catalog number: 107632, lot:B202803), anti-I-A/I-E-BV605 (M5/114.15.2, catalog number: 107639), anti-I-A/I-E-PE (BD Biosciences, M5/114.15.2, catalog number: 557000, lot:B31691), anti-CD3-AF421 (17A2, catalog number: 100226, lot:B214893), anti-CD45.1-PE.Cy7 (ThermoFisher, A20, catalog number: 25-0453-82, lot:E075571-1630), anti-CD45.2-PE (104, catalog number: 109808, lot:B126495), anti-CD45.2-PE.Dazzle 594 (104, catalog number: 109846, lot:B232132), anti-CD45.2-APC.Cy7 (104, catalog number: 109823, lot:B216060), anti-CD45.2-APC.700 (ThermoFisher, 104, catalog number: 56-0454-81, lot:E08993-1634), anti-CD80-PercpCy5.5 (16-10A1, catalog number: 104722, lot:B187361), anti-CD86-PE.Cy7 (ThermoFisher, GL1, catalog number: 25-0862-82, lot:E20041-102), anti-ICOSL-APC (2D3, catalog number: 309408), anti-CD-L1-BV711 (10F.9G2, catalog number: 124319, lot:B195358), anti-PD-L2-PE (Ty25, catalog number: 107206, lot:B204873), anti-LFA-1-APC (M17/4, catalog number: 101114, lot:B171752), anti-ICAM-1-PE (ThermoFisher, KAT-1, catalog number: 12-0542-81), Streptavidin-BV60 (catalog number: 405231, lot:B217346), Streptavidin-QDot525 (ThermoFisher, catalog number: Q10141MP, lot:1795048), purified anti-CD16/32 (93, catalog number: 101320, lot:13249154). Antibodies were purchased from Biolegend (San Diego, CA) unless otherwise stated. Anti-MCC88-103-I-Ek (D4) and anti-HEL46-61-I-Ak (AW3.14) antibodies were provided by Dr. Gouri Chattophadyay and Dr. Susan K. Pierce respectively. Antibodies were used at 1/200 dilution unless otherwise stated.

Validation

The commercially available primary antibodies were validated by the vendors on their official websites. Anti-MCC88-103-I-Ek (D4) and anti-HEL46-61-I-Ak (AW3.14) that were used to detect peptide-MHCII complexes have been validated as cited in the manuscript. Further details of antibody validation can be found at:
1) https://www.biolegend.com/reproducibility
2) https://www.thermofisher.com/us/en/home/life-science/antibodies/invitrogen-antibody-validation/independent-antibody-validation.html
3) Anti-MCC88-103-I-Ek (D4): https://doi.org/10.1038/nature01076
4) Anti-HEL46-61-I-Ak (AW3.14): https://doi.org/10.1016/S1074-7613(00)80448-3
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | 8-12 weeks old female mice were used for the study. Mouse strains include: DsRed.T3 (Jackson Laboratory), C57BL/6 (Charles River), SCCC7-FoxP3.GFP Rag2-/-, SCCC7-CTLA4-/-Rag2-/-, SCCC7-IL10-/-Rag2-/-, Hen Egg Lysozyme (HEL46-61) specific TCR transgenic 3A9 Rag2-/-, OT-II, OT-II-Rag2-/-, CD11c.YFP, FoxP3.GFP, B10A, B10A-Foxp3.GFP, SCCC7-FoxP3.GFP Rag2+/-, 3A9-Foxp3.GFP Rag2+/+mice were generated by two crosses into B10A-Foxp3.GFP background. OT-II-Foxp3.RFP mice were generated by a single cross between OT-II and Foxp3.RFP mice. Mice were obtained from Taconic Farms under the NIAID contract unless otherwise stated. SMARTA mice were obtained from the La Jolla Institute of Allergy and Immunology. OT-II-DsRed.T3 were generated in house. |
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve field-collected samples. |

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation | Cells were washed with MACS buffer, followed by FACS buffer and stained with fluorochrome-conjugated antibodies at 4 °C for 30 min in dark |
Instrument | LSR II (BD), Fortessa (BD), X20 (BD) |
Software | BD FACS Diva |
Cell population abundance | Purity of the samples were ensured by FACS sorting and magnetic isolation techniques as stated in the manuscript. Cell numbers in the experiments were matched for all the conditions tested. |
Gating strategy | Gating strategies were indicated in the figure legends. For all samples, preliminary FSC/SSC and live/dead gates were applied before gating the population of interest. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.